Journal of Medicinal Chemistry

Brief Article

pubs.acs.org/jmc

2-(Alkyl/Aryl)Amino-6-Benzylpyrimidin-4(3*H*)-ones as Inhibitors of Wild-Type and Mutant HIV-1: Enantioselectivity Studies

Dante Rotili,[†,#] Alberta Samuele,[‡,#] Domenico Tarantino,[†] Rino Ragno,[†] Ira Musmuca,[†] Flavio Ballante,[†] Giorgia Botta,[†] Ludovica Morera,[†] Marco Pierini,[†] Roberto Cirilli,[§] Maxim B. Nawrozkij,[∥] Emmanuel Gonzalez,[⊥] Bonaventura Clotet,[⊥] Marino Artico,[†] José A. Esté,*[,⊥] Giovanni Maga,*[,‡] and Antonello Mai*[,†]

[†]Istituto Pasteur—Fondazione Cenci Bolognetti, Dipartimento di Chimica e Tecnologie del Farmaco, Università degli Studi di Roma "La Sapienza", P.le A. Moro 5, 00185 Rome, Italy

[‡]Istituto di Genetica Molecolare IGM-CNR, via Abbiategrasso 207, 27100 Pavia, Italy

[§]Dipartimento del Farmaco, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy

[∥]Volgograd State Technical University, prospekt Lenina, 28, 400131 Volgograd, Russia

[⊥]Retrovirology Laboratory IrsiCaixa, Hospital Universitari Germans Trias i Pujol, Universitat Autònoma de Barcelona, 08916 Badalona, Spain

Supporting Information

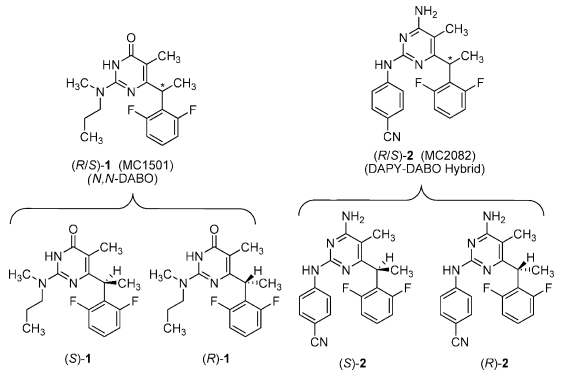
ABSTRACT: The single enantiomers of two pyrimidine-based HIV-1 non-nucleoside reverse transcriptase inhibitors, **1** (MC1501) and **2** (MC2082), were tested in both cellular and enzyme assays. In general, the *R* forms were more potent than their *S* counterparts and racemates and (*R*)-**2** was more efficient than (*R*)-**1** and the reference compounds, with some exceptions. Interestingly, (*R*)-**2** displayed a faster binding to K103N RT with respect to WT RT, while (*R*)-**1** showed the opposite behavior.

INTRODUCTION

Since 1992, our research team has discovered excellent dihydro-alkyloxy-benzyl-oxopyrimidine (DABO) classes of non-nucleoside reverse transcriptase (RT) inhibitors (NNRTIs) such as F_2-*S*-DABOs,[1] F_2-*NH*-DABOs,[2] and F_2-*N*,*N*-DABOs.[3] Such compounds had inhibitory potencies in the (sub)nanomolar range against wild-type (WT) HIV-1 without significant cytotoxicity at higher concentrations, and with potencies in the submicromolar range against clinically relevant mutant strains. The F_2-*N*,*N*-DABO derivative **1** (MC1501),[3] characterized by a *N*-methyl-*N*-propyl side chain at the C-2 pyrimidine ring position and by a double pyrimidine C-5/C-6 benzylic position methyl substitution, with its subnanomolar inhibitory potency against WT HIV-1 and the Y181C mutant strain, can be considered one of the most promising DABO compounds reported so far (Chart 1).

