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Synthesis and biological evaluation of novel 2-(substituted phenylamino-carbonylmethylthio)-6-(2,6-dichlorobenzyl)-pyrimidin-4(3*H*)-ones as potent HIV-1 NNRTIs

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ABSTRACT

A series of novel 2-(phenylaminocarbonylmethylthio)-6-(2,6-dichlorobenzyl)-pyrimidin-4(3*H*)-ones have been designed and synthesized. All of the new compounds were evaluated for their anti-HIV activities in MT-4 cells. Most of these new compounds showed moderate to potent activities against wild-type HIV-1 with an EC_{50} ranging from 4.48 μM to 0.18 μM. Among them, 2-[(4-bromophenylamino)carbonylmethyl-thio]-6-(2,6-dichlorobenzyl)-5-methylpyrimidin-4(3*H*)-one **4b3** was identified as the most promising compound (EC_{50} = 0.18 ± 0.06 μM, CC_{50} >243.56 μM, SI >1326). The structure–activity relationship (SAR) of these new congeners is discussed.

1. Introduction

The current therapy against the human immunodeficiency virus type 1 (HIV-1), which is the etiological agent of acquired immunodeficiency syndrome (AIDS), is based on six of categories drugs: nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs/NtRTIs); non-nucleoside reverse transcriptase inhibitors (NNRTIs); protease inhibitors (PIs); cell entry inhibitors [fusion inhibitors (FIs) and co-receptor inhibitors (CRIs)]; and integrase inhibitors (INIs).[1] Among them, the HIV-1 NNRTIs serve as a representative of most frontline AIDS combination therapies which are highly effective with less side effects.

NNRTIs are highly specific for HIV-1 and include more than 50 structurally different classes of molecules. Among NNRTIs, dihydroalkyloxybenzyloxopyrimidines (DABOs) are an interesting class of compounds active at nanomolar concentrations.[2,3] They were first disclosed in 1992[4,5] and further developed into three generations during the following years: dihydroalkyloxybenzyl-oxopyrimidines (*O*-DABOs); dihydroalkylthiobenzyloxopyrimidines (*S*-DABOs); dihydroalkylaminodifluorobenzyloxopyrimidines (*N*-DABOs)[6–9] (Fig. 1).

Compared to *O*-DABOs, the *S*-DABOs were more potent and selective, with the *C*-2 alkylthio chain being the most peculiar determinant for exhibition of anti-HIV-1 activity. 6-(2,6-Dichlorobenzyl) substituent of *S*-DABOs have been suggested to exert their favorable effect on anti-HIV-1 activity by enhancement of the putative charge-transfer interactions between the π-stacking aromatic rings of the inhibitor and Y188 and Y181 in HIV-1 RT.[10–15] On the basis of these results, we kept the 6-(2,6-dichlorobenzyl) substituent fixed and systematically modified the *C*-2 position by increasing the length of the linker connecting the *S* atom to the phenyl ring. A new series of *S*-DABOs (Fig. 1) was prepared, by the insertion of a phenylaminocarbonylmethylthio chain at *C*-2, and hydrogen or methyl substituents at the *C*-5 position of the pyrimidine ring. We proposed that the introduction of the *C*-2 side chain of *S*-DABOs might enhance the interaction between the inhibitors and the RT, and further exploration of the structure–activity relationships of *S*-DABOs may yield the discovery of new more potent HIV-1 inhibitors. Herein, the detailed synthesis, anti-HIV activity and preliminary SAR studies of these new congeners are described.

2. Results and discussion

2.1. Chemistry

The target compounds **4a1–4a9, 4b1–4b8** were prepared as depicted in Scheme 1. The β-ketoesters (**2a**, **2b**) were prepared by a simple and high-yielding method of Clay et al.[16] by the reaction of

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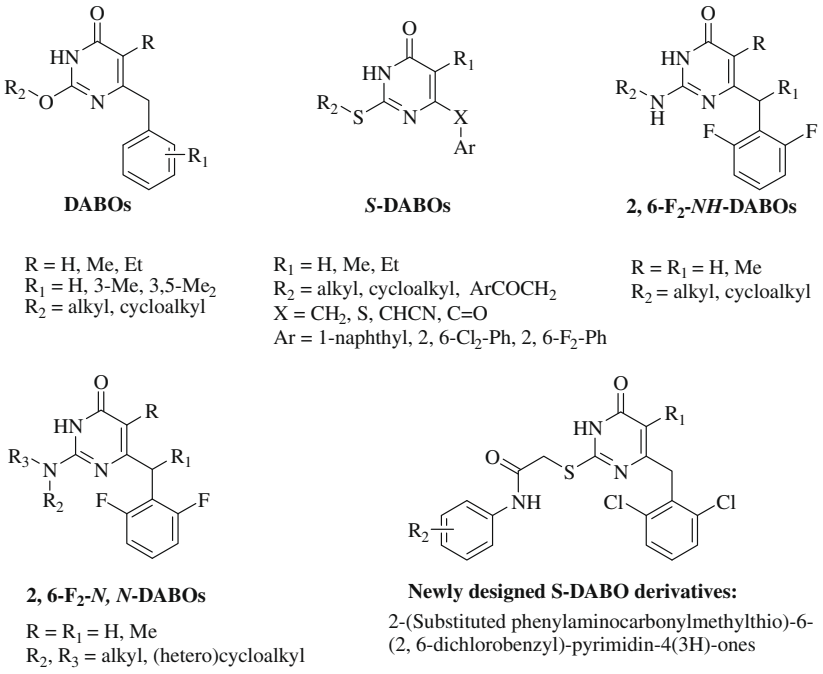
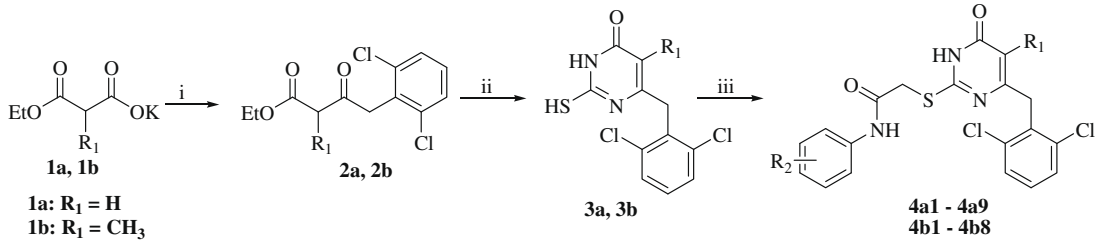


