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Review Article

Selected non-nucleoside reverse transcriptase inhibitors (NNRTIs): the DABOs family

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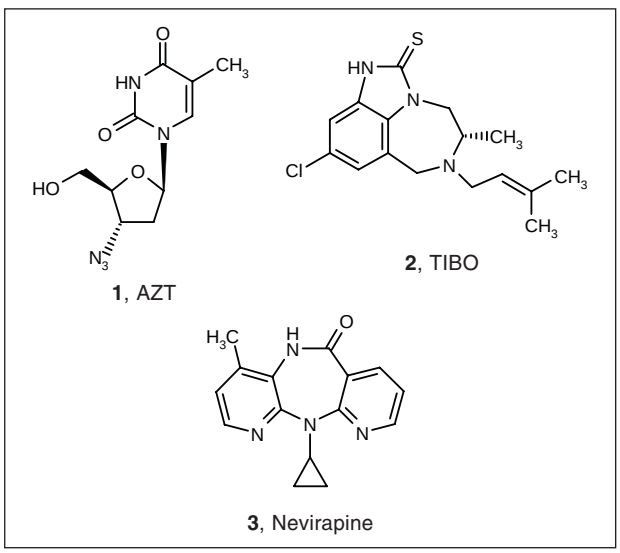


Fig. 1. NRT (AZT) and NNRT (TIBO, nevirapine) inhibitors.

Introduction

The worldwide spread of AIDS (acquired immunodeficiency syndrome), an epidemic disease in continuous development, has required potent antiretroviral chemotherapeutic agents for reducing the number of deaths caused by HIV-1 (human immunodeficiency virus type 1), the etiological agent of AIDS. Anti-HIV agents include inhibitors of reverse transcriptase (RT) (1-3) and protease (PR) (4), two enzymes highly determinant for viral replication of HIV-1. Another target in the viral reproduction cycle that has been investigated in more recent years is the enzyme integrase (IN) (5).

The era of anti-AIDS drugs started with the introduction in clinical practice of AZT (**1**, zidovudine), the prototype of nucleoside reverse transcriptase inhibitors (NRTIs) (Fig. 1). This event was soon followed by the discovery of TIBO (**2**) and nevirapine (**3**), two members of the new emerging class of non-nucleoside reverse transcriptase inhibitors (NNRTIs), which lacked the toxic effects shown by AZT and related congeners (ddI, ddC, d4T, PMEA).

NNRT agents comprise a number of structurally unrelated classes that share a common mode of action, binding to an allosteric site placed about 10 Å from the catalytic site. These agents include TIBO, nevirapine, BHAP (**4**), PETT (**5**), TSAO (**6**), α-APA (**7**), efavirenz (**8**) (Fig. 2), HEPT (**9**) and the related MKC-442 (**10**) and DABOs (**11**) (Fig. 3). Three compounds of these classes are actually available for clinical use: nevirapine (Viramune®), delarvidine (Rescriptor®) and efavirenz (Sustiva®). Other selected NNRTIs (MKC-442, DABOs, delavirdine) are undergoing preclinical/clinical investigations.

Reviews focused on the chemical and biological aspects of NNRT agents, including the mechanism of action and the role of NNRTIs in the therapy of patients infected by HIV-1, have been reported by Artico (6), Tucker *et al.* (7), De Clercq (8-12), Pedersen *et al.* (13) and Hajos *et al.* (14).

Among the most representative classes of NNRTIs studied in the last decade, DABOs occupy a relevant position. Their discovery and development are the object of the present review.

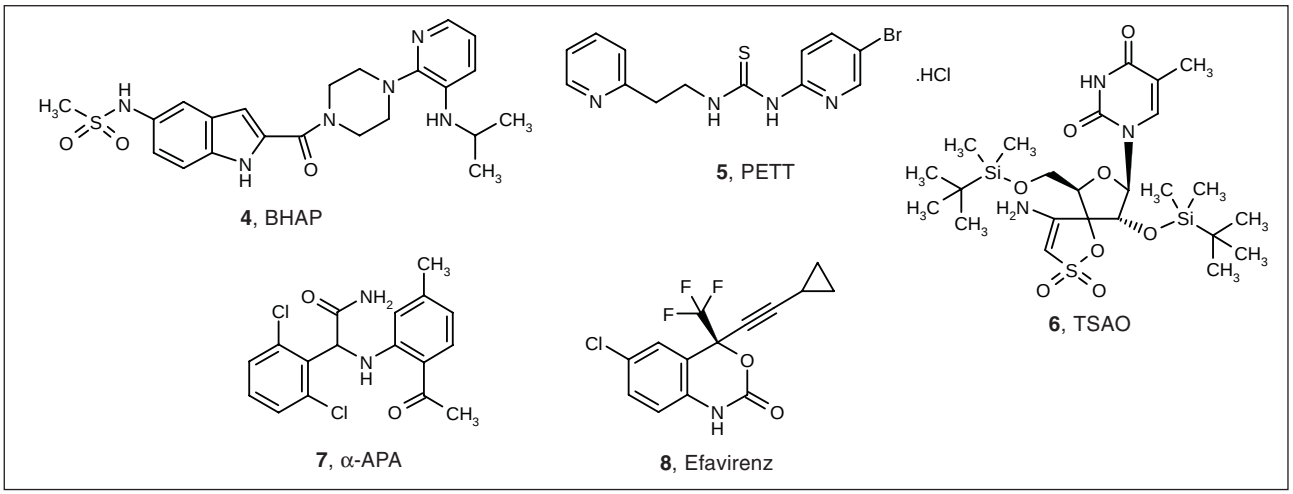


Fig. 2. NNRT inhibitors.