Diarylpyrimidine (DAPY) derivatives are one of the most successful classes of pyrimidine-based NNRTIs, as demonstrated by the recent approval of etravirine and rilpivirine for clinical use.[4,5] SAR studies on the DAPY class had also shown how the *para*-cyanoaniline substitution at the C-2 pyrimidine ring position is crucial for a strong inhibitory activity. Recently, combining the 2,6-difluorobenzyl or 1-(2,6-difluorophenyl)-ethyl group at the C-6 position characteristic of DABOs with the *para*-cyanoaniline group at the C-2 of the pyrimidine ring typical of DAPYs, and inserting at the C-4 pyrimidine ring position groups found in either DABOs (−OH) or DAPYs (−Cl, −H, $-NH_2$), a new series of highly potent anti-HIV-1 agents called DAPY-DABO hybrids has been developed.[6] Among those, the derivative **2** (MC2082) (Chart 1) was the most potent due to its (sub)nanomolar activity against WT and clinically relevant mutant HIV-1 strains, such as K103N and Y188L.[6]

Chart 1. Pyrimidine-Based NNRTIs That Are the Object of the Present Study



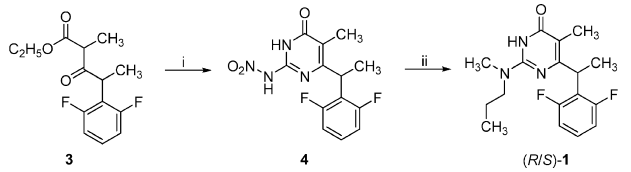
Both the pyrimidine-based NNRTIs **1** and **2** contain a stereogenic center at the C-6 benzylic position and, therefore, exist as racemic mixtures. Because of the high potency of these two lead compounds, we decided to perform a systematic investigation on their potential enantioselective anti-HIV-1 activity. Here we describe, through a multidisciplinary approach, the enantioseparation, the absolute configuration assignment, and the enzymatic and cellular evaluation of the

Received: October 11, 2011
Published: March 19, 2012

HIV-1 inhibitory activity of the single enantiomers in comparison with the corresponding racemic mixtures of both compounds. The application of a comparative binding energy (ComBinE) model built to quantitate the influence of single point HIV-RT mutations to modeled bound conformations of both **1** and **2** was in good agreement with the experimental findings and gave insights on the absolute configuration assignment of both **1** and **2** separated enantiomers.

Chemistry. The racemic mixture of **2** was prepared as previously described.[6] The racemic mixture of **1** was prepared starting from the β-oxoester **3**[1] and using, differently from previously reported,[3] a route characterized by only two steps and by a final nucleophilic displacement under mild conditions and with high yield. After condensation of the β-oxoester **3** with nitroguanidine, the resulting 6-[1-(2,6-difluorophenyl)-ethyl]-5-methyl-2-nitroamino-3*H*-pyrimidin-4-one **4** was heated in a sealed tube of a Parr high pressure reactor in the presence of an excess of *N*-methyl-*N*-propylamine to give directly the racemic **1** (Scheme 1).

Scheme 1[a]



[a](i) EtONa, nitroguanidine, dry EtOH, reflux; (ii) *N*-methyl-*N*-propylamine, 120 °C, Parr high pressure reactor.

RESULTS AND DISCUSSION

The direct resolution of **1** and **2** was achieved by HPLC on the coated-type Chiralpak AD chiral stationary phase (CSP) using pure ethanol in mixture with a small percentage (0.1%) of diethylamine (DEA) as eluent (see Supporting Information). Efforts to obtain the absolute configuration of **1** and **2** by X-ray crystallography were not successful, therefore stereochemical information on the four chiral compounds were obtained by analyzing their chiroptical properties. The (*S*) configuration was assigned to the dextrorotatory enantiomers of **1** and **2** ((*S*)-(+)-**1** and (*S*)-(+)-**2**, first-eluted enantiomers on the Chiralpak AD CSP) and the (*R*) configuration assigned to the levorotatory counterparts ((*R*)-(−)-**1** and (*R*)-(−)-**2**, second-eluted enantiomers).

The racemic compounds **1** and **2** and their enantiomers were tested in MT-4 cells to evaluate their cytotoxicity and their capability to inhibit by 50% the HIV-induced cytopathic effect (HIV-1 strain: NL4−3). The compounds were also tested against a panel of clinically relevant HIV-1 mutant strains (K103N, Y181C, and Y188L). Nevirapine (NVP), efavirenz (EFV), and dapivirine (TMC120) were also tested as reference drugs (Table 1).

