Arch. Pharm. Chem. Life Sci. 2016, *349*, 363–372

ARCH PHARM
Archiv der Pharmazie

Full Paper

Synthesis and Anti-HIV-1 Evaluation of Some Novel MC-1220 Analogs as Non-Nucleoside Reverse Transcriptase Inhibitors

Yasser M. Loksha[1,2], Erik B. Pedersen[1], Roberta Loddo[3], and Paolo La Colla[3]

[1] Department of Physics, Chemistry and Pharmacy, Nucleic Acid Centre, University of Southern Denmark, Odense M, Denmark
[2] Faculty of Pharmacy and Pharmaceutical Industries, Department of Pharmaceutical Chemistry, Sinai University, Al-Arish, North Sinai, Egypt
[3] Dipartimento di Scienze e Tecnologie Biomediche, Sezione di Microbiologia e Virologia Generale e Biotecnologie Microbiche, Universita di Cagliari, Monserrato, Italy

Some novel MC-1220 analogs were synthesized by condensation of 4,6-dichloro-*N*-methylpyrimidin-2-amine derivatives (**1a**,**b** and **15)** and/or 4-chloro-6-methoxy-*N*,*N*,5-trimethylpyrimidin-2-amine (**2a**) with the sodium salt of 2,6-difluorophenylacetonitrile followed by treatment with aqueous sodium hydroxide in methanol, alkylation, reduction, halogenation, and/or acidic hydrolysis. All synthesized compounds were evaluated for their activity against HIV-1. The most active compound in this study was compound **7**, which showed activity against HIV-1 comparable to that of MC-1220. The only difference in structure between compound **7** and MC-1220 is a fluoro atom instead of a CH_3 group.

Keywords: 4,6-Dichloro-*N*,*N*-dimethylpyrimidin-2-amine / 2,6-Difluorobenzyl cyanide / HIV-1 / MC-1220 analogs / Non-nucleoside reverse transcriptase inhibitor (NNRTI)

Received: January 11, 2016; Revised: February 23, 2016; Accepted: February 25, 2016

DOI 10.1002/ardp.201600008

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Introduction

Retroviruses such as HIV-1 and HIV-2 require a reverse transcriptase (RT) to convert viral RNA into proviral DNA [1, 2]. Non-nucleoside reverse transcriptase inhibitors (NNRTIs) [3] play an essential role in the therapy for AIDS due to their potent antiviral activity, high specificity, and low toxicity. Dihydroalkoxybenzyloxopyrimidines (DABOs) family is one representative of the reported potent HIV-1 NNRTIs, with robust anti-HIV-1 activity against both the wild-type (wt) and drug-resistant isolates carrying multiple RT gene mutations. Three generations of DABO analogs have been studied up to now, that is: dihydroalkyloxybenzyloxopyrimidines (O-DABOs), dihydroalkylthiobenzyloxopyrimidines (S-DABOs) and dihydroalkylaminodifluorobenzyloxopyrimidines (N-DABOs), from which many promising DABOs are under development [4].

A great number of DABOs have been synthesized and tested as anti-HIV-1 agents to obtain more potent and selective compounds. Structure–activity relationship (SAR) profiles of DABOs together with molecular modeling investigations of their putative binding modes have shown that the presence of a C2-alkoxy (DABOs) side chain is a structural determinant for the antiviral activity of their derivatives [5–7].

The concepts of vaginal microbicides are a potentially promising strategy to prevent the spread of HIV. Microbicides are self-administered, prophylactic products designed to protect against sexually transmitted pathogens, including HIV-1. A special subgroup of NNRTIs, the DABOs, have a proven capacity to irreversibly block HIV-1 replication [8–11]. This property correlates with their ability to bind HIV-1 RT with high affinity. One of the most important lead compounds of this series of tight-binding NNRTIs is MC-1220

Correspondence: Dr. Erik B. Pedersen, Department of Physics, Chemistry and Pharmacy, Nucleic Acid Center, University of Southern Denmark, Campusvej 55, DK-5230 Odense M, Denmark.
E-mail: erik@sdu.dk
Fax: +45 66158780

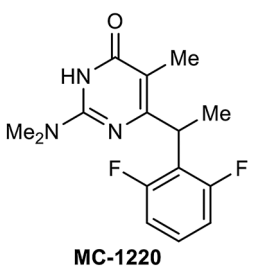
(Fig. 1), which is used in racemic form [12–16]. The synthesis of MC-1220 and its analogs was previously published by Mai et al. [12] and Bartolini et al. [13] by condensation of the corresponding β-keto esters with guanidine sulfate derivatives. The β-keto esters, however, were prepared through multistep reactions [12]. Recently, Radi et al. reported the synthesis of arylmethyl-functionalized *S*-DABOs and related analogs from C6-protected formyl pyrimidinone [17]. We have previously described a new and efficient synthetic route to racemic MC-1220 which can also be used for the preparation of its analogs [18, 19].

In continuation to our previous work [18], we report the synthesis and anti-HIV-1 evaluation of some novel MC-1220 analogs as NNRTIs.

Results and discussion

Chemistry

4,6-Dichloro-*N*,*N*-dimethylpyrimidin-2-amines (**1a**,**b**) were synthesized by the action of phosphorus oxychloride on the corresponding dihydroxy derivatives as previously described by Boon [20] for **1a** and Hofer et al. [21] for **1b**. Nucleophilic substitution of only one chlorine atom in compounds **1a**,**b** with a methoxy group was achieved by treatment with sodium methoxide in methanol to afford 4-chloro-6-methoxy-*N*,*N*-dimethylpyrimidin-2-amines (**2a**,**b**) as described by Boon [22] for synthesis of **2a**. Each of compounds **2a**,**b** was coupled with the sodium salt of 2,6-difluorobenzyl cyanide to furnish (2,6-difluorophenyl)[2-(dimethylamino)-6-methoxypyrimidin-4-yl]acetonitriles (**3a**,**b**). The sodium salt of (2,6-difluorophenyl)[2-(dimethylamino)-6-methoxy-5-methylpyrimidin-4-yl]acetonitrile (**3b**) was oxidized with a stream of oxygen bubbled through the reaction mixture for 3 h to afford (2,6-difluorophenyl)[2-(dimethylamino)-6-methoxy-5-methylpyrimidin-4-yl]methanone (**4**) in 21% yield. Hydrolysis of compound **4** with 4 M hydrochloric acid furnished 6-(2,6-difluorobenzoyl)-2-(dimethylamino)-5-methylpyrimidin-4(3*H*)-one (**5**) which was reduced to its corresponding alcohol **6** by sodium borohydride in methanol. 6-[(2,6-Difluorophenyl)-(hydroxy)methyl]-2-(dimethylamino)-5-methylpyrimidin-4(3*H*)-one (**6**) was fluorinated with (diethylamino)sulfur trifluoride (DAST) in dichloromethane to give 6-((2,6-difluorophenyl)-fluoromethyl)-2-(dimethylamino)-5-methylpyrimidin-4(3*H*)-one (**7**) (Scheme 1).



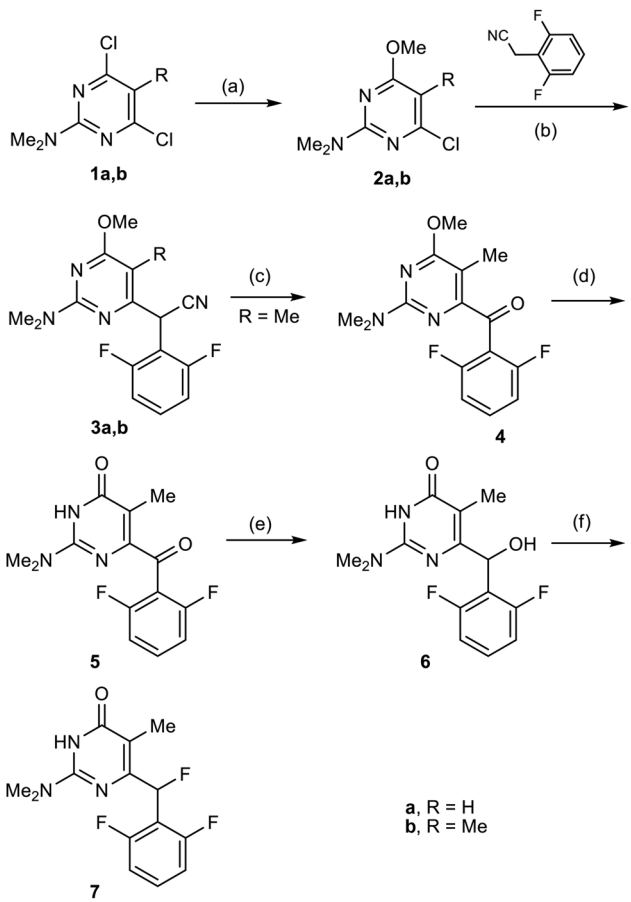
MC-1220

Figure 1. Structure of MC-1220.