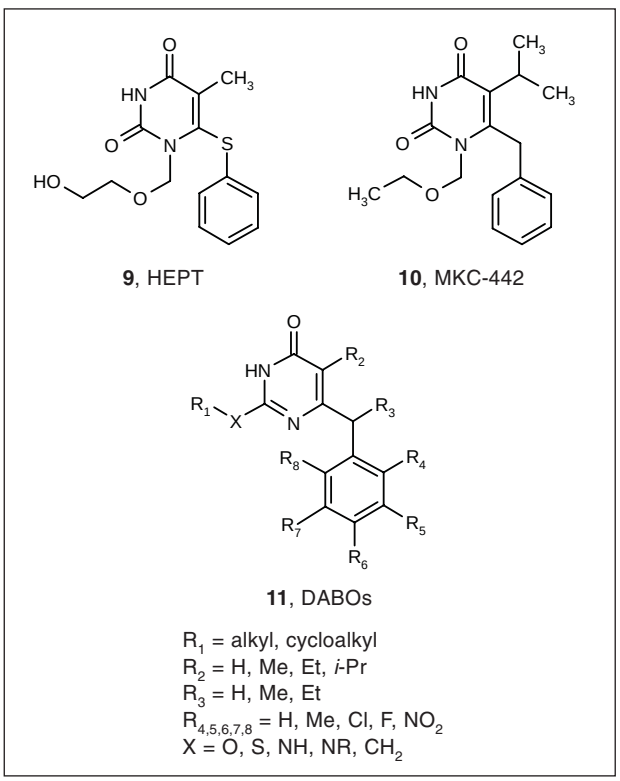


Fig. 3. DABO and HEPT inhibitors of RT.

The DABOs family

DABOs are a potent family of NNRTIs belonging to the 4-pyrimidinone series as well as HEPTs, the series discovered by Tanaka's group in 1989 (15). Both DABOs and HEPTs include uracil and thymine derivatives and related variants at position 5 of the pyrimidine ring. Different from the parent lead (**9**) of the HEPT series (15) but similar to MKC-442 (**10**) (Fig. 3), a selected preclinical variant of HEPTs (16-18), DABOs have as a salient chemical feature a benzyl chain at position 6. Another peculiar feature, which is a high determinant for the anti-HIV-1 activity of DABOs, is the O (S, NH) alkyl or cyloalkyl side-chain linked at the C-2 position of the pyrimidine ring. In the HEPT series the *seco*-sugar side-chain, which is needed for antiviral activity, is linked at the C-1 position.

Like other NNRTIs such as nevirapine, TIBO, TBZ, HEPT and α-APA, DABOs assume a "butterfly-like" spatial arrangement with the methylene as a bridge between the two wings of the benzene and pyrimidine rings. This configuration was determined by crystallographic analyses and molecular modeling studies of the overlapping of DABOs and HEPTs within the non-nucleoside binding site (NNBS) of reverse transcriptase (19-21).

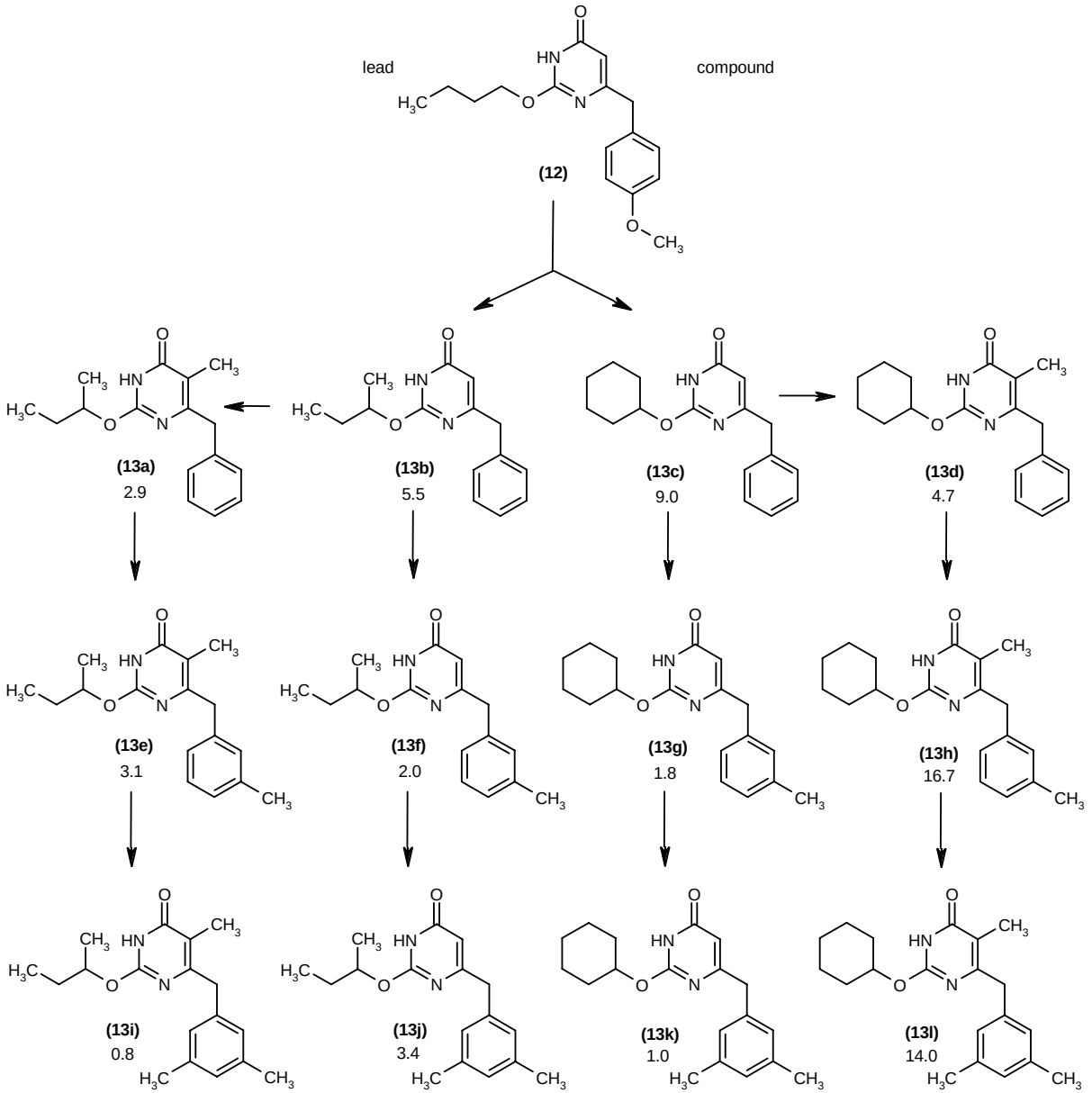
Like all the NNRT inhibitors, DABOs targeted the HIV-1 reverse transcriptase and were inactive when tested against HIV-2.