The enantiomer (*R*)-**1** was much more potent (ranging from about 2800- to 11000-fold) than the other enantiomer (*S*)-**1** in inhibiting WT HIV-1 and the three tested mutant strains. (*R*)-**1** was slightly better, both in terms of activity and of selectivity, than the corresponding racemic mixture. The enantiomer (*R*)-**2** inhibited WT and mutant HIV-1 strains from 900- to 10000-fold more efficiently than the other enantiomer and was slightly more potent than the racemic mixture. With the only exception of the mutant Y181C, (*R*)-**2** was always more efficient than (*R*)-**1** and TMC120 in the inhibition of both WT and mutant (K103N and Y188L) HIV-1 strains (Table 1). Moreover, (*R*)-**2** was 10-fold less toxic than TMC120, yielding an highly improved selectivity index ($SI_{(R)\text{-}2}$ = 300000; SI_{TMC120} = 5000).

We evaluated the RNA-dependent DNA polymerase activity of WT RT and those enzymes carrying the most common NNRTI-resistance mutations (K103N, L100I, Y181I, V106A, Y188L) in the presence of increasing concentrations of the **1** and **2** enantiomers (Table 2). In the inhibition assays, the enantiomers (*R*)-**1** and (*R*)-**2** showed higher potency than their corresponding *S* isomers toward RT WT and mutants. Moreover, (*R*)-**2** showed the lowest ID_{50} values with respect to the majority of the mutants evaluated, such as K103N, V106A, and Y188L, whereas its efficacy appeared slightly lesser than that observed for (*R*)-**1** in the case of L100I and Y181I. As illustrated in Table 2, in all cases both racemic compounds presented intermediate levels of potency compared to the corresponding pairs of enantiomers.

Steady-state kinetic assays were performed for the two most potent isomers, (*R*)-**1** and (*R*)-**2**, to assess the inhibition mechanism against RT WT and RT carrying K103N mutation. The enzyme activity was measured in the presence of fixed

Table 1. Cytotoxicity and Anti-HIV-1 Activity against WT (NL4-3) and Clinically Relevant HIV-1 Mutant Strains of Racemic Compounds 1 and 2 and their Enantiomers[a]

	EC_{50},[b] nM (*fold resistance*)[c]					
compd	NL4−3	K103N	Y181C	Y188L	CC_{50},[d] nM	selectivity index[e]
1	1.9 ± 0.2	16.5 ± 0.5 (*9*)	17 ± 1 (*9*)	49 ± 4 (*26*)	>78000	>41000
(*S*)-**1**	15000 ± 50	>78000	>78000	>78000	>78000	>5.2
(*R*)-**1**	1.3 ± 0.1	11 ± 0.7 (*8*)	13 ± 1 (*10*)	28 ± 2 (*22*)	75000 ± 50	58000
2	0.6 ± 0.1	35 ± 3 (*60*)	40 ± 2 (*70*)	27 ± 3 (*40*)	40000 ± 70	70000
(*S*)-**2**	1000 ± 10	4500 ± 10 (*4*)	>38000	6500 (*6*)	38000 ± 50	40
(*R*)-**2**	0.1 ± 0.02	0.8 ± 0.01 (*8*)	31 ± 2 (*300*)	7.1 ± 0.5 (*70*)	31000 ± 50	300000
TMC120	0.6 ± 0.07	1.2 ± 0.2 (*2*)	1.2 ± 0.2 (*2*)	330 ± 10 (*500*)	3000 ± 30	5000
NVP	130 ± 1	6100 ± 20 (*47*)	>7500	>7500	7500 ± 20	58
EFV	7.3 ± 0.5	340 ± 4 (*47*)	10 ± 1 (*1.4*)	1600 ± 5 (*220*)	3200 ± 8	440

[a]Values are means ± SD determined from at least three experiments. [b]Effective concentration 50, concentration needed to inhibit 50% HIV-induced cytopathic effect, evaluated with the MTT method in MT-4 cells. [c]Fold resistance: ratio of EC_{50} value against drug-resistant strain and EC_{50} of the WT NL4−3 strain. [d]Cytotoxic concentration 50, concentration to induce 50% death of noninfected cells, evaluated with the MTT method in MT-4 cells. [e]Selectivity index, CC_{50}/EC_{50}.