Figure 1. The DABOs family.



Scheme 1. Reagents and conditions: (i) (a) $MgCl_2$, Et_3N, CH_3CN, rt, 2 h; (b) 2-(2,6-dichlorophenyl)acetic acid, *N*,*N*′-carbonyldiimidazole, rt, overnight then reflux, 2 h; (ii) thiourea, EtONa, reflux, 6–12 h; (iii) appropriate *N*-phenylacetamide halides, K_2CO_3, DMF, rt, 12 h.

2-(2,6-dichlorophenyl)acetic acid with *N*,*N*′-carbonyldiimidazole (CDI) followed by treatment with potassium ethyl malonate or 2-methylmalonate in the presence of anhydrous magnesium chloride and triethylamine, which were sufficiently pure to be used for subsequent reactions without further purification. Subsequent condensation of β-ketoesters (**2a, 2b**) with thiourea in the presence of EtONa in refluxing ethanol gave the key intermediates 5-alkyl-6-substituted thiouracil **3a, 3b**.[17] Next, selective S-alkylation of **3a**, **3b** with the appropriate *N*-phenylacetamide halides (1:1.1) in the presence of K_2CO_3 in anhydrous DMF afforded the desirable target compounds **4a1–4a9, 4b1–4b8**. Both analytical and spectral data of all the compounds are in full agreement with the proposed structures.

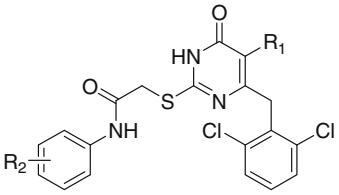
2.2. Anti-HIV evaluation

The activity and cytotoxicity of the newly designed and synthesized *S*-DABO analogues (compounds **4a1–4a9, 4b1–4b8**) were tested in MT-4 cells for their ability to inhibit HIV-1- and HIV-2-induced cytopathogenicities. The results, expressed as EC_{50}, CC_{50} and SI (selectivity index), are illustrated in Table 1. Nevirapine (NVP), delaviridine (DLV), efavirenz (EFV) and zidovudine (azidothymidine, AZT) were used as the reference drugs.

The majority of these compounds exhibited moderate to good activities against HIV-1 with EC_{50} values in the range of 4.48–0.18 μM. The most active and selectivity *S*-DABO derivative was compound **4b3** with an EC_{50} value of 0.18 ± 0.06 μM, and a CC_{50} value of >243.56 μM. The SI value was greater than 1326, which is much better than for the reference drugs of NVP and DLV. Some compounds, **4b2 and 4b6**, also had high anti-HIV-1 potency (EC_{50} = 0.32 and 0.69 μM, respectively) and good selectivity indices (SI >836 and >387), while compounds**4a7, 4a8, 4a9, 4b7, 4b8** were totally inactive.

The SARs analysis showed that the nature of the substituents on the *C*-2 side chain phenyl ring influenced the antiviral activity of these *S*-DABOs. The monosubstituted anilide analogues (**4a1–4a5, 4b1–4b4**) were more active than the disubstituted ones (**4a6–4a9, 4b5–4b8**). Disubstitutions at the *meta*-position and *para*-position of the anilide (**4a7, 4a8, 4a9, 4b7, 4b8**) led to compounds devoid of anti-HIV-1 activity, which might reflect a spatial restriction in the target site of the HIV-1 enzyme. This feature can be used in the design of new *S*-DABOs. Moreover, it was found that the nature of the substituents at the *C*-5 position of the pyrimidine ring also influenced the anti-HIV-1 activity of these new congeners. When the *C*-5 was substituted by a methyl group (series **4b**), a marked increase of anti-HIV-1 activity was observed for all the title compounds except for **6a1** and **6b1**. The active sequence of the

Table 1
Anti-HIV activities, cytotoxicities and selectivity indices of 2-(substituted phenylaminocarbonylmethylthio)-6-(2,6-dichlorobenzyl)-pyrimidin-4(3*H*)-ones (series **4a1–4a9**, **4b1–4b8**)