Since their discovery in 1993 (22), DABOs have been the object of great interest and have led in the last years to the identification of highly potent DABO derivatives with 50% inhibitory concentration (EC_{50}) (23, 24) at low nanomolar concentrations and without detectable cytotoxicity at concentrations as high as 200 μM.

First generation DABOs: dihydroalkyloxybenzyloxopyrimidines

In light of the appreciable results obtained by Tanaka with non-nucleoside HEPT derivatives (15, 25-27), in 1992 Artico and coworkers submitted to anti-HIV-1 screening some 4-pyrimidinones, which were initially synthesized as trimethoprim-like potential inhibitors of dihydrofolatoreductase (22). One of these pyrimidinones, namely 6-(4-methoxybenzyl)-2-butyloxy-3,4-dihydro-4-oxopyrimidine (**12**), when assayed on HIV-1-infected C8166 cells was shown to be a selective inhibitor of HIV-1 with some appreciable activity (ED_{90} = 25 μg/ml) and

*Table I: Chemical modulation of dihydroalkoxybenzyloxopyrimidines **13a-l** (DABOs): from lead compound to 2-*sec*-butyloxy-5-methyl-6-(3,5-dimethylbenzyl)-3,4-dihydro-4-oxopyrimidine (**13i**) active at 0.8 micromolar concentration.*



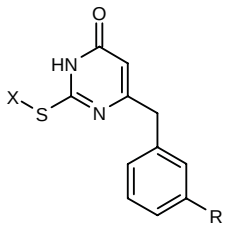
was chosen as a lead for further investigations on 6-benzyl substituted uracil and thymine congeners.

Chemical modifications aimed at increasing the antiviral activity of **12** were attempted, such as: i) transformation of the uracil moiety into thymine by introducing a methyl group at position 5 of the pyrimidine ring (28, 29); ii) replacing the *n*-butyl of the alkoxy chain linked at position 2 of pyrimidine with ramified (*i*-propyl, *i*-butyl, *s*-butyl) or cycloalkyl (*c*-pentyl, *c*-hexyl, *c*-heptyl) groups (28, 29); iii) introduction of methyl groups at positions 3- and 3,5- on the phenyl ring (30), substitutions that have been shown to enhance the potency of HEPT derivatives.

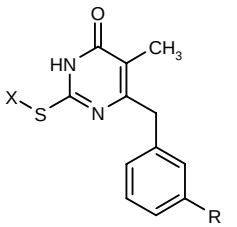
Starting from the lead compound **12** a progressive increase in potency was observed with the above modifications, the best results being obtained in the thymine series, with the *sec*-butyloxy and cyclohexyloxy chains and the 3,5-dimethylbenzyl moiety. Table I depicts such modifications together with activity expressed as EC_{50} values (μM) below the serial numbers.

Among the first generation DABOs (**13a-l**) assayed against HIV-1, the most active was 2-*sec*-butyloxy-5-methyl-6-(3,5-dimethylbenzyl)-3,4-dihydro-4-oxopyrimidine (**13i**, EC_{50} = 0.8 μM). This compound in an enzyme assay inhibited reverse transcriptase (IC_{50} = 1.8 μM) and

Table II: Antiviral activity of thio-DABOs.



14a-o (uracils)



14p-a' (thymines)

R	X	EC_{50} (μM)	R	X	EC_{50} (μM)
H	methyl (**14a**)	34.5	H	methyl (**14p**)	1.2
H	*iso*-propyl (**14b**)	7.7	H	*iso*-propyl (**14q**)	1.8
H	*iso*-butyl (**14c**)	5.1	H	*iso*-butyl (**14r**)	**0.8**
H	*sec*-butyl (**14d**)	1.2	H	*sec*-butyl (**14s**)	**0.6**
H	cyclopentyl (**14e**)	1.7	H	cyclopentyl (**14t**)	**0.6**
H	cyclohexyl (**14f**)	**0.8**	H	cyclohexyl (**14u**)	**0.6**
Me	methyl (**14g**)	17.0	Me	methyl (**14v**)	3.0
Me	*iso*-propyl (**14h**)	1.3	Me	*iso*-propyl (**14w**)	1.3
Me	*iso*-butyl (**14i**)	3.3	Me	*iso*-butyl (**14x**)	1.6
Me	*sec*-butyl (**14j**)	**0.6**	Me	*sec*-butyl (**14y**)	1.0
Me	cyclopentyl (**14k**)	1.2	Me	cyclopentyl (**14z**)	**0.6**
Me	cyclohexyl (**14l**)	1.5	Me	cyclohexyl (**14a'**)	**0.6**
Me	allyl (**14m**)	3.6			
Me	3-methylallyl (**14n**)	8.0	Nevirapine		**0.3**
Me	3,3-dimethylallyl (**14o**)	13.0	AZT		**0.01**

showed low cytotoxicity (CC_{50} >335) with a good selectivity index (SI >410). In comparison with HEPT (**9**, EC_{50} = 7 μM) it was about 9 times more potent.

On the basis of the above results, DABO derivatives were considered a novel class of specific inhibitors of HIV-1 replication in acutely infected cells, targeting HIV-1 RT. These compounds were then further pursued to evaluate the extent of structural and/or biological similarities with HEPT derivatives and other non-nucleoside RT inhibitors.

Second generation DABOs: dihydroalkylthiobenzyloxopyrimidines

The rapid development of HEPTs leading to potent modified derivatives, such as E-EPU, E-BPU and E-EBU-dM (31), further guided the approach of Artico's Italian group to novel DABO series. With the aim of correlating DABOs with the sulfur-containing structure of the HEPT prototype, a novel series was designed which differed from previous DABOs in the nature of the side-chain linked at the C-2 position that presents a sulfur atom instead of an oxygen. These new derivatives were referred to as thio-DABOs or *S*-DABOs (**14**) (32).