The product from the treatment of the sodium salt of compound **5** with dimethylsulfamoyl chloride in *N*,*N*-dimethylformamide was 6-(2,6-difluorobenzoyl)-2-(dimethylamino)-5-methylpyrimidin-4-yl dimethylsulfamate (**8**). *O*-Sulfamoylation occurred and not *N*1-sulfamoylation like previously elucidated in our recent article [23]. Reduction of compound **8** with sodium borohydride in methanol afforded 6-[(2,6-difluorophenyl)(hydroxy)methyl]-2-(dimethylamino)-5-methylpyrimidin-4-yl dimethylsulfamate (**9**). As previously published in our recent article for compound **10b** [18], condensation of **1a**,**b** with the sodium salt of 2,6-difluorobenzyl cyanide in *N*,*N*-dimethylformamide gave 2-[6-chloro-2-(dimethylamino)pyrimidin-4-yl]-2-(2,6-difluorophenyl)acetonitriles (**10a**,**b**) in very high yields (Scheme 2).

Treatment of compound **10b** with 25% aqueous sodium hydroxide in methanol at room temperature followed by passing stream of oxygen through the reaction mixture afforded three different compounds. Two of them were precipitated from the reaction mixture, separated from each other by column chromatography and were elucidated to be 4-aroyl-6-methoxy-*N*,*N*,5-trimethylpyrimidin-2-amine derivatives (**11a**,**b**). The third one 4-(2,6-difluorobenzyl)-6-methoxy-*N*,*N*,5-trimethylpyrimidin-2-amine (**11c**) was crystallized from the filtrate by addition of water. ^{13}C NMR showed the presence of C=O group in both of compounds **11a**,**b** at 194–197 ppm. ^{1}H and ^{13}C NMR showed the disappearance of CH–CN and CH_2 group. Compounds **11a**,**b** are benzoyl derivatives at position 6 of the pyrimidine ring with substitution of one fluoro atom with methoxy group on the benzene ring (**11a**) and substitution of the two fluoro atoms with two methoxy groups on the benzene ring (**11b**). For compound **11c**, ^{1}H and ^{13}C NMR have confirmed the existence of a CH_2 group (3.94 and 27.34 ppm, respectively) and the disappearance of CH–CN or C=O group in compound **11c**. Hydrolysis of compounds **11a–c** with 4 M hydrochloric acid furnished the corresponding pyrimidine-6-one derivatives **12a–c**. Compound **12c** was first synthesized by La Colla and Artico [24] and later on by us where we synthesized it by strong acidic hydrolysis of compound **10b** [18]. M.p., NMR (^{1}H and ^{13}C) for compound **12c** were identical to the compound prepared by acid hydrolysis of **10b** [18]. Bromination of the CH_2 group in compound **12c** was achieved by treatment with *N*-bromosuccinimide and benzoyl peroxide as a catalyst in ethanol to give 6-[bromo(2,6-difluorophenyl)-methyl]-2-(dimethylamino)-5-methylpyrimidin-4(3*H*)-one (**13**) in 70% yield as the sole product (Scheme 3).

Additional analogs of MC-1220 were synthesized by coupling the sodium salt of 4,6-dichloro-*N*,5-dimethylpyrimidin-2-amine (**14**) [25] with 4-iodobenzyl bromide at room temperature in dry *N*,*N*-dimethylformamide to give 4,6-dichloro-*N*-(4-iodobenzyl)-*N*,5-dimethylpyrimidin-2-amine (**15**) which was condensed with the sodium salt of 2,6-difluorobenzyl cyanide to afford 2-(6-chloro-2-[(4-iodobenzyl)(methyl)amino]-5-methylpyrimidin-4-yl)-2-(2,6-difluorophenyl)acetonitrile (**16**)



Scheme 1. Synthesis of compounds **2**–**7**. Reagents and conditions: (a) NaOMe, MeOH, RT; (b) NaH, DMF, RT; (c) NaH, DMF, O_2, RT; (d) 4 M HCl, reflux; (e) $NaBH_4$, MeOH, 0°C; (f) DAST, CH_2Cl_2, −5°C.

in 99% yield. Acid hydrolysis of compound **16** using 7 M hydrochloric acid and acetic acid afforded 6-(2,6-difluorobenzyl)-2-[(4-iodobenzyl)(methyl)amino]-5-methylpyrimidin-4(3*H*)-one (**17**) (Scheme 4).

Antiviral activity

The HIV-1 strain HTLV-IIIB in MT-4 cells was used in our assay to investigate the anti-HIV-1 activity of MC-1220 analogs synthesized in the present study. The results are summarized in Table 1. Most of the new compounds showed moderate activity or no activity against HIV-1 wt. Only compound **7** was close to the activity of MC-1220. It was active against HIV-1 wt at 0.02 μM and against the mutant Y181C at 2.8 μM. The only difference in structure between compound **7** and MC-1220 is a fluoro atom instead of CH_3 group. Although smaller, the fluoro atom is close in size to a methyl group. It is clear that the size of methyl group as a substituent on the benzylic position is crucial for the activity against HIV-1 for MC-1220. When the size of the substituent was increased by using a bromine atom as a substituent in compound **13**, the activity as expressed by EC_{50} was reduced to 0.4 μM against HIV-1 wt and it was absent against the other mutants. On the other hand, complete removal of the substituent resulted in a reduced activity with 0.07 μM against HIV-1 wt and no activity against the other mutants for compound **12c**. Assuming an easy hydrolysis of the chloro and dimethylaminosulfamate groups in compounds **8**, **9**, **10a**,**b**, and **16**, these compounds could be considered produgs of MC-1220 analogs. However, the dimethylaminosulfamate group was detrimental to activity against HIV as deduced when the activity of compounds **8** and **9** was compared to those of **5** and **6**, respectively. Interestingly, a weak activity against HIV was observed for the chloro compounds **10a**,**b**.

Conclusion

The most interesting structural variations of MC-1220 synthesized in this work are the new compounds **7** and **13** because they give a unique opportunity to evaluate the effect of the size of the substituent on the activity against HIV when a methyl group in MC-1220 is replaced by another substituent. When the methyl group is replaced with a small fluorine atom, the activity is slightly reduced whereas replacement with hydrogen or a large bromine atom has either a larger negative effect or nearly a detrimental effect on the activity against HIV, respectively.