Compound	R_1	R_2	EC_{50}[a] (μM)		CC_{50}[b] (μM)	SI[c] (III_B)
			HIV-1 III_B	HIV-2 ROD		
4a1	H	4-OCH_3	0.92 ± 0.16	>25.21	25.52 ± 2.75	28
4a2	H	4-Cl	0.95 ± 0.11	>24.80	24.80 ± 2.35	23
4a3	H	4-Br	0.74 ± 0.08	>22.84	22.84 ± 1.96	31
4a4	H	4-CH_3	0.88 ± 0.28	>47.43	>or = 47.43	>or = 54
4a5	H	4-NO_2	2.07 ± 1.29	>268.64	>268.64	>130
4a6	H	3-OCH_3, 4-OCH_3	4.48 ± 1.62	>165.67	165.67 ± 11.22	28
4a7	H	3-F, 4-F	>273.95	>273.95	>273.95	X1
4a8	H	3-Cl, 4-Cl	>or = 84.63	>19.58	19.59 ± 1.23	
4a9	H	3-Cl, 4-F	>264.41	>264.41	>264.41	<1
4b1	$-CH_3$	4-OCH_3	2.74 ± 3.00	>10.84	10.84 ± 7.00	4
4b2	$-CH_3$	4-Cl	0.32 ± 0.11	>266.65	>266.65	>836
4b3	$-CH_3$	4-Br	0.18 ± 0.06	>243.56	>243.56	>1326
4b4	$-CH_3$	4-NO_2	0.99 ± 0.27	⩾45.80	>261.49	>264
4b5	$-CH_3$	3-OCH_3, 4-OCH_3	1.45 ± 1.13	>252.84	>252.84	>175
4b6	$-CH_3$	3-F, 4-F	0.69 ± 0.02	>265.78	>265.78	>387
4b7	$-CH_3$	3-Cl, 4-Cl	⩾2.82	>248.40	>248.40	>or X88
4b8	$-CH_3$	3-Cl, 4-F	⩾41.09	>256.79	>256.79	X1
NVP[d]			0.21		>15.02	>72
DLV[d]			0.32		>3.83	>12
EFV[d]			0.0044		>6.34	>1434
AZT[d]			0.015		>93.55	>6192

[a] EC_{50}: concentration of compound required to achieve 50% protection of MT-4 cells against HIV-induced cytotoxicity, as determined by the MTT method.
[b] CC_{50}: concentration required to reduce the viability of mock-infected cells by 50%, as determined by the MTT method.
[c] SI: selectivity index (CC_{50}/ EC_{50}). The SI values: X1 stand for ⩾1 or <1.
[d] The antiviral properties of these compounds were previously described.[18]

substituents at the anilide moiety was as follows: 4-Br > 4-Cl > 3-F, 4-F > 4-NO_2 > 3-OCH_3, 4-OCH_3 > 4-OCH_3.

In addition, all the title compounds were also evaluated for their capability to inhibit the HIV-2 (strain ROD) replication in MT-4 cells, but none was found effective (Table 1). These findings showed that this new series of *S*-DABOs was specific for HIV-1 and belonged to typical NNRTIs.

2.3. Molecular modeling analysis

With the aim to investigate the binding mode of our newly synthesized compounds, molecular modeling study was performed by means of Autodock Vina for docking. Compound **4b3** was chosen to be docked into the NNRTIs binding pocket (NNIBP) of HIV-1 RT. Three-dimensional coordinates of the HIV-1 RT/6-benzyl-1-(benzyloxymethyl)-5-isopropylpyrimidine-2,4(1*H*,3*H*)-dione (TNK -651) complex (Brookhaven Protein Data Bank entry 1RT2) were used as the input structure for docking calculations because of the high degree of similarity between TNK-651 and DABOs.[19] Default parameters were used as described in the Autodock Vina manual unless otherwise specified. The theoretical binding mode of **4b3** to the NNIBP is shown in Figure 2.

The docking simulation showed the binding mode of the **4b3** into the NNIBP. Results showed that the pyrimidine *NH* moiety at position 3 was engaged in a hydrogen bond with the *C*═*O* moiety of Lys101. The 2-phenylaminocarbonylmethylthio substituent was well accommodated in the large pocket mainly defined by Val106, Pro225, Pro236, and Phe227. A hydrogen bond is formed between the *C*═*O* group of the *C*-2 side chain and *NH* group of Lys103 backbone. Lengthening the *C*-2 side chain led the *C*-2 side chain to go beyond the opening defined by Pro225 and Pro236. Thus, the end of *C*-2 side chain was exposed to the solvent, causing the lack of its hydrophobic interactions with the NNIBP. The 2,6-

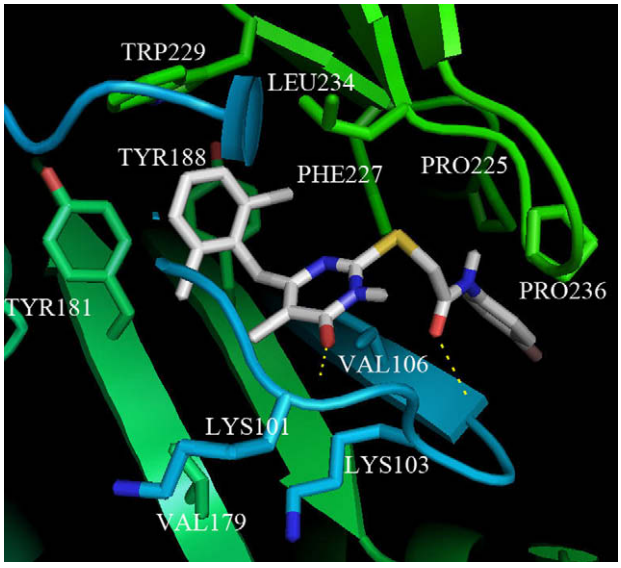


Figure 2. Model of **4b3** docked into the RT non-nucleoside binding site (PDB code: 1RT2) using Autodock Vina [http://vina.scripps.edu]. The docking result of **4b3** is showed by PyMOL [http://pymol.sourceforge.net].