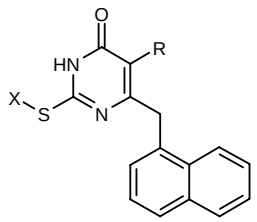
A number of *S*-DABOs were noncytotoxic in MT-4 cells at doses higher than 200 μM and most of them were selective inhibitors of HIV-1 replication (Table II). Maximum activity (EC_{50} = 0.6 μM) was obtained mainly with derivatives of the thymine series bearing as substituents at the C-2 position of the pyrimidine ring *sec*-butylthio (**14s**), cyclopentylthio (**14t**) and cyclohexylthio (**14u**) moieties. Introduction of a methyl group at the *meta* position of the phenyl ring (**14z** and **14a'**) did not increase the maximum potency. The best inhibitor was 2-*sec*-butylthio-3,4-dihydro-6-(3-methylbenzyl)-4-oxopyrimidine (CC_{50} >347 μM, EC_{50} = 0.6 μM, IC_{50} = 1.2 μM and SI >578) (**14j**), a uracil derivative 2-fold and 60-fold less potent than nevirapine and MKC-442, respectively.

Compared to DABOs, the thio-DABOs were more potent and selective, with the C-2 alkylthio chain being the most peculiar determinant for exhibition of anti-HIV-1 activity. Contrary to that observed in the DABOs series, the presence of methyl groups in the phenyl of benzyl moiety did not introduce any benefit in terms of potency and/or selectivity. The last observation was crucial for the further developments in the DABOs field leading research towards substituents in the phenyl ring different from methyl groups.

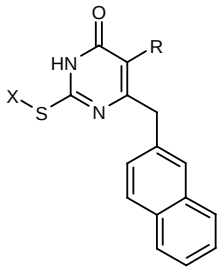
DATNOs: dihydroalkylthionaphthyloxopyrimidines

During molecular modeling studies on HEPTs it was postulated by Hopkins *et al.* (33) that the replacement of the phenylthio group linked at C-6 of the pyrimidine ring with a 1-naphthylmethyl group would improve anti-HIV activity. However, Tanaka's team did not attempt to synthesize the hypothesized naphthylmethyl-HEPTs. In the same year our laboratories synthesized a novel class of

Table III: Antiviral activity of DATNOs.



15a-n



16a-f

R	X	EC_{50} (μM)	Ref	R	X	EC_{50} (μM)	Ref.
H	*iso*-propyl (**15a**)	1.0	34	H	*sec*-butyl (**16a**)	2.8	34
H	*sec*-butyl (**15b**)	**0.33**	34	H	cyclopentyl (**16b**)	3.7	34
H	2-pentyl (**15c**)	4.6	34	H	cyclohexyl (**16c**)	1.2	34
H	3-pentyl (**15d**)	4.0	34	Me	*sec*-butyl (**16d**)	1.2	34
H	cyclopentyl (**15e**)	2.0	34	Me	cyclopentyl (**16e**)	15.0	34
H	cyclohexyl (**15f**)	3.1	34	Me	cyclohexyl (**16f**)	125.0	34
H	cycloheptyll (**15g**)	3.0	34				
H	benzyl (**15h**)	12.6	34	Nevirapine		0.25	6
H	*nor*-undecyl (**15i**)	>200	34	AZT		0.01	6
Me	*sec*-butyl (**15j**)	1.0	34				
Me	cyclopentyl (**15k**)	6.0	34				
Me	cyclohexyl (**15l**)	63.0	34				
Et	Me-thiomethyl (**14m**)	4.6	35				
Et	Et-thyomethyl (**14n**)	2.7	35				

uracil and thymine thio-DABOs with the C-6 phenylmethyl moiety replaced by the 1-naphthylmethyl or 2-naphthylmethyl groups. Most of the new derivatives, termed DATNOs (**15**, **16**) (34), were selective inhibitors of HIV-1-induced cytopathogenicity in MT-4 cells (Table III). Maximum inhibitory potency (EC_{50} = 0.33 μM) was obtained in the uracil series, with *sec*-butylthio and 1-naphthylmethyl groups as substituents at positions C-2 and C-6, respectively.

As a rule, DATNOs bearing a 2-naphthylmethyl moiety were less potent than the related 1-naphthylmethyl counterparts, and introduction of alkyl groups at position 5 of the pyrimidine ring always reduced the activity. In fact, 5-methyl-DATNOs (thymine series) (**15j**,**k**,**l**) prepared by Mai *et al.* (34) showed moderate activity against HIV-1 compared with the uracil series (**15b**,**e**,**f**). Also, 5-ethyl-DATNOs (**15m**,**n**), synthesized together with some 6-naphthyl-HEPTs by Danel *et al.* (35), showed low activity and selectivity.

Replacement of the naphthyl ring with pyridine, thiophene, indole or benzothiophene led to compounds less potent and selective than the DATNOs (36).

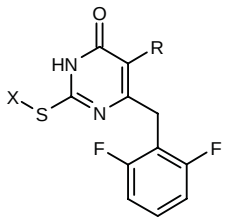
F_2-*S*-DABOs: dihydroalkylthiodifluorobenzyloxopyrimidines

With the aim of obtaining novel DABOs with improved potency and selectivity, various derivatives containing electron-withdrawing groups, such as Cl, F and NO_2, were synthesized and tested against HIV-1 (37). Both the nature of the substituent and its position in the phenyl ring markedly influenced the antiviral activity of the newly synthesized DABOs. Fluoroderivatives were more potent than chloro and nitro, with *ortho* substitution playing a fundamental role. SAR evaluations and the high activity shown by NNRTIs containing a 3,5-dichlorophenyl (α-APA and S-1153) (6, 38) or a 2,6-difluorophenyl moiety (TBZ) (6), led to the design of dihalophenylmethy *S*-DABOs as a novel class of highly potent HIV-1 RT inhibitors (37).