Experimental

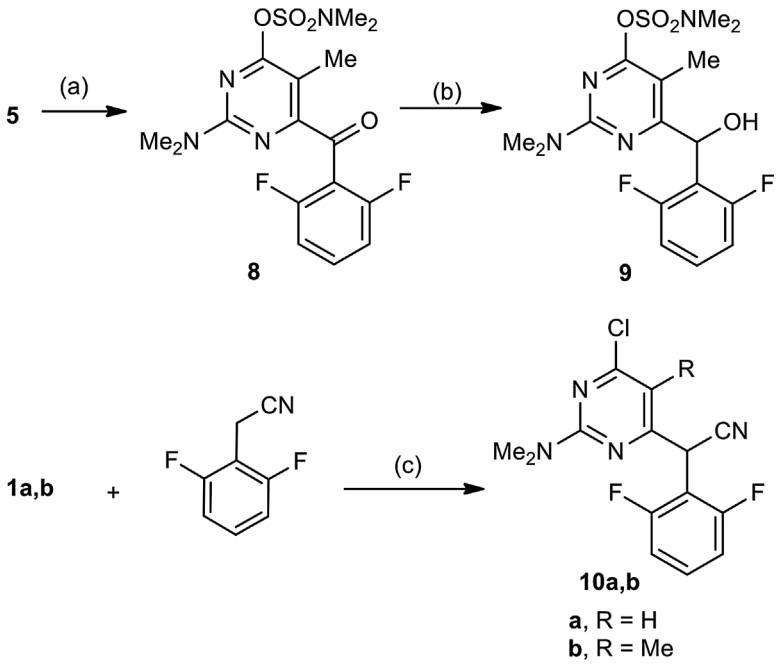
Chemistry

NMR spectra were recorded on a Varian Gemini 2000 NMR spectrometer at 300 MHz for ^1H and 75 MHz for ^{13}C with TMS as internal standard. MALDI mass spectra were recorded on a 4.7 Tesla Ultima (IonSpec, Irvine, CA) Fourier transform ion cyclotron resonance (FTICR) mass spectrometer. Melting points were determined on a Büchi melting point apparatus. Elemental analyses were performed at H.C. Ørsted Institute, University of Copenhagen. Silica gel (0.040–0.063 mm) used for column chromatography and analytical silica gel TLC plates 60 F_{254} were purchased from Merck. Solvents for chromatography were bought as HPLC grade or distilled prior to use.

The InChI codes of the synthesized compounds together with selected biological activities are listed in the Supporting Information.

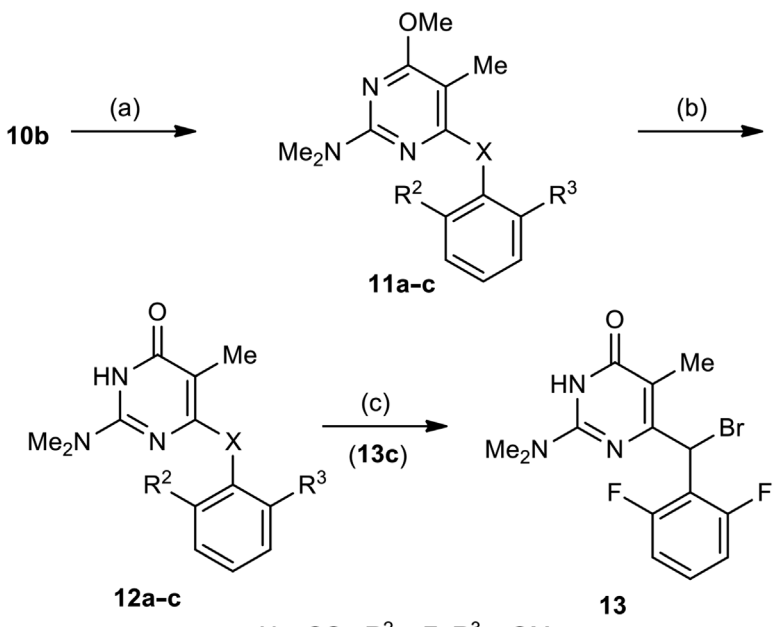
4-Chloro-6-methoxy-N,N-dimethylpyrimidin-2-amines ***2a,b***
To a stirred suspension of **1a**,**b** (30 mmol) in methanol (30 mL) was added dropwise sodium methoxide solution (0.76 g, 33 mmol Na in 20 mL MeOH). The reaction mixture was stirred for 16 h, the solvent was removed under reduced pressure. Water (80 mL) was added and the solid product formed was filtered off, washed with water, and dried to afford **2a**,**b**.

4-Chloro-6-methoxy-N,N-dimethylpyrimidin-2-amine ***2a***
Yield 91%; m.p. 60–62°C. Lit. m.p. 62°C [22].

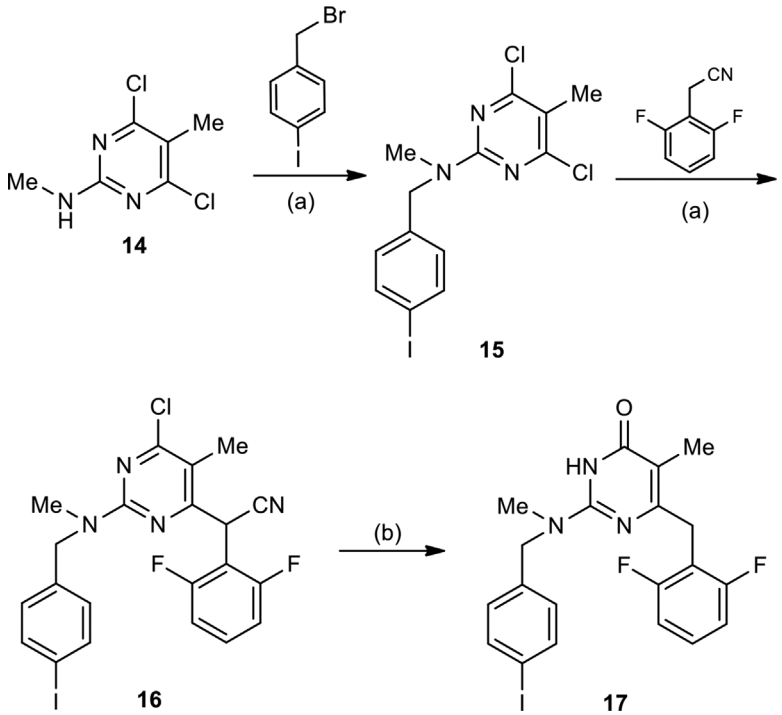


Scheme 2. Synthesis of compounds **8**–**10a**,**b**. Reagents and conditions: (a) $(CH_3)_2N–SO_2–Cl$, NaH, DMF, RT; (b) $NaBH_4$, MeOH, 0°C; (c) NaH, DMF, RT.

4-Chloro-6-methoxy-N,N,5-trimethylpyrimidin-2-amine ***2b*** Yield 98%; as a white solid; m.p. 63–65°C; ^{1}H NMR ($CDCl_3$) δ [ppm]: 2.05 (s, 3H, CH_3), 3.13 [s, 6H, $(CH_3)_2N$], 3.92 (s, 3H, OCH_3); ^{13}C NMR ($CDCl_3$) δ [ppm]: 10.67 (CH_3), 36.84 [$(CH_3)_2N$], 53.73 (OCH_3), 101.47 (C4), 103.92 (C5), 159.33 (C2), 168.54 (C6); EI-MS: *m/z* 201 (100%, M^+). Anal. calcd. for $C_8H_{12}ClN_3O$ (201.65): C, 47.65; H, 6.00; N, 20.84. Found: C, 47.71; H, 5.98; N, 20.93.



Scheme 3. Synthesis of compounds **11a–c** to **13**. Reagents and conditions: (a) i) 25% aq. NaOH, MeOH, ii) O_2, 6 h, iii) reflux, 30 h; (b) 4 M HCl, reflux 16 h; (c) NBS, EtOH, $(PhCO_2)_2$, RT.



Scheme 4. Synthesis of compounds **15**–**17**. Reagents and conditions: (a) NaH, DMF, RT; (b) 7 M HCl, AcOH, reflux, 50 h.

Table 1. Cytotoxicity and anti-HIV-1 activity of compounds 3–13, 16, 17, and the reference compound MC-1220.