dichlorobenzyl substituent at position 6 of the pyrimidinone ring accommodated in a hydrophobic pocket mainly defined by the aromatic side chains of Tyr181, Tyr188, Phe227, and Trp229 as well as by Leu234. In particular, the phenyl ring interacts favorably with the Tyr188 side chain, giving rise to a positive π-stacking interaction. Moreover, the methyl group at *C*-5 position was positioned in the hydrophobic cavity formed by the Val179 side chains.

In summary, the results of the AutoDocking analysis supported our newly designed and synthesized 2-(phenylaminocarbonylmethylthio)-6-(2,6-dichlorobenzyl)-pyrimidin-4(3*H*)-ones. Further optimization of **4b3** will consider these aspects in further design attempts.

3. Conclusions

In summary, we designed and synthesized a series of novel 2-(substituted phenylaminocarbonylmethylthio)-6-(2,6-dichlorobenzyl)-pyrimidin-4(3*H*)-ones (*S*-DABOs), which were structurally confirmed by IR, ^{1}H NMR and MS spectral analysis and evaluated for their anti-HIV (HIV-1 III_B and HIV-2 ROD) activities by inhibition of HIV-induced cytopathogenicity in MT-4 cell cultures.

Some of the new compounds displayed anti-HIV-1 activity in the submicromolar range along with low cytotoxicity. Among them, the most potent HIV-1 inhibitor was **4b3** (EC_{50} = 0.18 ± 0.06 μM), which was more effective than the reference drugs nevirapine and efavirenz. The preliminary SAR among the newly synthesized congeners and docking studies provided useful indications for guiding the further rational design of new *S*-DABO analogues as more active and selective HIV-1 inhibitors.

4. Experimental section

4.1. Chemistry

All melting points were determined on a micromelting point apparatus and are uncorrected. Infrared spectra (IR) were recorded with a Nexus 470FT-IR Spectrometer. NMR spectra were obtained on a Brucker Avance-600 NMR-spectrometer in the indicated solvents. Chemical shifts are expressed in δ units and TMS as internal reference. Mass spectra were taken on a LC Autosampler Device: Standard G1313A instrument. TLC was performed on silica gel GF254 for TLC (Merck) and spots were visualized by iodine vapors or by irradiation with UV light (254 nm). Solvents were reagent grade and, when necessary, were purified and dried by standard methods. Concentration of the reaction solutions involved the use of rotary evaporator at reduced pressure.

4.1.1. General procedure for the preparation of β-ketoesters 2a and 2b

To a well stirred solution of substituted diethyl malonate (500 mmol) in anhydrous EtOH (400 mL) was added dropwise a solution of KOH (28 g, 500 mmol) in EtOH (400 mL) at room temperature over 4 h. Then the resulting mixture was allowed to stand overnight until the pH of the final mixture had a value between 7 and 8. After removing the solvent, the residue was washed with a small amount of ether and suspended in anhydrous CH_3CN (800 mL), Et_3N (106 mL, 761 mmol) and $MgCl_2$ (57 g, 595 mmol) were added and the mixtures were stirred at room temperature for 2 h. Then were added the solutions of arylacetyl imidazolide, prepared 15 min before by reaction between 2-(2,6-dichlorophenyl)acetic acid (49 g, 238 mmol) and *N,N′*-carbonyldiimidazole (CDI, 46 g, 286 mmol) in CH_3CN (400 mL). The reaction mixtures were stirred overnight at room temperature and then refluxed for 2 h. After the mixture was cooled, a solution of 13% HCl (800 mL) was added slowly while the temperature was kept below 25 °C, and the resulting clear mixtures were stirred for a further 20 min. The organic layer was separated and concentrated, and the residue was treated with EtOAc (200 mL). The aqueous layer was extracted with EtOAc (3 × 150 mL), and the combined organic layers were washed with saturated $NaHCO_3$ (3 × 350 mL) and brine (3 × 350 mL), dried (Mg_2SO_4), filtered and concentrated to give the crude products **2a** and **2b**, respectively, which were directly used in the following step without further purification.

4.1.2. General procedure for the preparation of 5-alkyl-6-substituted thiouracil 3a, 3b

Sodium metal (8.2 g, 356 mmol) was dissolved in 50 mL of absolute ethanol, and thiourea (19 g, 249 mmol) and β-ketoesters **2a**, **2b** (178 mmol) were added to the clear solution at room temperature. The reaction mixture was refluxed for 10–12 h (checked by TLC) under a nitrogen atmosphere. The reaction mixture was cooled to room temperature. Then, solvent was evaporated and the residues were dissolved in H_2O and were precipitated by addition of concd aq HCl and subsequent acidification to pH 4 with glacial AcOH. The resulting precipitate was filtered under reduced pressure. The solid was washed sequentially with H_2O, EtOH, and Et_2O, then dried to give **3a, 3b**, which is directly used in the next step without further purification.