dx.doi.org/10.1021/jm201308v | *J. Med. Chem.* 2012, 55, 3558−3562

Table 2. Comparison of Inhibition Potencies, Expressed as ID_{50} (nM), between the Racemic Compounds 1 and 2 and their Enantiomers, Tested against RT Wild-Type (WT) and K103N, L100I, Y181I, V106A, Y188L RT Mutants[a]

	ID_{50},[b] nM (*fold resistance*)[c]					
compd	WT	K103N	L100I	Y181I	V106A	Y188L
1	30 ± 3	174 ± 4 (*6*)	177 ± 9 (*6*)	1260 ± 8 (*40*)	19 ± 1 (*0.6*)	1430 ± 20 (*50*)
(*S*)-**1**	975 ± 5	>20000	350 ± 8 (*0.4*)	>20000	120 ± 8 (*0.1*)	>20000
(*R*)-**1**	2 ± 0.2	96 ± 9 (*48*)	96 ± 1 (*50*)	450 ± 9 (*200*)	3 ± 0.2 (*1.5*)	195 ± 5 (*100*)
2	14 ± 2	22 ± 3 (*1.6*)	280 ± 3 (*20*)	1700 ± 10 (*120*)	ND[d]	150 ± 6 (*11*)
(*S*)-**2**	180 ± 8	240 ± 9 (*1.3*)	>15000	>20000	64 ± 4 (*0.3*)	500 ± 8 (*3*)
(*R*)-**2**	8 ± 1	8 ± 1 (*1*)	150 ± 9 (*20*)	750 ± 8 (*90*)	0.3 ± 0.02 (*0.04*)	3 ± 0.2 (*0.4*)
TMC120	7 ± 1	100 ± 10 (*14*)	90 ± 1 (*13*)	>1000	ND	380 ± 3 (*50*)
NVP	400 ± 10	7000 ± 100 (*20*)	ND	35000 ± 100 (*87*)	ND	ND
EFV	30 ± 3	3000 ± 50 (*100*)	ND	80 ± 3 (*3*)	ND	ND

[a]Values are means ± SD determined from at least three experiments. [b]Inhibitory dose 50, compound dose required to inhibit the HIV-1 rRT activity by 50%. [c]Fold resistance: ratio of ID_{50}mut/ID_{50}WT values. [d]ND: not determined.

concentrations of the compounds and variable concentrations of one of the two substrates (either nucleic acid or nucleotide), while the other was maintained at saturating doses. The kinetic parameters V_{max} and K_m were derived by fitting the experimental data to the appropriate rate equations (Table S1 in Supporting Information). For both compounds, either varying the nucleic acid (NA) or the nucleotide (dNTP) concentrations, the V_{max} values of RT WT or K103N decreased as the inhibitor concentrations increased while the K_m values did not change. Thus, the mechanism of action of (*R*)-**1** and (*R*)-**2** against RT WT and K103N was fully noncompetitive. The (*R*)-**2** showed K_i values lower than those of (*R*)-**1**. Binding experiments were subsequently carried out, with RT WT and K103N to evaluate the association (k_{on}) and the dissociation rate (k_{off}) values for each inhibitor with respect to the different catalytic forms of the enzyme (free, binary complex, ternary complex) along the reaction pathway (Table S2 in Supporting Information). The k_{on} rate values were used to calculate the relative association index (RAI = k_{on} WT/k_{on} mut). RAI values were >1 for (*R*)-**1** toward the RT binary and ternary complexes, while they scored <1 for (*R*)-**2** (toward the free RT and binary complex). This indicated a different interaction mechanism of the two compounds with RT: (*R*)-**1** experienced a slower binding to the mutated form with respect to RT WT, while (*R*)-**2**, surprisingly, associated faster to the mutant RT (Figure 1A). Such unexpected behavior could be related to the presence of the cyano group into the (*R*)-**2** structure and its ability to disrupt the K103–Y188 hydrogen bond in the apo form of the mutated enzyme.[7] k_{off} values were used to derive the relative dissociation index (RDI = k_{off} mut/k_{off} WT). RDI values were, for both inhibitors, >1 toward the free enzyme and <1 in the case of the binary and ternary complexes, indicating that the binding of the compounds to the K103N RT was more stable than with WT RT once the enzymes were bound to the substrates (Figure 1B).