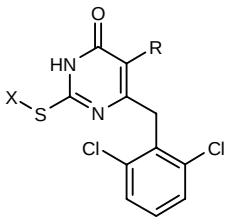
Molecular modeling investigations on the binding mode of *S*-DABOs to the NNBS of HIV-1 RT, based on the crystal structure of the RT/HEPT complex (39), supported the idea that DABOs bearing fluorine (**17**) or chlorine (**18**) atoms at the 2- and 6-positions of the phenyl ring would strongly increase the antiviral activity, with 2,6-difluorination to be preferred. In fact, in light of the molecular modeling studies, the 2,6-dihalogenation was predicted to strongly improve a putative π-staking interaction between the electron-deficient benzene ring of the ligand and the electron-rich benzene ring of Tyr 188 positioned in the non-nucleoside binding cleft of RT. The results of assays on MT-4 chronically infected cells reported in Table IV confirmed 2,6-difluoroderivatives **17** as the most potent and selective among all of the investigated series of *S*-DABOs.

Various difluorothio-DABOs (**17b**,**e**,**f**,**g**,**i**,**j**,**l**,**m**,**n**) were active at concentrations ranging between 40 and

Table IV: Cytotoxicity and anti-HIV-1 activity of dihalo-S-DABOs.



17a-n



18a-n

R	X	CC_{50} (μM)	EC_{50} (μM)	R	X	CC_{50} (μM)	EC_{50} (μM)
H	Me (**17a**)	>200	0.81	H	Me (**18a**)	>200	3.2
H	*iso*-propyl (**17b**)	>200	**0.05**	H	*iso*-propyl (**18b**)	>200	1.9
H	*nor*-butyl (**17c**)	162	0.18	H	*nor*-butyl (**18c**)	>200	0.44
H	*iso*-butyl (**17d**)	182	0.14	H	*iso*-butyl (**18d**)	>200	0.45
H	*sec*-butyl (**17e**)	>200	**0.04**	H	*sec*-butyl (**18e**)	>200	0.14
H	cyclopentyl (**17f**)	>200	0.08	H	cyclopentyl (**18f**)	>200	0.4
H	cyclohexyl (**17g**)	>200	0.08	H	cyclohexyl (**18g**)	>200	0.6
Me	Me (**17h**)	>200	0.19	Me	Me (**18h**)	>200	38
Me	*iso*-propyl (**17i**)	>200	**0.05**	Me	*iso*-propyl (**18i**)	>200	1.3
Me	*nor*-butyl (**17j**)	>200	0.08	Me	*nor*-butyl (**18j**)	>200	1.1
Me	*iso*-butyl (**17k**)	164	0.1	Me	*iso*-butyl (**18k**)	>200	1.2
Me	sec-butyl (**17l**)	>200	**0.05**	Me	*sec*-butyl (**18l**)	>200	**0.05**
Me	cyclopentyl (**17m**)	>200	0.08	Me	cyclopentyl (**18m**)	>200	1.8
Me	cyclohexyl (**17n**)	>200	0.09	Me	cyclohexyl (**18n**)	>200	22
Nevirapine		>200	0.3				
MKC-442		200	0.01				

90 nM and were noncytotoxic for MT-4 cells at doses as high as 200 μM, thus displaying selectivity indexes from >2,500 to >5,000. Such difluoro derivatives were comparable in potency and selectivity to MKC-442 (CC_{50} = 200 μM, EC_{50} = 30 nM and SI = 6,666).

MTM-*S*-DABOs: dihydromethylthiomethylthiobenzyloxopyrimidines

In 1998 Uckun and coworkers performed a study which combined structural information from several RT-NNI complexes to generate a composite molecular surface revealing a larger than presumed NNI binding pocket. As a result of their research they designed and synthesized some 5-alkyl-2-[(methylthiomethyl)thio]-6-(benzyl)-pyrimidin-4(1*H*)-ones (**19**) as novel and highly potent non-nucleoside reverse transcriptase inhibitors of the *S*-DABO series (40-42).

Their computational approach allowed the identification of 5-*iso*-propyl-2-[(methylthiomethyl)thio]-6-(benzyl)-pyrimidin-4(1*H*)-one (**19d**), which was claimed to show potent anti-HIV activity with an IC_{50} value of < 1 nM for inhibition of HIV replication, without any evident cytotoxicity and an unprecedented selectivity index of >100,000.

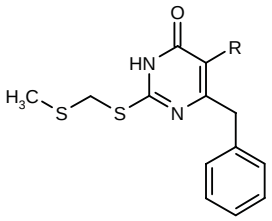
Due to our recent success in obtaining compounds with anti-HIV potency in the low nanomolar range following the introduction of the 2,6-difluorophenylmethyl moiety at the C-6 position of the pyrimidine ring in the *S*-DABO structure, we decided to synthesize methylthiomethyl (MTM) derivatives of the *S*-DABO (**19**) and F_2-*S*-DABO (**20**) series (43) and to compare their anti-HIV-1 activities with those of the related C-2 *sec*-butylthio counterparts (**21** and **22**) (Table V) (32, 37).

When evaluated for anti-HIV-1 activity by the MTT (EC_{50} values) or the p24 assay (EC_{90} values), MTM-*S*-DABOs (**19a-d**) and *sec*-butyl-*S*-DABOs (**21a-d**) proved to be active at micromolar concentrations with potencies falling in the same range. The only exception was the 5-*iso*-propyl-2-MTM-*S*-DABO (**19d**), which in our tests was about three orders of magnitude less potent than that found by Vig *et al.* (40) in their experiments.

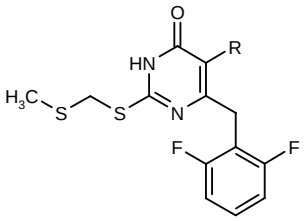
As expected, MTM-*S*-DABOs behaved as previous DABOs. In fact, their potency did not change as a result of a 3,5-dimethyl substitution (40) in the phenyl ring of the phenylmethyl moiety, whereas it strongly increased following a 2,6-difluoro substitution (43). Accordingly to the HEPT series, MTM-*S*-DABOs (**19a-d**) increase their potency with the size of the C-5 substituent from hydrogen to *iso*-propyl, whereas the difluoro-MTM-*S*-DABOs (**20a-d**) behave inversely in that they show maximum activity (EC_{50}) when the H atom is present at the C-5 position of the pyrimidine ring.