4.1.3. General procedure for the preparation of target compounds 4a1–4a9, 4b1–4b8

Compounds **3a, 3b** (5 mmol) and appropriate *N*-phenylacetamide halides (5.5 mmol) were suspended in anhydrous DMF (25 mL) in the presence of anhydrous potassium carbonate (0.759 g, 5.5 mmol) at room temperature. The mixtures were irradiated at room temperature for 12 h. The reaction mixture was poured into cold H_2O (200 mL), the resulting precipitate was collected by filtration under reduced pressure and washed sequentially with H_2O, EtOH and Et_2O and then dried to give the corresponding crude product, which was purified by crystallization to give the pure target compounds **4a1–4a9, 4b1–4b8**.

4.1.3.1. 2-[(4-Methoxyphenylamino)carbonylmethylthio]-6-(2,6-dichlorobenzyl)-pyrimidin-4(3*H*)-one (4a1). Recrystallized from EtOH/DMF as a white crystal, Yield: 25.4%, mp: 238–240 °C (dec). ^{1}H NMR (DMSO-d_6, ppm) δ: 12.80 (s, 1H, NH), 10.08 (s, 1H, NH), 7.45–7.29 (m, 7H), 5.41 (s, 1H, CH═C═O), 4.05 (s, 2H, S-CH_2), 3.99 (s, 2H, CH_2), 3.69 (s, OCH_3, 3H); IR (KBr, cm^{-1}): 3276 (ν_{NH}), 3075 (ν_{NH}), 1661 ($\nu_{C=O}$), 1247 (ν_{C-N}). ESI-MS: *m*/*z* 450.6 (M+1), 472.7 (M+Na). $C_{20}H_{17}Cl_2N_3O_3S$ (449.04).

4.1.3.2. 2-[(4-Chlorophenylamino)carbonylmethylthio]-6-(2,6-dichlorobenzyl)-pyrimidin-4(3*H*)-one (4a2). Recrystallized from EtOH–DMF as a white crystal, Yield: 23.6%, mp: 223–225 °C (dec). ^{1}H NMR (DMSO-d_6, ppm) δ: 12.77 (s, 1H, NH), 10.37 (s, 1H, NH), 7.57–7.24 (m, 7H), 5.44 (s, 1H, CH═C═O), 4.00 (s, 2H, S-CH_2), 3.95 (s, 2H, CH_2). IR (KBr, cm^{-1}): 3317 (ν_{NH}), 3052 (ν_{NH}), 1657 ($\nu_{C=O}$), 1240 (ν_{C-N}), 1206 (ν_{C-N}). ESI-MS: *m*/*z* 456.3 (M+1), 478.3 (M+Na). $C_{19}H_{14}Cl_3N_2S_2O$ (454.96).

4.1.3.3. 2-[(4-Bromophenylamino)carbonylmethylthio]-6-(2,6-dichlorobenzyl)-pyrimidin-4(3*H*)-one (4a3). Recrystallized from EtOH–DMF as a white crystal, Yield: 23.1%, mp: 232–233 °C (dec). ^{1}H NMR (DMSO-d_6, ppm) δ: 12.82 (s, 1H, NH), 10.34 (s, 1H, NH), 7.54 (d, *J* = 9.0 Hz, 2H), 7.49 (d, *J* = 9.0 Hz, 2H), 7.44 (d, *J* = 8.4 Hz, 2H), 7.26 (d, *J* = 8.4 Hz, H), 5.44 (s, 1H, CH═C═O), 4.03 (s, 2H, S-CH_2), 3.97 (s, 2H, CH_2); IR (KBr, cm^{-1}): 3320 (ν_{NH}), 3051 (ν_{NH}), 1656 ($\nu_{C=O}$), 1239 (ν_{C-N}), 1206 (ν_{C-N}). ESI-MS: *m*/*z* 498.3 (M+1), 520.2 (M+Na). $C_{19}H_{14}BrCl_2N_3O_2S$ (496.94).

4.1.3.4. 2-[(4-Methylphenylamino)carbonylmethylthio]-6-(2,6-dichlorobenzyl)-pyrimidin-4(3*H*)-one (4a4). Recrystallized from EtOH–DMF as a white crystal, Yield: 26.7%, mp: 235–237 °C (dec). ^{1}H NMR (DMSO-d_6, ppm) δ: 12.84 (s, 1H, NH), 10.11 (s, 1H, NH), 7.45–7.12 (m, 7H), 5.45 (s, 1H, CH═C═O), 4.05 (s, 2H, S-CH_2), 3.95 (s, 2H, CH_2), 2.25 (S, 3H, CH_3); IR (KBr, cm^{-1}): 3287 (ν_{NH}), 3034 (ν_{NH}), 1663 ($\nu_{C=O}$), 1241 (ν_{C-N}). ESI-MS: *m*/*z* 434.7 (M+1), 456.5 (M+Na). $C_{20}H_{17}Cl_2N_3O_2S$ (433.34).

4.1.3.5. 2-[(4-Nitrophenylamino)carbonylmethylthio]-6-(2,6-dichlorobenzyl)-pyrimidin-4(3*H*)-one (4a5). Recrystallized from EtOH–DMF as a white crystal, Yield: 29.6%, mp: 227–229 °C.