Compound	CC_{50} (μM)[b]	SI[c]	EC_{50} (μM)[a]			
			Wild-type	EFV^R	Y181C	K103N + Y181C
3a	40 ± 8	13	3 ± 0.4	>40	>40	>40
3b	10	>1	>10	>10	>10	>10
4	20	>1	>20	>20	>20	>20
5	>100	>333	0.3 ± 0.1	>100	96	>100
6	50 ± 1	50	1 ± 0.2	>50	>50	>50
7	>100	>5000	0.02	>100	2.8	≥100
8	41	>1	>41	>41	>41	>41
9	85	>1	>85	>85	>85	>85
10a	80 ± 8	40	2 ± 0.5	>80	>80	>80
10b	34 ± 7	4	8 ± 0.1	>34	>34	>34
11a	7.8	>1	>7.8	>7.8	>7.8	>7.8
11b	8	>1	>8	>8	>8	>8
11c	67	>1	>67	>67	>67	>67
12a	>100	>200	0.5 ± 0.1	>100	54	>100
12b	>100	>2.5	40 ± 6	>100	>100	>100
12c	>100	>1429	0.07	>100	≥100	≥100
13	43	108	0.4 ± 0.1	>43	>43	>43
16	>100	>2	53 ± 0.5	>100	>100	70 ± 20
17	>100	>500	0.2 ± 0.05	>100	11 ± 1	>100
MC-1220	>100	>10000	0.01 ± 0.003	>100	0.7 ± 0.2	90 ± 10

[a] Compound dose required to reduce the viability of mock-infected cells by 50%, as determined by the MTT method.
[b] Compound dose required to achieve 50% protection of MT-4 cells from HIV-1-induced cytopathogenicity, as determined by the MTT method. The symbol (>) indicates that the CC_{50} was not reached at the highest concentration tested.
[c] Selectivity index: ratio CC_{50}/EC_{50}. EC_{50} and CC_{50} are expressed as the mean values of at least two separate experiments.

*(2,6-Difluorophenyl)[2-(dimethylamino)-6-methoxypyrimidin-4-yl]acetonitriles **3a,b***

Under stream of nitrogen, sodium hydride (2.9 g, 66 mmol, 55% susp. in paraffin oil) was added portionwise to a stirred solution of **2a**,**b** (20 mmol) and 2,6-difluorobenzyl cyanide (22 mmol) in dry *N*,*N*-dimethylformamide (50 mL) at 0°C. The reaction mixture is allowed to reach room temperature gradually and left to be stirred for 12 h, then poured on ice-cold water (200 mL) and extracted with ether (3 × 50 mL). The ethereal extracts were dried using sodium sulfate and the solvent was removed under reduced pressure. The residual material was chromatographed on a column of silica gel using petroleum ether/ether (5:1, v/v) as an eluent to afford **3a**,**b**.

*(2,6-Difluorophenyl)[2-(dimethylamino)-6-methoxypyrimidin-4-yl]acetonitrile **3a***

Yield 33%; as yellow crystals; m.p. 102–104°C; ^{1}H NMR ($CDCl_3$) δ [ppm]: 3.09 [s, 6H, $(CH_3)_2N$], 3.88 (s, 3H, OCH_3), 5.36 (s, 1H, H5), 6.03 (s, 1H, CH–CN), 6.95 (t, 2H, $J = 8.1$ Hz, H_{arom}), 7.26–7.38 (m, 1H, H_{arom}); ^{13}C NMR ($CDCl_3$) δ [ppm]: 32.99 (CH–CN), 36.57 [$(CH_3)_2N$], 53.16 (OCH_3), 92.16 (C5), 111.58–111.91 (m, C_{arom}), 116.58 (CN), 130.64 (t, $J = 10.5$ Hz, C_{arom}), 160.74 (dd, $J = 6.6$, 251.6 Hz, C_{arom}), 161.81 (C4), 161.92 (C2), 170.78 (C6); EI-MS: $m/z = 204$ [100%, M^+]. Anal. calcd. for $C_{15}H_{14}F_2N_4O$ (304.29): C, 59.21; H, 4.64; N, 18.41. Found: C, 59.25; H, 4.53; N, 18.35.

*(2,6-Difluorophenyl)[2-(dimethylamino)-6-methoxy-5-methylpyrimidin-4-yl]acetonitrile **3b***

Yield 30%; as a yellow solid; m.p. 104–106°C; ^{1}H NMR ($CDCl_3$) δ [ppm]: 2.01 (s, 3H, CH_3), 3.05 [s, 6H, $(CH_3)_2N$], 3.89 (s, 3H, OCH_3), 5.50 (s, 1H, CH), 6.93 (t, 2H, $J = 8.3$ Hz, H_{arom}), 7.26–7.36 (m, 1H, H_{arom}); ^{13}C NMR ($CDCl_3$) δ [ppm]: 9.11 (CH_3), 31.12 (CH–CN), 36.45 [$(CH_3)_2N$], 53.47 (OCH_3), 101.11 (C5), 111.41–111.75 (m, C_{arom}), 130.33 (t, $J = 10.3$ Hz, C_{arom}), 157.33 (C4), 160.86 (dd, $J = 6.6$, 251.6 Hz, C_{arom}), 159.80 (C2), 168.62; HRMS-MALDI: $m/z = 341.1184$ ($C_{16}H_{16}F_2NaN_4O$ [MNa^+]), requires 341.1188.

*(2,6-Difluorophenyl)[2-(dimethylamino)-6-methoxy-5-methylpyrimidin-4-yl]methanone **4***

Compound **3b** (1.6 g, 5 mmol) was added portionwise to a solution of sodium hydride (0.65 g, 15 mmol, 55% susp. in paraffin oil) in dry *N*,*N*-dimethylformamide (30 mL). Stream of oxygen was bubbled through the reaction mixture for 3 h. The solution was neutralized with 4 M hydrochloric acid then poured on ice-cold water (150 mL). The solid product formed was filtered off, washed with water, and dried to afford 320 mg (21% yield) of compound **4** as greenish yellow crystals.

M.p. 88–90°C; ^{1}H NMR ($CDCl_3$) δ [ppm]: 2.26 (s, H, CH_3), 2.97 [s, 6H, $(CH_3)_2N$], 3.96 (s, 3H, OCH_3), 6.90 (t, 2H, $J = 8.0$ Hz, H_{arom}), 7.31–7.41 (m, 1H, H_{arom}); ^{13}C NMR ($CDCl_3$) δ [ppm]: 9.48 (CH_3), 36.48 [$(CH_3)_2N$], 53.67 (OCH_3), 104.69 (C5), 111.10–111.43 (m, C_{arom}), 131.60 (t, $J = 10.2$ Hz, C_{arom}), 157 (C4), 160.32 (dd, $J = 7.6$, 252.3 Hz, C_{arom}), 159.77 (C2), 169.54 (C6), 191.23 (C=O); EI-MS: $m/z = 307$ [M^+] (100%). Anal. calcd. for $C_{15}H_{15}F_2N_3O_2$ (307.30): C, 58.63; H, 4.92; N, 13.67. Found: C, 58.91; H, 4.94; N, 13.49.

*6-(2,6-Difluorobenzoyl)-2-(dimethylamino)-5-methylpyrimidin-4(3H)-one **5***

Compound **4** (0.92 g, 3 mmol) was refluxed in 4 M hydrochloric acid (20 mL) for 16 h. The reaction mixture was cooled and the solvent was removed under reduced pressure. Water (20 mL) was added and the solution was neutralized with 5% aq. sodium hydroxide. The crystallized product from the solvent was filtered off, washed with water, and dried to give 703 mg (80% yield) of compound **5** as a pale yellow solid.

M.p. 202–204°C; ^{1}H NMR (DMSO-d_6) δ [ppm]: 2.05 (s, 3H, CH_3), 2.85 [s, 6H, $(CH_3)_2N$], 7.19 (t, 2H, $J = 8.3$ Hz, H_{arom}), 7.56–7.65 (m, 1H, H_{arom}), 11.41 (bs, 1H, NH); ^{13}C NMR (DMSO-d_6) δ [ppm]: 9.77 (CH_3), 36.59 [$(CH_3)_2N$], 111.57–111.90 (m, C_{arom}), 132.99 (t, $J = 10.3$ Hz, C_{arom}), 117.46 (C5), 159.18 (dd, $J = 7.6$, 250.0 Hz, C_{arom}), 155.96 (C4), 159.68 (C2), 163.96 (C6), 190.94 (C=O), EI-MS: $m/z = 293$ [M^+] (100%). Anal. calcd. for $C_{14}H_{13}F_2N_3O_2$ (293.27): C, 57.34; H, 4.47; N, 14.33. Found: C, 57.45; H, 4.38; N, 14.05.