In conclusion, our experiments provided evidence that the introduction of a 2-MTM-side chain is not sufficient *per se* to significantly increase the DABOs' potency. Nevertheless, although potent MTM-*S*-DABOs can be obtained following a 2,6-difluorophenylmethyl group as a C-6 substituent, it is evident that the C-2 *sec*-butylthio

Table V: Cytotoxicity, anti-HIV-1 and anti-rRT activities of MTM-S-DABOs.

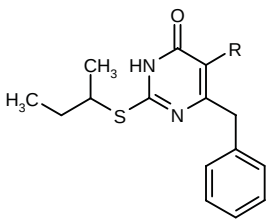


19a-d

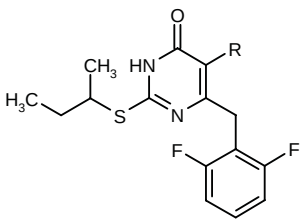


20a-d

R	CC_{50} (μM)	EC_{50} (μM)	EC_{90} (μM)	IC_{50} (μM)	SI	R	CC_{50} (μM)	EC_{50} (μM)	EC_{90} (μM)	IC_{50} (μM)	SI
H (**19a**)	>200	20	27	31	>10	H (**20a**)	>200	0.80	1.4	0.90	>250
Me (**19b**)	160	7.0	11	3.2	23	Me (**20b**)	30	0.12	0.20	0.13	250
Et (**19c**)	123	3.1	3.5	0.5	40	Et (**20c**)	53	0.11	0.15	0.10	450
i-Pr (**19d**)[a]	175	1.6	2.3	0.3	110	*i*-Pr (**20d**)	≥200	0.10	0.17	0.03	>2,000



21a-d



22a-d

R	CC_{50} (μM)	EC_{50} (μM)	EC_{90} (μM)	IC_{50} (μM)	SI	R	CC_{50} (μM)	EC_{50} (μM)	EC_{90} (μM)	IC_{50} (μM)	SI
H (**21a**)	150	1.2	2.0	1.8	125	H (**22a**)	≥200	0.04	0.04	0.05	≥5,000
Me (**21b**)	86	0.6	1.2	1.4	140	Me (**22b**)	≥200	0.05	0.03	0.05	≥4,000
Et (**21c**)	>200	1.0	1.2	0.8	>200	Et (**22c**)	≥200	0.08	0.10	0.12	≥2,500
i-Pr (**21d**)	>200	2.7	0.9	1.3	≥74	*i*-Pr (**22d**)	>200	0.10	0.12	0.16	>2,000
MKC-442	>200	0.03	0.02	0.04	>6,666						

[a]Vig (40) reported: IC_{50}(MTA) > 100 μM (cytotoxicity), IC_{50}(P24) < 0.001 μM (p24 production), IC_{50}(rRT) = 6.1 μM (inhibition of recombinant reverse transcriptase) and SI = >100,000.

chain is the major determinant for anti-HIV-1 activity in the difluoro-*S*-DABO series (compare derivatives **20a-d** with the **22a-d** counterparts).

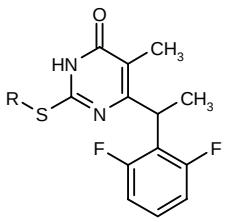
Conformationally restricted F_2-*S*-DABOs

In pursuing our lead optimization efforts we have recently investigated a novel series of *S*-DABOs (**23**) (44) designed as conformationally restricted analogues of the difluoro-*S*-DABOs featuring a methyl group at the benzylic methylene and at the pyrimidine C-5 position. Studies based on conformational analyses and docking simulations suggested that the presence of both methyls would strongly reduce the conformational flexibility without compromising, in the (*R*)-enantiomers, the capability of fitting into the RT non-nucleoside binding pocket (NNBP).

All of the newly described compounds were evaluated for their cytotoxicity and HIV-1 inhibitory activity in MT-4 cells and some of them were assayed against highly purified recombinant wild-type HIV-1 RT using homopolymeric template primers. The results of assays, expressed as CC_{50} (cytotoxicity), EC_{50} (anti-HIV-1 activity), SI (selectivity index, given by the CC_{50}/EC_{50} ratio) and IC_{50} (RT inhibitory activity) values, are reported in Table VI for the C-6 2,6-difluorophenylmethylthymines, the most active series. In this series, methylation of the α-benzylic carbon strongly improved anti-HIV-1 and RT inhibitory activities together with selectivity. The C-2 cyclopentylthio derivative **23f** turned out to be the most potent and selective (CC_{50} > 200 μM, EC_{50} = 6 nM, IC_{50} = 5 nM and SI >33,333) among the thio-DABOs described to date.

Chiral resolution (45) of *rac*-2-cyclopentyl-6-[1-(2,6-difluorophenyl)ethyl]-3,4-dihydro-5-methylpyrimidin-

Table VI: Conformationally restricted F_2-S-DABOs: cytotoxicity and antiviral activities.



23a-g

R	CC_{50} (μM)	EC_{50} (μM)	IC_{50} (μM)	SI
Me (**23a**)	>200	0.04	0.02	>5,000
i-Pr (**23b**)	200	0.007	0.019	>28,570
n-Bu (**23c**)	112	0.008	ND	14,000
i-Bu (**23d**)	>200	0.01	ND	>20,000
s-Bu (**2e**)	>200	0.03	0.02	6,666
cyclopentyl (**23f**)	>200	0.006	0.005	>33,333
cyclohexyl (**23g**)	>200	0.018	ND	>11,111
[+] cyclopentyl	>200	0.002	0.008	>100,000
[–] cyclopentyl	>200	0.07	1.0	>2,867
MKC-442	200	0.03	0.04	6,666
Nevirapine	>200	0.3	0.3	>666
Efavirenz	35	ND	0.048	729

ND: not determined

4(3*H*)-one (**23f**) by chiral HPLC and anti-HIV-1 assay performed on the pure (+)-enantiomer, which was much more active than the (–)-enantiomer, furnished unprecedented results such as CC_{50} >200,000 nM, EC_{50} = 2 nM, IC_{50} = 8 nM and SI >100,000, under conditions wherein the Mitsubishi Kasei Corporation Laboratories compound MKC-442 was less active and selective (CC_{50} = 200 μM, EC_{50} = 30 nM, IC_{50} = 40 nM and SI = 6,666).