^{1}H NMR (DMSO-d_6, ppm) δ: 12.78 (s, 1H, NH), 10.82 (s, 1H, NH), 7.56 (dd, J_1 = 9.6 Hz, J_2 = 2.4 Hz, 2H), 7.8 (dd, J_1 = 9.6 Hz, J_2 = 2.4 Hz, 2H), 7.36 (d, J = 7.8 Hz, 2H), 7.23 (d, J = 7.8 Hz, H), 5.52 (s, 1H, CH═C═O), 4.08 (s, 2H, *S*-CH_2), 4.02 (s, 2H, CH_2). IR (KBr, cm^{-1}) 3323 (ν_{NH}), 3077 (ν_{NH}), 1655 ($\nu_{C=O}$), 1241 (ν_{C-N}), 1205 (ν_{C-N}). ESI-MS: *m*/*z* 465.5 (M+1). $C_{19}H_{14}Cl_2N_4O_4S$ (464.01).

4.1.3.6. 2-[(3,4-Dimethoxyphenylamino)carbonylmethylthio]-6-(2,6-dichlorobenzyl)-pyrimidin-4(3*H*)-one (4a6). Recrystallized from EtOH–DMF as a white crystal, Yield: 22.6%, mp: 221–223 °C (dec). ^{1}H NMR (DMSO-d_6, ppm) δ: 12.80 (s, 1H, NH), 10.03 (s, 1H, NH), 7.51–6.86 (m, 6H), 5.47 (s, 1H, CH═C═O), 4.08 (s, 2H, *S*-CH_2), 4.02 (s, 2H, CH_2), 3.72 (2 × OCH_3, 6H); IR (KBr, cm^{-1}) 3292 (ν_{NH}), 3136 (ν_{NH}), 1664 ($\nu_{C=O}$), 1231.65 (ν_{C-N}), 1218 (ν_{C-N}). ESI-MS: *m*/*z* 482.4 (M+3). $C_{21}H_{19}Cl_2N_3O_4S$ (479.05).

4.1.3.7. 2-[(3,4-Difluorophenylamino)carbonylmethylthio]-6-(2,6-dichlorobenzyl)-pyrimidin-4(3*H*)-one (4a7). Recrystallized from EtOH–DMF as a white crystal, Yield: 24.2%, mp: 228–230 °C (dec). ^{1}H NMR (DMSO-d_6, ppm) δ: 12.78 (s, 1H, NH), 10.45 (s, 1H, NH), 7.74–7.28 (m, 6H), 5.52 (s, 1H, CH═C═O), 4.05 (s, 2H, S-CH_2), 3.93 (s, 2H, CH_2); IR (KBr, cm^{-1}): 3297 (ν_{NH}), 3085 (ν_{NH}), 1659 ($\nu_{C=O}$), 1241 (ν_{C-N}), 1210 (ν_{C-N}). ESI-MS: *m*/*z* 456.5 (M+1), 478.5 (M+Na). $C_{19}H_{13}Cl_2F_2N_3O_2S$ (455.01).

4.1.3.8. 2-[(3,4-Dichlorophenylamino)carbonylmethylthio]-6-(2,6-dichlorobenzyl)-pyrimidin-4(3*H*)-one (4a8). Recrystallized from EtOH–DMF as a white crystal, Yield: 26.8%, mp: 236–238 °C (dec) ^{1}H NMR (DMSO-d_6, ppm) δ: 12.87 (s, 1H, NH), 10.50 (s, 1H, NH), 7.95 (d, J = 2.4 Hz, H), 7.58 (d, J = 8.4 Hz, H), 7.45 (dd, J_1 = 8.4 Hz, J_2 = 2.4 Hz, H), 7.42 (d, J = 7.8 Hz, 2H), 7.25 (d, J = 7.8 Hz, 1H), 5.44 (s, 1H, CH═C═O), 4.04 (s, 2H, S-CH_2), 3.95 (s, 2H, CH_2); IR (KBr, cm^{-1}): 3291 (ν_{NH}), 3091 (ν_{NH}), 1660 ($\nu_{C=O}$), 1234 (ν_{C-N}), 1206 (ν_{C-N}). ESI-MS: *m*/*z* 488.3 (M+1), 510.1 (M+Na). $C_{19}H_{13}Cl_4N_3O_2S$ (486.95).

4.1.3.9. 2-[(3-Chloro-4-fluorophenylamino)carbonylmethylthio]-6-(2,6-dichlorobenzyl)-pyrimidin-4(3*H*)-one (4a9). Recrystallized from EtOH–DMF as a white crystal, Yield: 21.8%, mp: 237–239 °C (dec). ^{1}H NMR (DMSO-d_6, ppm) δ: 12.75 (s, 1H, NH), 10.41 (s, 1H, NH), 7.87–7.25 (m, 6H), 5.50 (s, 1H, CH═C═O), 4.02 (s, 2H, S-CH_2), 3.93 (s, 2H, CH_2); IR (KBr, cm^{-1}): 3290 (ν_{NH}), 3042 (ν_{NH}), 1658 ($\nu_{C=O}$), 1221 (ν_{C-N}). ESI-MS: *m*/*z* 472.6 (M+1), 494.6 (M+Na). $C_{19}H_{13}Cl_3FN_3O_2S$ (470.98).