Biosensor-based analysis of binding of different NNRTIs to HIV-1 RT either WT or carrying drug-resistance mutants[8,9] supported the notion that the inherent flexibility of HIV-1 RT might explain the existence of high-affinity and low-affinity forms of RT with respect to NNRTI binding. Moreover, those studies revealed that the resistance induced by some mutations (such as K103N) was due to effects on both the association (k_{on}) and dissociation (k_{off}) rates of NNRTIs in an inhibitor-specific manner. In particular, the K103N mutation was found to stabilize the low-affinity conformation of the enzyme, thereby increasing both the k_{on} and k_{off} rates of some NNRTIs.

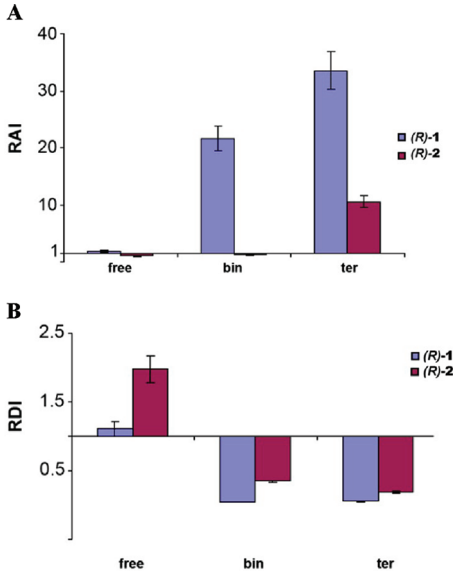


Figure 1. (A) Relative association index (RAI) derived from the association rates (k_{on}) of the inhibitors (*R*)-**1** and (*R*)-**2** to RT K103N and RT WT, expressed as ratio k_{on} WT/k_{on} mut. (B) Relative dissociation index (RDI) derived from the dissociation rates (k_{off}) of the inhibitors from RT K103N and RT WT, expressed as ratio k_{off} mut/k_{off} WT. free, free enzyme; bin, binary complex; ter, ternary complex. Error bars indicate the standard deviations (±SD).

Binding of substrates to RT is known to stabilize specific conformations of the enzyme, thus narrowing its range of inherent flexibility. According to the studies mentioned above, this implies that a given mutation will exert its effects on a different set of possible enzyme conformations, depending on whether the enzyme is unliganded, bound to nucleic acid, or in the ternary complex conformation. Our data suggest that such substrate-induced conformational changes are capable of influencing the ability of a certain mutation to affect the different kinetic steps of NNRTI interaction.

A structure-base study by means of ComBinE[10]-like protocol was undertaken (see Supporting Information) to further support the experimental evidence on the absolute configuration of the most- and the least-active **2** enantiomers and to provide a quantitative assessment of the mutation influence on the activity potency. The statistically most robust ComBinE model CM4, (previously developed without consideration of these compounds, Ballante, F., et al. *J. Comput.-Aided Mol. Des.*,

dx.doi.org/10.1021/jm201308v | *J. Med. Chem.* 2012, 55, 3558–3562

submitted for publication) was used to verify the mutation role on the activity profile of (*R*)- and (*S*)-**1** and of (*R*)- and (*S*)-**2**.

It could be argued that the use of static unrefined complexes cannot account for the dynamic aspect of ligand binding, nevertheless the CM4 models is able to highlight some aspects that would be otherwise ignored to interpret the experimental data. Application of the CM4 model to the inhibitors successfully reproduced the experimental eudismic ratio of (*R*) and (*S*) enantiomers as well as the increase of potency of **2** compared to **1**. In particular, among the residues around the RT NNBP a main role can be quantitatively attributed to Lys101 that was not considered in the RT mutated list. Other important residues for the binding strength were the highly conserved Trp229 and Tyr188 residues. The latter (Tyr188 in the WT isoform) serves as a steric anchor point to fulfill the concomitant lack of interactions that occurs in other mutation points such as in the case of V106A (Figure 2).