*6-[(2,6-Bifluorophenyl)(hydroxy)methyl]-2-(dimethylamino)-5-methylpyrimidin-4(3H)-one **6***

Under ice cooling bath, sodium borohydride (84 mg, 2.2 mmol) was added portionwise to a stirred solution of compound **5** (0.5 g, 1.7 mmol) in methanol (15 mL). The reaction mixture was left to reach room temperature gradually with stirring for 1 h. Acetic acid (1 mL) was added to the reaction mixture followed by addition of water (40 mL). The crystallized product from the mixture of solvents was filtered off and dried to afford 550 mg (85% yield) of compound **6** as a white solid.

M.p. 212–214°C; ^{1}H NMR (DMSO-d_6) δ [ppm]: 1.67 (s, 3H, CH_3), 3.02 [s, 6H, $(CH_3)_2N$], 5.74 (d, 1H, $J = 5.4$ Hz, OH), 5.83 (d, 1H, $J = 5.4$ Hz, C*H*–OH), 7.04 (t, 1H, $J = 8.7$ Hz, H_{arom}), 7.32–7.43 (m, 1H, H_{arom}); ^{13}C NMR (DMSO-d_6) δ [ppm]: 8.57 (CH_3), 36.85 [$(CH_3)_2N$], 63.31 (CH–OH), 111.44–111.77 (m, C_{arom}), 118.07 (C5), 118.21 (t, $J = 16.8$ Hz, C_{arom}), 129.95 (t, $J = 10.5$ Hz, C_{arom}), 147.51 (C4), 160.59 (dd, $J = 8.6$, 248.5 Hz, C_{arom}), 160.35 (C2), 164.67 (C6); HRMS-MALDI: $m/z = 318.1025$ ($C_{14}H_{15}F_2NaN_3O_2$ [MNa^+]), requires 318.1013.

*6-[(2,6-Difluorophenyl)fluoromethyl]-2-(dimethylamino)-5-methylpyrimidin-4(3H)-one **7***

Under nitrogen, to a stirred solution of compound **6** (0.41 g, 1.4 mmol) in dry methylene chloride (5 mL) was added DAST (0.27 mL in 1 mL CH_2Cl_2, 2 mmol) dropwise at −5°C. The reaction mixture is allowed to reach room temperature gradually and stirred for 3 h. A 5% aq. sodium bicarbonate (0.5 mL) was added to the reaction mixture and the solvents were removed under reduced pressure, then water (20 mL) was added to the residual material. The solid product formed was filtered off, washed with water, and dried to give 374 mg (90% yield) of compound **7** as a white solid.

M.p. 162–164°C; ^{1}H NMR (DMSO-d_6) δ [ppm]: 1.99 (s, 3H, CH_3–C5), 2.85 [s, 6H, $(CH_3)_2N$], 6.80 (d, 1H, $J_{H,F} = 45.3$ Hz, CH–F),

7.13 (t, 2H, $J = 8.6$ Hz, H_{arom}), 7.44–7.54 (m, 1H, H_{arom}), 11.16 (bs, 1H, NH); ^{13}C NMR (DMSO-d_6) δ [ppm]: 9.17 (CH_3), 36.43 [(CH_3)N], 85.03 (d, $J = 174.7$ Hz, CH–F), 103.18 (d, $J = 7.2$ Hz, C5), 111.45–111.78 (m, C_{arom}), 131.38 (t, $J = 10.7$ Hz, C_{arom}), 158.62–162.12 (m, C_{arom}, C2, C4), 164.92 (C6). EI-MS: $m/z = 297$ [M^+] (100%). Anal. calcd. for $C_{14}H_{14}F_3N_3O$ (297.28): C, 56.56; H, 4.75; N, 14.14. Found: C, 56.97; H, 4.48; N, 13.90.

6-(2,6-Difluorobenzoyl)-2-(dimethylamino)-5-methylpyrimidin-4-yl dimethylsulfamate ***8***

Sodium hydride (610 mg, 14 mmol, 55% susp. in paraffin oil) was added portionwise to a stirred solution of compound **5** (2.93 g, 10 mmol) in dry *N*,*N*-dimethylformamide (30 mL) at room temperature and left to be stirred for 15 min. Dimethylsulafomyl chloride (1.3 mL, 12 mmol) was added dropwise to the reaction mixture and the stirring was continued for 3 h. The reaction mixture was poured on ice-cold water (20 mL) and the solid product formed was filtered off, dried, and purified by column chromatography of silica gel using petroleum ether/ether (5:1, v/v) as an eluent to afford 2.2 g (55%) of compound **8** as green crystals.

M.p. 110–112°C; ^{1}H NMR ($CDCl_3$) δ [ppm]: 2.31 (s, 3H, CH_3), 3.01 [s, 6H, $(CH_3)_2NSO_2$], 3.08 [s, 6H, $(CH_3)_2$N-C2], 6.93 (t, 2H, $J = 8.1$ Hz, H_{arom}), 7.36–7.46 (m, 1H, H_{arom}); ^{13}C NMR ($CDCl_3$) δ [ppm]: 9.67 (CH_3), 36.67 [($CH_3)_2$N-C2], 38.84 [$(CH_3)_2NSO_2$], 105.13 (C5), 111.29–111.63 (m, C_{arom}), 132.39 (t, $J = 10.3$ Hz, C_{arom}), 160.45 (dd, $J = 7.3$, 253.5 Hz, C_{arom}), 159.47 (C2), 160.98 (C4), 164.57 (C6), 189.85 (C=O); HRMS-MALDI: $m/z = 401.1090$ ($C_{16}H_{19}F_2N_4O_4S$ [MH^+]), requires 401.1071.

6-[(2,6-Difluorophenyl)(hydroxy)methyl]-2-(dimethylamino)-5-methylpyrimidin-4-yl dimethylsulfamate ***9***

Under ice cooling bath, sodium borohydride (150 mg, 4 mmol) was added portionwise to a stirred solution of compound **8** (1 g, 2.5 mmol) in methanol (15 mL). The reaction mixture was left to reach room temperature gradually with stirring for 1 h. Acetic acid (1 mL) was added followed by addition of water (40 mL). The solid product formed was filtered off and dried to afford 855 mg (85% yield) of compound **9** as a pure pale yellow solid.

M.p. 93–95°C; ^{1}H NMR ($CDCl_3$) δ [ppm]: 1.78 (s, 3H, CH_3), 3.03 [$(CH_3)_2NSO_2$], 3.23 [$(CH_3)_2$N], 5.85 (d, 1H, $J = 5.7$ Hz, OH), 5.98 (d, 1H, $J = 5.7$ Hz, CH–OH), 6.85 (t, 2H, $J = 8.3$ Hz, H_{arom}), 7.20–7.30 (m, 1H, H_{arom}); ^{13}C NMR ($CDCl_3$) δ [ppm]: 8.46 (CH_3), 37.12 [$(CH_3)_2$N-C2], 38.78 [$(CH_3)_2NSO_2$], 63.28 (t, $J = 3.3$ Hz, CH–OH), 102.01 (C5), 111.55–111.89 (m, C_{arom}), 116.96 (t, $J = 16.6$ Hz, C_{arom}), 130.09 (t, $J = 10.6$ Hz, C_{arom}), 158.49 (C2), 161.24 (dd, $J = 7.7$, 251.0 Hz, C_{arom}), 163.36 (C4), 167.86 (C6); EI-MS: $m/z = 402$ [M^+] (86%), 294 (100%); HRMS-MALDI: $m/z = 403.1246$ ($C_{16}H_{20}F_2N_4O_4S$ [MH^+]), requires 403.1257.

2-[6-Chloro-2-(dimethylamino)pyrimidin-4-yl]-2-(2,6-difluorophenyl)acetonitriles ***10a,b***

Sodium hydride (0.55 g, 12.5 mmol, 55% susp. in paraffin oil) was added portionwise to a stirred solution of compound **1** (5 mmol) and 2,6-difluorobenzyl cyanide (0.84 g, 5.5 mmol) in dry *N*,*N*-dimethylformamide (20 mL) at room temperature. The reaction mixture was stirred for 1 h, then poured on ice-cold water (100 mL). The solid product formed was filtered off, washed with water, and dried to afford **10a**,**b**.