SAR studies on 43 newly synthesized derivatives of the constrained series pointed out some important features: i) the 2,6-difluorophenylethylthymines were generally endowed with higher potency compared with the uracil counterparts; ii) in the 2,6-difluorophenylpropylthymines series (methyl replaced by ethyl at the benzylic carbon) the activity was retained or diminished slightly, thus suggesting that groups larger than ethyl would not be compatible with dimensions of the cavity within the RT hosting the alpha-benzylic substituent; iii) in accordance with the favorable electronic effect exerted by the 2,6-fluorine atoms on a putative charge-transfer interaction between the aromatic moieties of the inhibitor and Tyr 188, aryl groups different from 2,6-difluorophenyl, such as phenyl, 2,6-dichlorophenyl and 1-naphthyl, were detrimental for activity; iv) in the 2,6-difluorophenylmethyl series the best performance in potency was displayed by 2-cyclopentylthio (**23f**) and 2-*iso*-propylthio (**23b**) side-chains, followed by *iso*-butylthio (**23d**) and *nor*-butylthio (**23c**), respectively.

Hybrids between *S*-DABO and HEPT analogues

Among NNRTIs of clinical interest, both *S*-DABOs and HEPT occupy a determinant place as reference models for further developments in pursuing anti-HIV-1 agents. Therefore, as a variant of research on DABOs the synthesis of derivatives which could be considered as hybrids between *S*-DABOs and HEPT was carried out. These include the conformationally restricted 2,3-dihydro-7*H*-thiazolo[3,2-*a*]pyrimidin-7-ones (46), various uracil and cytosine derivatives (47, 48) featuring similarities with both HEPT and *S*-DABOs and some 4-benzylpyridinones (49), which are pyridinone hybrids of HEPT/*S*-DABO derivatives.

Figure 4 shows, in comparison with **23f** and MKC-442 (**10**), the most active compounds together with their

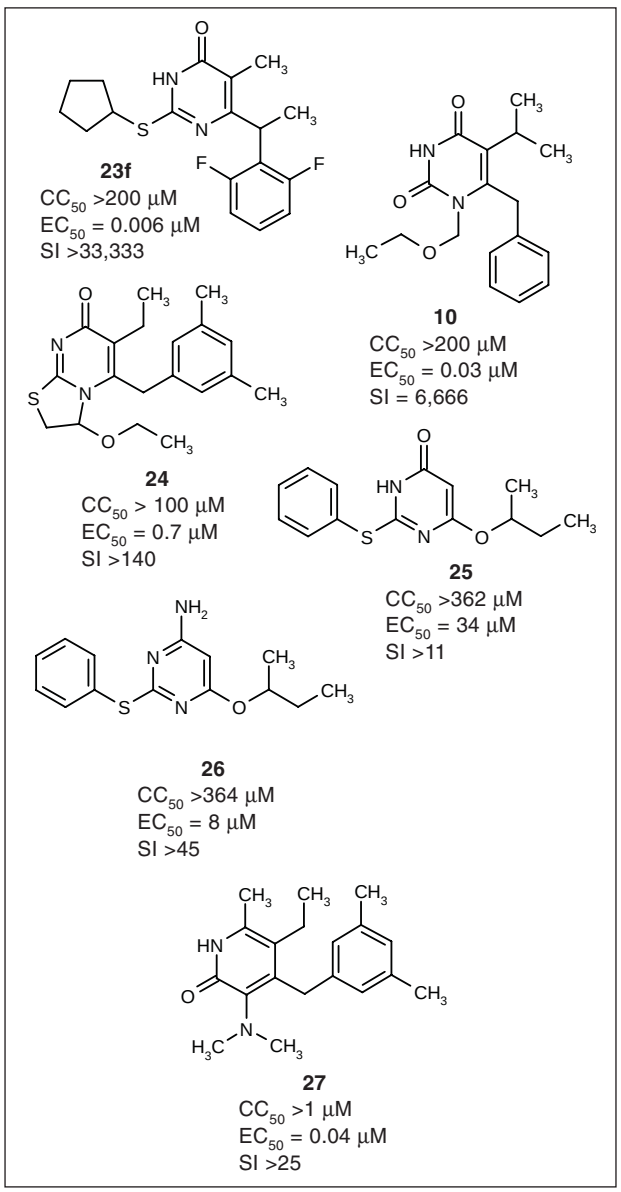


Fig. 4. Hybrids between DABO and HEPT inhibitors.