4.1.3.10. 2-[(4-Methoxyphenylamino)carbonylmethylthio]-6-(2,6-dichlorobenzyl)-5-methylpyrimidin-4(3*H*)-one (4b1). Recrystallized from EtOH–DMF as a white crystal, Yield: 54.0%, mp: 248–251 °C (dec). ^{1}H NMR (DMSO-d_6, ppm) δ: 12.71 (s, 1H, NH), 9.72 (s, 1H, NH), 7.40–6.68 (m, 7H), 4.05 (s, 2H, *S*-CH_2), 3.99 (s, 2H, CH_2), 2.04 (s, CH_3, 3H); IR (KBr, cm^{-1}): 3323 (ν_{NH}), 3041 (ν_{NH}), 1654 ($\nu_{C=O}$), 1248 (ν_{C-N}). ESI-MS: *m*/*z* 464.5 (M+1), 502.6 (M+K). $C_{21}H_{19}Cl_2N_3O_3S$ (463.05).

4.1.3.11. 2-[(4-Chlorophenylamino)carbonylmethylthio]-6-(2,6-dichlorobenzyl)-5-methylpyrimidin-4(3*H*)-one (4b2). Recrystallized from EtOH–DMF as a white crystal, Yield: 27.6%, mp: 256–258 °C (dec). ^{1}H NMR (DMSO-d_6, ppm) δ: 12.71 (s, 1H, NH), 10.00 (s, 1H, NH), 7.53 (d, J = 8.4 Hz, 2H), 7.37 (d, J = 8.4 Hz, 2H), 7.24 (d, J = 7.8 Hz, 2H), 7.04 (d, J = 7.8 Hz, H), 4.11 (s, 2H, *S*-CH_2), 3.72 (s, 2H, CH_2), 2.04 (s, CH_3, 3H); IR (KBr, cm^{-1}) 3327 (ν_{NH}), 3053 (ν_{NH}), 1660 ($\nu_{C=O}$), 1260 (ν_{C-N}), 1243 (ν_{C-N}). ESI-MS: *m*/*z* 468.5 (M+1). $C_{20}H_{16}Cl_3N_3O_2S$ (467).

4.1.3.12. 2-[(4-Bromophenylamino)carbonylmethylthio]-6-(2,6-dichlorobenzyl)-5-methylpyrimidin-4(3*H*)-one (4b3). Recrystallized from EtOH–DMF as a white crystal, Yield: 29.2%, mp: 259–261 °C (dec). ^{1}H NMR (DMSO-d_6, ppm) δ: 12.70 (s, 1H, NH), 9.99 (s, 1H, NH), 7.49–7.04 (m, 7H), 4.11 (s, 2H, *S*-CH_2), 3.72 (s, 2H, CH_2), 2.04 (s, CH_3, 3H); IR (KBr, cm^{-1}): 3307 (ν_{NH}), 3051 (ν_{NH}), 1656 ($\nu_{C=O}$), 1256 (ν_{C-N}), 1242 (ν_{C-N}). ESI-MS: *m*/*z* 514.5 (M+3). $C_{20}H_{16}BrCl_2N_3O_2S$ (510.95).

4.1.3.13. 2-[(4-Nitrophenylamino)carbonylmethylthio]-6-(2,6-dichlorobenzyl)-5-methylpyrimidin-4(3*H*)-one (4b4). Recrystallized from EtOH–DMF as a white crystal, Yield: 24.8%, mp: 250–252 °C (dec). ^{1}H NMR (DMSO-d_6, ppm) δ: 12.76 (s, 1H, NH), 10.47 (s, 1H, NH), 8.25–6.97 (m, 7H), 4.10 (s, 2H, *S*-CH_2), 3.79 (s, 2H, CH_2), 2.03 (s, CH_3, 3H); IR (KBr, cm^{-1}): 3412 (ν_{NH}), 3085 (ν_{NH}), 1649 ($\nu_{C=O}$), 1620 ($\nu_{C=O}$), 1331 (ν_{C-N}), 1307 (ν_{C-N}). ESI-MS: *m*/*z* 479.2 (M+1), 501.2 (M+Na). $C_{20}H_{16}Cl_2N_4O_4S$ (478.03).

4.1.3.14. 2-[(3,4-Dimethoxyphenylamino)carbonylmethylthio]-6-(2,6-dichlorobenzyl)-5-methylpyrimidin-4(3*H*)-one (4b5). Recrystallized from EtOH–DMF as a white crystal, Yield: 52.3%, mp: 246–249 °C (dec). ^{1}H NMR (DMSO-d_6, ppm) δ: 12.68 (s, 1H, NH), 9.75 (s, 1H, NH), 7.29–6.89 (m, 6H), 4.13 (s, 2H, *S*-CH_2), 3.73–3.72 (2 × OCH_3, 6H), 3.67 (s, 2H, CH_2), 2.04 (s, CH_3, 3H); IR (KBr, cm^{-1}): 3272 (ν_{NH}), 3050 (ν_{NH}), 1671 ($\nu_{C=O}$), 1651 ($\nu_{C=O}$), 1262 (ν_{C-N}), 1234 (ν_{C-N}). ESI-MS: *m*/*z* 494.3 (M+1), 516.3 (M+Na). $C_{22}H_{21}Cl_2N_3O_4S$ (493.06).