2-[6-Chloro-2-(dimethylamino)pyrimidin-4-yl]-2-(2,6-difluorophenyl)acetonitrile ***10a***

Yield 98%; m.p. 93–95°C; ^{1}H NMR ($CDCl_3$) δ [ppm]: 3.12 [s, 6H, $(CH_3)_2$N], 5.40 (s, 1H, CH-CN), 6.58 (s, 1H, H5), 6.98 (t, 2H, $J = 8.1$ Hz, H_{arom}), 7.33–7.43 (m, 1H, H_{arom}); ^{13}C NMR ($CDCl_3$) δ [ppm]: 36.88 [$(CH_3)_2$N], 104.82 (C5), 111.78–112.11 (m, C_{arom}), 115.80 (CN), 131.18 (t, $J = 10.3$ Hz, C_{arom}), 160.61 (dd, $J = 6.6$, 252.2 Hz, C_{arom}), 161.56 (C4), 162.15 (C2), 162.92 (C6); HRMS-MALDI: $m/z = 309.0713$ ($C_{14}H_{12}ClF_2N_4$ [MH^+]), requires 309.0718.

2-(6-Chloro-2-(dimethylamino)-5-methylpyrimidin-4-yl)-2-(2,6-difluorophenyl)acetonitrile ***10b***

Yield 97%; m.p. 120–122°C. Lit. 120–122°C [18].

4-Aroyl (and/or arylmethyl)-6-methoxy-N,N,dimethylpyrimidin-2-amines ***11a–c***

Compound **10b** (1.6 g, 5 mmol) was refluxed in a solution of 30% aq NaOH (8 mL) and methanol (20 mL) for 30 h. The reaction mixture was cooled to room temperature and the precipitate was filtered off, dried, and chromatographed on a column of silica gel using petroleum ether/ether (5:1, v/v) as an eluent to give two separated compounds **11a**,**b**. Water (50 mL) was added to the filtrate with stirring and the solid product formed was filtered off, washed with water, and dried to afford compound **11c**.

[2-(Dimethylamino)-6-methoxy-5-methylpyrimidin-4-yl]-(2-fluoro-6-methoxyphenyl)methanone ***11a***

Yield 13%; as a white solid; m.p. 108–110°C; ^{1}H NMR ($CDCl_3$) δ [ppm]: 2.26 (s, 3H, CH_3), 2.97 [s, 6H, $(CH_3)_2$N], 3.73 (s, 3H, OCH_3), 3.95 (s, 3H, OCH_3), 6.67–6.73 (m, 2H, H_{arom}), 7.26–7.34 (m, 1H, H_{arom}); ^{13}C NMR ($CDCl_3$) δ [ppm]: 9.47 (CH_3), 36.45 [$(CH_3)_2$N)], 53.54 (OCH_3), 56.24 (OCH_3), 104.04 (C5), 106.68 (d, $J = 3$ Hz, C_{arom}), 107.83 (d, $J = 22$ Hz, C_{arom}), 131.08 (d, $J = 10.3$ Hz, C_{arom}), 158.44, (d, $J = 7.8$ Hz, C_{arom}), 158.66 (C_{arom}) 159.74 (C4), 161.96 (C2), 169.42 (C6), 194.18 (C=O); HRMS-MALDI: $m/z = 342.1224$ ($C_{16}H_{18}FNaN_3O_3$ [MNa^+]), requires 342.1220.

(2,6-Dimethoxyphenyl)[2-(dimethylamino)-6-methoxy-5-methylpyrimidin-4-yl]methanone ***11b***

Yield 6%; as a white solid; m.p. 134–136°C; ^{1}H NMR ($CDCl_3$) δ [ppm]: 2.25 (s, 3H, CH_3), 2.96 [s, 6H, $(CH_3)_2$N)], 3.71 (s, 6H, 2OCH_3), 3.94 (s, 3H, OCH_3), 6.55 (d, 2H, $J = 8.7$ Hz, H_{arom}), 7.27 (t, 1H, $J = 8.7$ Hz, H_{arom}); ^{13}C NMR ($CDCl_3$) δ [ppm]: 9.45 (CH_3), 36.43 [$(CH_3)_2$N], 53.42 (OCH_3), 56.02 (2xOCH_3), 103.61 (C4), 104.04, 130.54, 157.96 (C_{arom}), 159.68 (C4), 159.73 (C2), 169.32 (C6), 197.16 (C=O); HRMS-MALDI: $m/z = 354.1424$ ($C_{17}H_{21}NaN_3O_4$ [MNa^+]), requires 354.1424.

*4-(2,6-Difluorobenzyl)-6-methoxy-N,N,5-trimethylpyrimidin-2-amine **11c***

Yield 14%; as a white solid; m.p. 75–77°C; ^{1}H NMR (DMSO-d_6) δ [ppm]: 2.07 (s, 3H, CH_3), 2.95 [s, 6H, $(CH_3)_2N$], 3.88 (s, 3H, OCH_3), 3.94 (s, 2H, CH_2Ar), 6.84 (t, 2H, $J = 7.8$ Hz, H_{arom}), 7.09–7.19 (s, 1H, H_{arom}); ^{13}C NMR (DMSO-d_6) δ [ppm]: 9.31 (CH_3), 27.34 (CH_2Ar), 36.35 [$(CH_3)_2N$], 53.05 (OCH_3), 101.02 (C5), 110.37–110.72 (m, C_{arom}), 115.24 (t, $J = 20.2$ Hz, C_{arom}) 127.49 (t, $J = 10.2$ Hz, C_{arom}), 159.92 (C4), 161.84 (dd, $J = 8.9$, 246.7 Hz, C_{arom}), 163.76 (C2), 168.03 (C6); HRMS-MALDI: $m/z = 294.1413$ ($C_{15}H_{18}F_2N_3O$ [MH^+]), requires 294.1403. Anal. calcd. for $C_{15}H_{17}F_2N_3O$ (293.31): C, 61.42; H, 5.84; N, 14.33. Found: C, 60.30; H, 5.78; N, 13.70.

*6-Aroyl (and/or arylmethyl)-2-(dimethylamino)-5-methylpyrimidin-4(3H)-ones **12a–c***

Each of compound **11a–c** (0.5 mmol) was refluxed in 4 M hydrochloric acid (10 mL) for 16 h, the reaction mixture was cooled and the solvent was evaporated under reduced pressure. The residual material was dissolved in water (10 mL) and neutralized with 10% aq. sodium bicarbonate. The solid product formed was filtered off, washed with water, and dried to afford **12a–c**.

*2-(Dimethylamino)-6-(2-fluoro-6-methoxybenzoyl)-5-methylpyrimidin-4(3H)-one **12a***

Yield 80%; as a white solid; m.p. 202–204°C; ^{1}H NMR (DMSO-d_6) δ [ppm]: 2.04 (s, 3H, CH_3), 2.84 [s, 6H, $(CH_3)_2N$], 3.72 (s, 3H, OCH_3), 6.83–6.97 (m, 2H, H_{arom}), 7.40–7.48 (m, 1H, H_{arom}), 11.33 (bs, 1H, NH); ^{13}C NMR (DMSO-d_6) δ [ppm]: 9.79 (CH_3), 36.51 [$(CH_3)_2N$], 56.29 (OCH_3), 107.42 (d, $J = 17.7$ Hz, C_{arom}), 107.56 (d, $J = 6.5$ Hz, C_{arom}), 107.55 (C5), 131.70 (d, $J = 10.3$ Hz, C_{arom}), 157.44 (C4), 157.74 (d, $J = 8.0$ Hz, C_{arom}), 160.69 (C2), 165.31 (C6), 193.95 (C=O); HRMS-MALDI: $m/z = 328.1068$ ($C_{15}H_{16}FNaN_3O_3$ [MNa^+]), requires 328.1060.