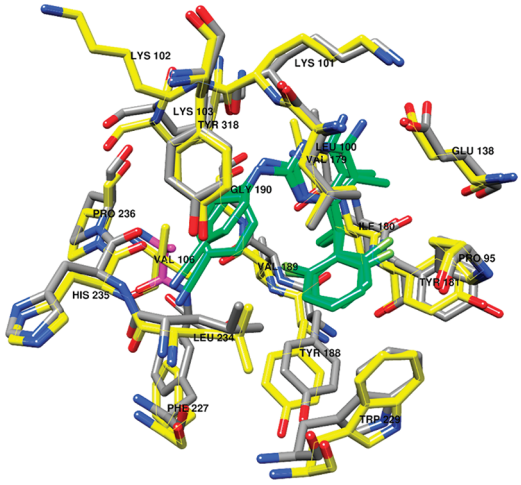


Figure 2. Comparison between (*R*)-**2** (green carbon atoms) docked into WT (yellow) and V106A mutated (gray) RT. The only residue that appreciably moves is Tyr188. The mutated Ala106 is displayed in magenta.

CONCLUSION

Among the most potent pyrimidine derivatives described by us as anti-HIV-1 agents, the *N*,*N*-DABO **1** and the DABO−DAPY hybrid **2**, both carrying a stereogenic center at the C6-benzylic position and previously prepared and tested as racemates, were resolved and characterized for their enantioselective activities in HIV-1-infected cells as well as by enzyme assays. In both cellular and enzyme assays, the *R* enantiomers of the two compounds were significantly more potent than the *S* counterparts, the racemates having an intermediate behavior compared to the corresponding single enantiomers. (*R*)-**2** was typically more efficient than (*R*)-**1** as well as than the reference compounds TMC120, NVP, and EFV in inhibiting the cytopathic effect of HIV-1 strains in MT-4 cells with the exception of the Y181C mutant, against which TMC120 and EFV showed the highest effects. However, (*R*)-**2** was one magnitude order less toxic than TMC120 and EFV, thus reaching a very high selectivity index. In enzyme assays, (*R*)-**2** displayed the highest inhibitory activities against RT WT, K103N, V106A, and Y188L, while against L100I and Y181I (joined in this case to EFV) (*R*)-**1** was more efficient. The effect of K103N RT mutation on the (*R*)-**1** and (*R*)-**2** activities was further characterized. A ComBinE model based on 14 complexes between RT and EFV and NVP without any knowledge of the herein reported compounds, reproduced with acceptable errors of prediction quantitatively the differences between different pyrimidinone derivatives and their enantiomers and was used to gain insight into the role of RT mutations on the inhibitors' biological activities.

EXPERIMENTAL SECTION

Chemistry. Melting points were determined on a Buchi 530 melting point apparatus and are uncorrected. ^{1}H NMR spectra were recorded at 400 MHz on a Bruker AC 400 spectrometer; chemical shifts are reported in δ units relative to the internal reference tetramethylsilane (Me_4Si). All of the compounds were routinely checked by TLC and ^{1}H NMR. TLC was performed on aluminum backed silica gel plates (Merck DC, Alufolien Kieselgel 60 F254) with spots visualized by UV light. All of the solvents were reagent grade and, when necessary, were purified and dried by standard methods. Concentration of solutions after reactions and extractions involved the use of a rotary evaporator operating at a reduced pressure of ca. 20 Torr. Organic solutions were dried over anhydrous sodium sulfate. Elemental analysis has been used to determine purity of the described compounds, that is >95%. Analytical results are within ±0.40% of the theoretical values. All chemicals were purchased from Aldrich Chimica, Milan (Italy), or from Lancaster Synthesis GmbH, Milan (Italy), and were of the highest purity.

Preparation of 6-[1-(2,6-Difluorophenyl)-ethyl]-5-methyl-2-nitroamino-3*H*-pyrimidin-4-one (4). See Supporting Information.