4.1.3.15. 2-[(3,4-Difluorophenylamino)carbonylmethylthio]-6-(2,6-dichlorobenzyl)-5-methylpyrimidin-4(3*H*)-one (4b6) . Recrystallized from EtOH–DMF as a white crystal, Yield: 29.6%, mp: 247–250 °C (dec). ^{1}H NMR (DMSO-d_6, ppm) δ: 12.71 (s, 1H, NH), 10.10 (s, 1H, NH), 7.68–7.03 (m, 6H), 4.11 (s, 2H, *S*-CH_2), 3.72 (s, 2H, CH_2), 2.04 (s, CH_3, 3H); IR (KBr, cm^{-1}): 3313 (ν_{NH}), 3060 (ν_{NH}), 1675 ($\nu_{C=O}$), 1647 ($\nu_{C=O}$), 1257 (ν_{C-N}), 1206 (ν_{C-N}). ESI-MS: *m*/*z* 470.3 (M+1), 492.1 (M+Na). $C_{20}H_{15}$ $Cl_2F_2N_3O_2S$ (469.02).

4.1.3.16. 2-[(3,4-Dichlorophenylamino)carbonylmethylthio]-6-(2,6-dichlorobenzyl)-5-methylpyrimidin-4(3*H*)-one (4b7). Recrystallized from EtOH–DMF as a white crystal, Yield: 25.7%, mp: 256–258 °C (dec). ^{1}H NMR (DMSO-d_6, ppm) δ: 12.74 (s, 1H, NH), 10.14 (s, 1H, NH), 7.87 (d, J = 7.8 Hz, 1H), 7.58 (d, J = 9 Hz, 1H), 7.38 (dd, J_1 = 9 Hz, J_2 = 1.8 Hz, 1H), 7.21 (d, J = 7.8 Hz, 2H), 7.02 (d, J = 7.8 Hz, 1H), 4.11 (s, 2H, *S*-CH_2), 3.73 (s, 2H, CH_2), 2.04 (s, CH_3, 3H); IR (KBr, cm^{-1}): 3283 (ν_{NH}), 3040 (ν_{NH}), 1673 ($\nu_{C=O}$), 1646 ($\nu_{C=O}$), 1257 (ν_{C-N}), 1236 (ν_{C-N}). ESI-MS: *m*/*z* 504.3 (M+3). $C_{20}H_{15}Cl_4N_3O_2S$ (500.96).

4.1.3.17. 2-[(3-Chloro-4-fluorophenylamino)carbonylmethylthio]-6-(2,6-dichlorobenzyl)-5-methylpyrimidin-4(3*H*)-one (4b8). Recrystallized from EtOH–DMF as a white crystal, Yield: 26.6%, mp: 259–261 °C (dec). ^{1}H NMR (DMSO-d_6, ppm) δ: 12.68 (s, 1H, NH), 10.06 (s, 1H, NH), 7.96–7.02 (m, 6H), 4.11 (s, 2H, *S*-CH_2), 3.72 (s, 2H, CH_2), 2.04 (s, CH_3, 3H); IR (KBr, cm^{-1}): 3294 (ν_{NH}), 3052

(ν_{NH}), 1673 ($\nu_{C=O}$), 1648 ($\nu_{C=O}$), 1260 (ν_{C-N}), 1221 (ν_{C-N}). ESI-MS: m/z 488.2 (M+3). $C_{20}H_{15}Cl_3FN_3O_2S$ (484.99).

4.2. Anti-HIV activity assays

Evaluation of the antiviral activity of the compounds against HIV-1 strain III_B and HIV-2 strain (ROD) in MT-4 cells was performed using the MTT assay as previously described.[20,21] Stock solutions (10 × final concentration) of test compounds were added in 25 μL volumes to two series of triplicate wells so as to allow simultaneous evaluation of their effects on mock-and HIV-infected cells at the beginning of each experiment. Serial fivefold dilutions of test compounds were made directly in flat-bottomed 96-well microtiter trays using a Biomek 3000 robot (Beckman instruments, Fullerton, CA). Untreated control HIV-and mock-infected cell samples were included for each sample.

HIV-1(III_B)[22] or HIV-2 (ROD)[23] stock (50 μL) at 100–300 $CCID_{50}$ (cell culture infectious dose) or culture medium was added to either the infected or mock-infected wells of the microtiter tray. Mock-infected cells were used to evaluate the effect of test compound on uninfected cells in order to assess the cytotoxicity of the test compound. Exponentially growing MT-4 cells[24] were centrifuged for 5 min at 1000 rpm and the supernatant was discarded. The MT-4 cells were resuspended at 6×10^5 cells/mL, and 50-μL volumes were transferred to the microtiter tray wells. Five days after infection, the viability of mock- and HIV-infected cells was examined spectrophotometrically by the MTT assay.

The MTT assay is based on the reduction of yellow colored 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Acros Organics, Geel, Belgium) by mitochondrial dehydrogenase of metabolically active cells to a blue-purple formazan that can be measured spectrophotometrically. The absorbances were read in an eight-channel computer-controlled photometer (Multiscan Ascent Reader, Labsystems, Helsinki, Finland), at two wavelengths (540 and 690 nm). All data were calculated using the median OD (optical density) value of tree wells. The 50% cytotoxic concentration (CC_{50}) was defined as the concentration of the test compound that reduced the absorbance (OD540) of the mock-infected control sample by 50%. The concentration achieving 50% protection from the cytopathic effect of the virus-infected cells was defined as the 50% effective concentration (EC_{50}).

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