*6-(2,6-Dimethoxybenzoyl)-2-(dimethylamino)-5-methylpyrimidin-4(3H)-one **12b***

Yield 83%; as a white solid; m.p. 250–252°C; ^{1}H NMR (DMSO-d_6) δ [ppm]: 2.01 (s, 3H, CH_3), 2.82 [s, 6H, $(CH_3)_2N$], 3.67 (s, 6H, $2OCH_3$), 6.66 (d, 2H, $J = 8.2$ Hz, H_{arom}), 7.31 (t, 1H, $J = 8.2$ Hz, H_{arom}), 11.23 (bs, 1H, NH); ^{13}C NMR (DMSO-d_6) δ [ppm]: 9.81 (CH_3), 36.44 [$(CH_3)_2N$], 55.77 ($2\times OCH_3$), 104.09, 130.60, 157.01, 119.17 (C_{arom}), 104.13 (C5), 157.02 (C4), 162.23 (C2), 165.64 (C6), 196.84 (C=O). EI-MS: $m/z = 317$ [M^+] (68%), 286 (100%). Anal. calcd. for $C_{16}H_{19}N_3O_4 \cdot 0.7H_2O$ (329.96): C, 58.24; H, 6.23; N, 12.73. Found: C, 58.13; H, 5.69; N, 12.44.

*6-(2,6-Difluorobenzyl)-2-(dimethylamino)-5-methylpyrimidin-4(3H)-one **12c***

Yield 84%; as a white solid; m.p. 208–210°C; Lit. [18] 208–210°C.

*6-[Bromo(2,6-difluorophenyl)methyl]-2-(dimethylamino)-5-methylpyrimidin-4(3H)-one **13***

Few crystals of benzoyl peroxide were added to a stirred solution of compound **12c** (200 mg, 0.66 mmol) and *N*-bromosuccinimide (130 mg, 0.73 mmol) in ethanol (10 mL) at room temperature. The reaction mixture was stirred for 1 h. During this time, it changed from a yellow suspension to clear a yellow solution and then to a colorless suspension. The crystallized product from ethanol was filtered off, washed with ethanol (2 mL), and dried to afford 165 mg (70% yield) of compound **13** as a white solid.

M.p. 194–196°C; ^{1}H NMR (DMSO-d_6) δ [ppm]: 1.95 (s, 3H, CH_3–C5), 2.93 [s, 6H, $(CH_3)_2N$], 6.43 (s, 1H, CH), 7.12 (t, 2H, $J = 8.9$ Hz, H_{arom}), 7.41–7.51 (m, 1H, H_{arom}), 11.13 (bs, 1H, NH); ^{13}C NMR (DMSO-d_6) δ [ppm]: 9.62 (CH_3–C5), 36.53 [$(CH_3)_2N$], 38.60 (CH), 105.19 (C5), 111.52–111.86 (m, C_{arom}), 114.70 (t, $J = 15.5$ Hz, C_{arom}), 130.96 (t, $J = 10.9$ Hz, C_{arom}), 157.50 (C4), 160.19 (dd, $J = 6.7$, 250.9 Hz, C_{arom}), 162.63 (C2), 164.71 (C6); EI-MS: $m/z = 257$ [M^+, ^{79}Br] (82%), 258 [M^++1, ^{79}Br] (14%), 359 [M^+, ^{81}Br] (77%), 278 (100%). Anal. calcd. for $C_{14}H_{14}BrF_2N_3O$ (358.18): C, 46.95; H, 3.94; N, 11.73. Found: C, 46.95; H, 3.78; N, 11.48.

*4,6-Dichloro-N-(4-iodobenzyl)-N,5-dimethylpyrimidin-2-amine **15***

Sodium hydride (0.26 g, 6 mmol, 55% susp. in paraffin oil) was added portionwise to a stirred solution of compound **14** (0.77 g, 4 mmol) and 4-iodobenzyl bromide (1.2 g, 4 mmol) in dry *N*,*N*-dimethylformamide (15 mL) at room temperature. The reaction mixture was stirred for 1 h then poured on ice-cold water (100 mL) and the solid product formed was filtered off, washed with water, and dried to afford 1.6 g (98% yield) of compound **15** as a white solid.

M.p. 100–102°C; ^{1}H NMR ($CDCl_3$) δ [ppm]: 2.28 (s, 3H, CH_3–C5), 3.07 (s, 3H, CH_3–N), 4.75 (s, 2H, CH_2–N), 6.99 (d, 2H, $J = 8.1$ Hz, H_{arom}), 7.63 (d, 2H, $J = 8.1$, H_{arom}); ^{13}C NMR ($CDCl_3$) δ [ppm]: 15.05 (CH_3–C5), 34.91 (CH_3–N), 52.07 (CH_2–N), 114.25 (C5), 92.68, 129.61, 137.15, 137.61 (C_{arom}), 161.54 (C2), 165.69 (C4 and C6); HRMS-MALDI: $m/z = 407.9526$ ($C_{13}H_{13}Cl_2IN_3$ [MH^+]), found 407.9534.

*2-{6-Chloro-2-[(4-iodobenzyl)(methyl) amino]-5-methylpyrimidin-4-yl}-2-(2,6-difluorophenyl)acetonitrile **16***

Sodium hydride (0.38 g, 9 mmol, 55% susp. in paraffin oil) was added portionwise to a stirred solution of compound **15** (1.42 g, 3.5 mmol) and 2,6-difluorobenzyl cyanide (0.612 g, 4 mmol) in dry *N*,*N*-dimethylformamide (15 mL) at room temperature. The reaction mixture was stirred for 1 h, and then poured on ice-cold water (100 mL). The solid product formed was filtered off, washed with water, and dried to afford 2.08 g (99% yield) of compound **16** as a white solid.

M.p. 108–110°C; ^{1}H NMR ($CDCl_3$) δ [ppm]: 2.23 (s, 3H, CH_3–C5), 3.04 (s, 3H, CH_3–N), 4.52 (bs, 1H, *H*CH–N), 4.78 (d, 1H, $J = 15$ Hz, HC*H*–N), 5.54 (s, 2H, CH–CN), 6.90 (bs, 4H, H_{arom}), 7.28–7.33 (m, 1H, H_{arom}), 7.57 (d, 2H, $J = 7.8$ Hz, H_{arom}); ^{13}C NMR ($CDCl_3$) δ [ppm]: 13.31 (CH_3–C5), 32.07 (CH–CN), 34.71 (CH_3–N), 51.99 (CH_2–N), 113.19 (C5), 115.57 (CN), 92.52, 129.65, 137.33, 137.44 (C_{arom}), 109.56 (t, $J = 16.5$ Hz, C_{arom}), 111.63–111.96 (m, C_{arom}), 131.02 (t, $J = 10.5$ Hz, C_{arom}), 160.65 (dd, $J = 6.6$, 252.1 Hz, C_{arom}), 159.25 (C2), 159.83 (C4), 162.67

(C6); HRMS-MALDI: $m/z = 525.0149$ ($C_{21}H_{17}ClF_2IN_4$ [MH^+]), requires 525.0141.

6-(2,6-Difluorobenzyl)-2-[(4-iodobenzyl)(methyl)amino]-5-methylpyrimidin-4(3H)-one ***17***

Compound **16** (240 mg, 0.5 mmol) was refluxed in a solution of 7 M hydrochloric acid (15 mL) and acetic acid (5 mL) for 50 h. The reaction mixture was cooled to room temperature and the solvents were removed under reduced pressure. Water (10 mL) was added to the residual material and neutralized with 5% aq. sodium bicarbonate solution. The solid product formed was filtered off, washed with water, and dried to afford 29 mg (12% yield) of compound **17** as a white solid.

M.p. 208–210°C; ^{1}H NMR (DMSO-d_6) δ [ppm]: 1.96 (s, 3H, CH_3–C5), 2.84 (s, 3H, CH_3–N), 3.81 (s, 2H, CH_2–C6), 4.42 (s, 2H, CH_2–N), 6.79 (d, 2H, $J = 8.3$ Hz, H_{arom}), 6.99 (t, 2H, $J = 7.8$ Hz, H_{arom}), 7.22–7.32 (m, 1H, H_{arom}), 7.56 (d, $J = 8.3$ Hz, H_{arom}), 10.99 (bs, 1H, NH); ^{13}C NMR (DMSO-d_6) δ [ppm]: 9.66 (CH_3–C5), 26.97 (CH_2Ar), 34.32 (CH_3–N), 50.85 (CH_2–N), 104.59 (C5), 72.74, 129.90, 136.85 (C_{arom}), 110.65–110.99 (m, C_{arom}), 114.48 (t, $J = 20.1$ Hz, C_{arom}), 128.45 (t, $J = 10.3$ Hz, C_{arom}), 161.05 (dd, $J = 9.7$, 244.8 Hz, C_{arom}), 159.99 (C4), 160.70 (C2), 164.07 (C6). HRMS-MALDI: $m/z = 482.0535$ ($C_{20}H_{19}F_2IN_3O$ [MH^+]), requires 482.0544.