Preparation of 6-[1-(2,6-Difluorophenyl)-ethyl]-5-methyl-2-(methyl-*n*-propyl-amino)-3*H*-pyrimidin-4-one (1). A mixture of 6-[1-(2,6-difluorophenyl)-ethyl]-5-methyl-2-nitroamino-3*H*-pyrimidin-4-one **4** (100 mg, 0.322 mmol) and *N*-methyl-*N*-propylamine (707.2 mg, 9.66 mmol, 0.99 mL) was heated in a sealed tube of a Parr apparatus at 120 °C for 5 h. After cooling, the crude residue was dissolved in ethyl acetate (15 mL) and water (15 mL). The aqueous phase was extracted with ethyl acetate (3 × 15 mL). The organic extracts were washed with brine (1 × 20 mL), dried, evaporated under reduced pressure, and purified by column chromatography (silica gel, ethyl acetate/chloroform 1:2) to give the desired product **1** as a white powder (75.1 mg, 72.6%); mp 128−130 °C (dichloromethane/diethyl ether). ^{1}H NMR ($CDCl_3$) δ 10.70 (s, 1H, N*H*), 7.13 (m, 1H, *H* benzene ring), 6.81 (m, 2H, *H* benzene ring), 4.54 (q, 1H, C*H*CH_3), 3.50 (m, 1H, NC*H*HCH_2CH_3), 3.37 (m, 1H, NCH*H*CH_2CH_3), 3.06 (s, 3H, NCH_3), 1.93 (s, 3H, CH_3), 1.64 (d, 3H, CHCH_3), 1.54 (m, 2H, $NCH_2CH_2CH_3$), 0.88 (s, 3H, $NCH_2CH_2CH_3$). Anal. C, H, N, F: % Calcd C, 63.54; H, 6.59; N, 13.08; F, 11.82, Percent found C, 63.22; H, 6.48; N, 13.25; F, 12.01.

Enantioseparation and Chiroptical Characterization. See Supporting Information.

Molecular Modeling: ComBinE and Docking Calculations. See Supporting Information.

Biology: Anti-HIV Activity in Lymphoid Cells. Biological activity of the compounds was tested in the lymphoid MT-4 cell line (received from the NIH AIDS Reagent Program) against the WT HIV-1 NL4−3 strain and three different HIV-1 strains, as described before.[11,12] For a brief description, see Supporting Information.

Anti-HIV Reverse Transcriptase Assays. RNA-dependent DNA polymerase activity was assayed as described[13] in the presence of 0.5 μg of poly(rA)/oligo(dT)$_{10:1}$ (0.3 μM 3′-OH ends), 10 μM [^{3}H]-dTTP (1 Ci/mmol), and 2−4 nM RT in the presence of 8% final concentration of DMSO. For a brief description, see Supporting Information.

ASSOCIATED CONTENT

Supporting Information

Details on enantioseparation and absolute configuration assignment, biochemistry and molecular modeling studies,

dx.doi.org/10.1021/jm201308v | *J. Med. Chem.* 2012, 55, 3558−3562

and experimental section. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*For J.A.E.: phone, +34-934656374; fax, +34-934653968; E-mail, jaeste@irsicaixa.es. For G.M.: phone, +39 0382 546354; fax, +39 0382 422286; E-mail: maga@igm.cnr.it. For A.M.: phone, +39 06 49913392; fax, +39 06 49693268; E-mail, antonello.mai@uniroma1.it.

Author Contributions

#Equal contribution.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was partially supported by MICINN (BFU2009-06958 and SAF2010-21617-C02 to J.A.E. and B.C.) and by the Italian National Program for AIDS Research Grant 40H26 (to G.M.).

ABBREVIATIONS USED

CC_{50}, compound concentration toxic for 50% of cells; CSP, chiral stationary phase; DABOs, dihydro-alkoxy-benzyl-oxopyrimidines; DAPYs, diarylpyrimidines; DEA, diethylamine; EC_{50}, effective concentration able to protect 50% of cells from the HIV-1 induced cytopathogenicity; EFV, efavirenz; F_2-*N*,*N*-DABOs, 5-alkyl-2-(*N*,*N*-disubstituted)amino-6-(2,6-difluorophenylalkyl) pyrimidin-4(3*H*)ones; HIV, human immunodeficiency virus; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; *NH*-DABOs, dihydro-alkylamino-benzyl-oxopyrimidines; NNBS, non-nucleoside binding site; NNRTIs, non-nucleoside reverse transcriptase inhibitors; NVP, nevirapine; RT, reverse transcriptase; SAR, structure−activity relationship; *S*-DABOs, dihydro-alkylthio-benzyl-oxopyrimidines; WT, wild-type

REFERENCES

(1) Mai, A.; Sbardella, G.; Artico, M.; Ragno, R.; Massa, S.; Novellino, E.; Greco, G.; Lavecchia, A.; Musiu, C.; La Colla, M.; Murgioni, C.; La Colla, P.; Loddo, R. Structure-based design, synthesis, and biological evaluation of conformationally restricted novel 2-alkylthio-6-[1-(2,6-difluorophenyl)alkyl]-3,4-dihydro-5-alkylpyrimidin-4(3*H*)-ones as non-nucleoside inhibitors of HIV-1 reverse transcriptase. *J. Med. Chem.* **2001**, *44*, 2544−2554.