Antiviral assay procedures

Compound preparation

Compounds were dissolved in DMSO at 100 μM and then diluted in culture medium.

Cells and viruses

MT-4, C8166, and H9/IIIB cells were grown at 37°C in a 5% CO_2 atmosphere in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 100 IU/mL penicillin G, and 100 mg/mL streptomycin. Cell cultures were checked periodically for the absence of mycoplasma contamination with a MycoTect Kit (Gibco). Human immunodeficiency viruses type 1 (HIV-1, IIIB strain) was obtained from supernatants of persistently infected H9/IIIB cells. The HIV-1 stock solutions had titers of 4.5×10^6 50% cell culture infectious dose ($CCID_{50}$)/mL. The K103R + V179D + P225H mutant (EFVR) was derived from an IIIB strain passage in C8166 cells in the presence of efavirenz (up to 2 μM). The Y181C mutant (NIH N119) was derived from an AZT-sensitive clinical isolate passage initially in CEM and then in MT-4 cells in the presence of nevirapine (10 μM). The K103N Y181C (NIH A17) was derived from the IIIB strain passaged in H9 cells in the presence of BI-RG 587 (1 μM). K103R + V179D P225H (EFVR), Y181C, and K103N Y181C stock solutions had titers of 3.0×10^5, 1.3×10^6, and 2.5×10^5 $CCID_{50}$/mL, respectively.

HIV titration

Titration of HIV was performed in C8166 cells by the standard limiting dilution method (dilution 1:2, four replica wells per dilution) in 96-well plates. The infectious virus titer was determined by light microscope scoring of syncytia after 4 days of incubation. Virus titers were expressed as $CCID_{50}$/mL.

Anti-HIV assays

The activity of test compounds against multiplication of wt HIV-1, Y181C, and K103N Y181C in acutely infected cells was based on inhibition of virus-induced cytopathicity in MT-4 cells. The activity of the compounds against the EFVR multiplication in acutely infected cells was based on inhibition of p24 antigen in C8166 cells. Briefly, an amount of 50 mL of culture medium containing 1×10^4 cells was added to each well of flat-bottom microtiter trays containing 50 mL of culture medium with or without various concentrations of test compounds. Then an amount of 20 mL of HIV suspensions (containing the appropriate amount of $CCID_{50}$ to cause complete cytopathicity at day 4) was added. After incubation at 37°C, cell viability was determined by the 3-(4,5-dimethylthiazol-1-yl)-2,5-diphenyltetrazolium bromide (MTT) method [26]. Alternatively, p24 levels were determined by an immunoenzymatic kit (Abbott). The cytotoxicity of test compounds was evaluated in parallel with their antiviral activity and was based on the viability of mock-infected cells, as monitored by the MTT method.

This work received funding from the European Community's Sixth Framework Programme under contract number LSHP-CT-2004-503162 (Selection and development of microbicides for mucosal use to prevent sexual HIV transmission/acquisition).

The authors have declared no conflict of interest.

References

[1] A. Basu, S. Basu, M. J. Modak, *Biochem. Biophys. Res. Comm*. **1992**, *183*, 1131–1138.
[2] H. Mitsuya, S. Broder, *Proc. Natl. Acad. Sci. U. S. A*. **1986**, *83*, 1911–1915.
[3] E. De Clercq, *Antiviral Res*. **1998**, *38*, 153–179.
[4] M. Yu, E. Fan, J. Wu, X. Liu, *Curr. Med. Chem*. **2011**, *18*, 2376–2385.
[5] M. Artico, S. Massa, A. Mai, M. E. Marongiu, G. Piras, E. Tramontano, P. La Colla, *Antiviral Chem. Chemother*. **1993**, *4*, 361–368.
[6] E. Tramontano, M. E. Marongiu, A. De Montis, A. G. Loi, M. Artico, S. Massa, A. Mai, P. La Colla, *New Microbiol*. **1994**, *17*, 269–279.
[7] S. Massa, A. Mai, M. Artico, G. Sbardella, E. Tramontano, A. G. Loi, P. Scano, P. La Colla, *Antivir. Chem. Chemother*. **1995**, *6*, 1–8.
[8] G. Sbardella, A. Mai, M. Artico, P. Chimenti, S. Massa, R. Loddo, M. E. Marongiu, P. La Colla, A. Pani, *Antivir. Chem. Chemother*. **2001**, *12*, 37–50.
[9] M. Artico, *Drugs Future* **2002**, *27*, 159–175.

[10] A. Pani, A. G. Loi, M. Mura, T. Marceddu, P. La Colla, M. E. Marongiu, *Curr. Drug Targets Infect. Disord*. **2002**, *2*, 17–32.
[11] A. Mai, M. Artico, R. Ragno, G. Sbardella, P. Turini, O. Befani, V. L. Dalla, E. Agostinelli, *Bioorg. Med. Chem*. **2005**, *13*, 2065–2077.
[12] A. Mai, M. Artico, D. Rotili, D. Tarantino, I. Clotet-Codina, M. Armand-Ugon, R. Ragno, S. Simeoni, G. Sbardella, M. B. Nawrozkij, A. Samuele, G. Maga, J. A. Este, *J. Med. Chem*. **2007**, *50*, 5412–5424.
[13] S. Bartolini, A. Mai, M. Artico, N. Paesano, D. Rotili, C. Spadafora, G. Sbardella, *J. Med. Chem*. **2005**, *48*, 6776–6778.
[14] M. Caron, G. Besson, S. L.-D. Etenna, A. Mintsa-Ndong, S. Mourtas, A. Radaelli, C. Morghen, R. Loddo, P. La Colla, S. G. Antimisiaris, M. Kazanji, *Virology* **2010**, *405*, 225–233.
[15] N. Stolte-Leeb, R. Loddo, S. Antimisiaris, T. Schultheiss, U. Sauermann, M. Franz, S. Mourtas, C. Parsy, R. Storer, P. La Colla, C. Stahl-Hennig, *Aids Res. Human Retrovir*. **2011**, *27*, 933–943.
[16] S. M. Fetherston, L. Geer, R. S. Veazey, L. Goldman, D. J. Murphy, T. J. Ketas, P. J. Klasse, S. Blois, P. La Colla, J. P. Moore, R. K. Malcolm, *J. Antimicrob. Chemother*. **2013**, *68*, 394–403.
[17] M. Radi, L. Contemori, D. Castagnolo, R. Spinosa, J. A. Este, S. Massa, M. Botta, *Org. Lett*. **2007**, *9*, 3157–3160.
[18] Y. M. Loksha, D. Globisch, R. Loddo, G. Collu, P. La Colla, E. B. Pedersen, *Chem. Med. Chem*. **2010**, *5*, 1847–1849.
[19] Y. M. Loksha, E. B. Pedersen, P. La Colla, R. Loddo, *Org. Biomol. Chem*. **2016**, *14*, 940–946.
[20] W. R. Boon, *J. Chem. Soc*. **1952**, 1532.
[21] W. Hofer, F. Maurer, H. J. Riebel, I. Hammann, W. Behrenz, DE 2620089, **1977**.
[22] W. R. Boon, *J. Chem. Soc*. **1957**, 2146–2158.
[23] Y. M. Loksha, E. B. Pedersen, P. La Colla, A. D. Bond, B. Secci, R. Loddo, G. Collu, *Synthesis* **2009**, 3589–3592.
[24] P. La Colla, M. Artico, PCT Int. Appl. **2000**, WO 2000003998.
[25] C. Guérémy, F. Audiau, A. Uzan, G. Le Fur, J.-M. Léger, A. Carpy, *J. Med. Chem*. **1982**, *25*, 1459–1465.
[26] R. Pawels, J. Balzarini, M. Baba, R. Snoeck, D. Schols, P. Herdewijn, J. Desmyster, E. De Clercq, *J. Virol. Methods* **1988**, *20*, 309–321.