(2) Mai, A.; Artico, M.; Ragno, R.; Sbardella, G.; Massa, S.; Musiu, C.; Mura, M.; Marturana, F.; Cadeddu, A.; Maga, G.; La Colla, P. 5-Alkyl-2-alkylamino-6-(2,6-difluorophenylalkyl)-3,4-dihydropyrimidin-4(3*H*)-ones, a new series of potent, broad-spectrum non-nucleoside reverse transcriptase inhibitors belonging to the DABO family. *Bioorg. Med. Chem.* **2005**, *13*, 2065−2077.

(3) Mai, A.; Artico, M.; Rotili, D.; Tarantino, D.; Clotet-Codina, I.; Armand-Ugon, M.; Ragno, R.; Simeoni, S.; Sbardella, G.; Nawrozkij, M. B.; Samuele, A.; Maga, G.; Este, J. A. Synthesis and biological properties of novel 2-aminopyrimidin-4(3*H*)-ones highly potent against HIV-1 mutant strains. *J. Med. Chem.* **2007**, *50*, 5412−5424.

(4) Johnson, L. B.; Saravolatz, L. D. Etravirine, a next-generation nonnucleoside reverse-transcriptase inhibitor. *Clin. Infect. Dis.* **2009**, *48*, 1123−1128.

(5) de Bethune, M. P. Non-nucleoside reverse transcriptase inhibitors (NNRTIs), their discovery, development, and use in the treatment of HIV-1 infection: a review of the last 20 years (1989−2009). *Antiviral Res.* **2010**, *85*, 75−90.

(6) Rotili, D.; Tarantino, D.; Artico, M.; Nawrozkij, M. B.; Gonzalez-Ortega, E.; Clotet, B.; Samuele, A.; Este, J. A.; Maga, G.; Mai, A. Diarylpyrimidine−dihydrobenzyloxopyrimidine hybrids: new, wide-spectrum anti-HIV-1 agents active at (sub)-nanomolar level. *J. Med. Chem.* **2011**, *54*, 3091−3096.

(7) Rodriguez-Barrios, F.; Balzarini, J.; Gago, F. The molecular basis of resilience to the effect of the Lys103Asn mutation in non-nucleoside HIV-1 reverse transcriptase inhibitors studied by targeted molecular dynamics simulations. *J. Am. Chem. Soc.* **2005**, *127*, 7570−7578.

(8) Geitmann, M.; Unge, T.; Danielson, U. H. Interaction kinetic characterization of HIV-1 reverse transcriptase non-nucleoside inhibitor resistance. *J. Med. Chem.* **2006**, *49*, 2375−2387.

(9) Geitmann, M.; Unge, T.; Danielson, U. H. Biosensor-based kinetic characterization of the interaction between HIV-1 reverse transcriptase and non-nucleoside inhibitors. *J. Med. Chem.* **2006**, *49*, 2367−2374.

(10) Ortiz, A. R.; Pisabarro, M. T.; Gago, F.; Wade, R. C. Prediction of drug binding affinities by comparative binding energy analysis. *J. Med. Chem.* **1995**, *38*, 2681−2691.

(11) Gonzalez, E.; Ballana, E.; Clotet, B.; Este, J. A. Development of resistance to VIR-353 with cross-resistance to the natural HIV-1 entry virus inhibitory peptide (VIRIP). *AIDS* **2011**, *25*, 1557−1583.

(12) Gonzalez-Ortega, E.; Mena, M. P.; Permanyer, M.; Ballana, E.; Clotet, B.; Este, J. A. ADS-J1 inhibits HIV-1 entry by interacting with gp120 and does not block fusion-active gp41 core formation. *Antimicrob. Agents Chemother.* **2010**, *54*, 4487−4492.

(13) Maga, G.; Amacker, M.; Ruel, N.; Hubscher, U.; Spadari, S. Resistance to nevirapine of HIV-1 reverse transcriptase mutants: loss of stabilizing interactions and thermodynamic or steric barriers are induced by different single amino acid substitutions. *J. Mol. Biol.* **1997**, *274*, 738−747.