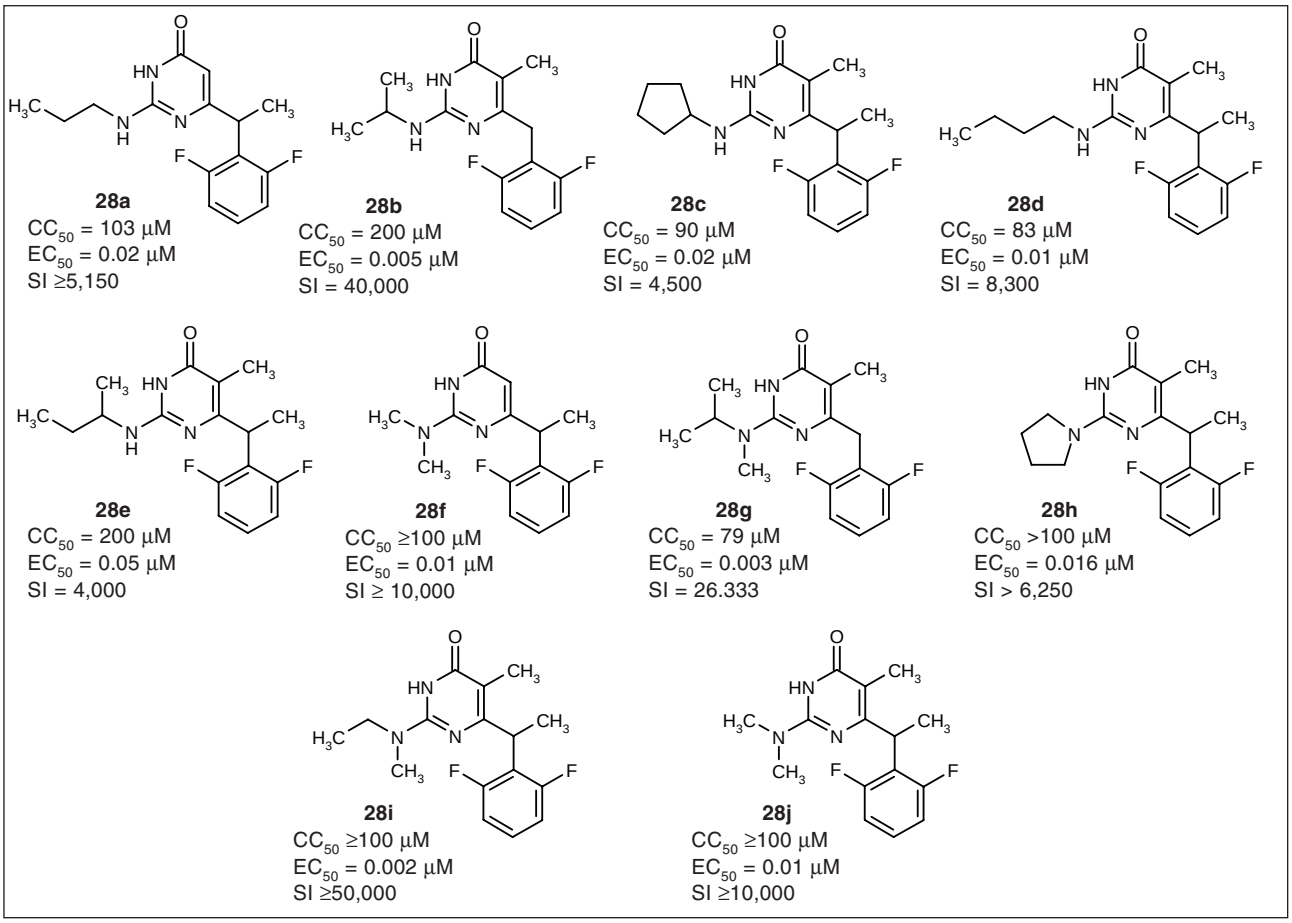


Fig. 5. Mono- and disubstituted amino-DABOs.

experimental CC_{50}, EC_{50} and SI values. Due to their low activity, research on 2,3-dihydro-7*H*-thiazolo[3,2-*a*]pyrimidin-7-ones (**24**) and on uracil (**25**) and cytosine (**26**) HEPT-like derivatives was abandoned. Only 4-benzylpyridinone derivatives (**27**) are still under development.

Third generation DABOs: dihydroalkylaminodifluorobenzyloxopyrimidines

Despite the low anti-HIV-1 activity shown by some isocytosine derivatives described by Botta *et al.* (50), our group was interested in investigating isocytosine-DABOs (**28a-j**), which differ from the *S*-DABOs series in that they replace the C-2 alkylthio/cycloalkylthio side-chain with the bioisosteric alkylamino/cycloalkylamino moieties at the same position. This chemical modification in our mind would help further interactions between the substrate (amino-DABOs) and the enzyme at the NNBS of RT.

A large number of derivatives, including alkylamino, dialkylamino, cycloalkylamino, heterocycloalkylamino and arylamino members, were planned, synthesized and tested against HIV-1 in chronically infected MT-4 cells as well as against wild-type rRT. Assays against Y181C-resistant mutant were also performed (51).

Various amino-DABO derivatives in the cell-based assay were highly active at concentrations falling in the low nanomolar range, without exhibiting any cytotoxicity at concentrations as high as 100 μM. The most representative examples of highly active compounds of this class are reported in Figure 5, together with their experimentally determined CC_{50}, EC_{50} and SI values.

The anti-HIV-1 activity of amino-DABOs were similar to the activities of the previously reported *S*-DABO series, including DATNOs and difluoro-*S*-DABOs. This is exemplified in Figure 6, which reports some *N*-DABOs and the related *S*-DABO counterparts bearing 2,6-difluorophenylmethyl and cyclopentylthio or cyclopentylamino as substituents at the C-6 and C-2 positions, respectively, and having hydrogen or methyl as substituents at the C-5 position of pyrimidine and at the α-benzylic position of the C-6 side-chain.

Table VII gives the results of assays against Y181C-resistant mutant of some conformationally restricted DABOs belonging to the thio and amino series in comparison with nevirapine and efavirenz as reference compounds. Two derivatives, 2-cyclopentylthio-5-methyl-6-

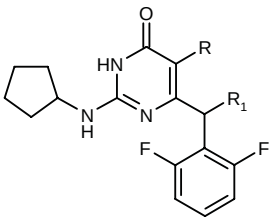
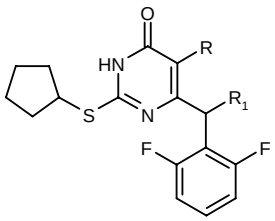
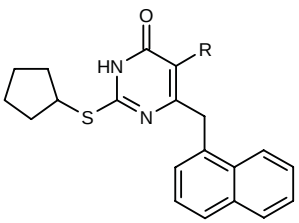
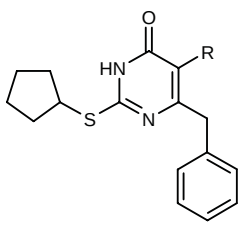
DABOs	R	R^1	CC_{50} (μM)	EC_{50} (μM)	SI
	H	H	>200	0.09	>2,222
	Me	H	>200	0.02	>10,000
	H	Me	76	0.03	2,533
	Me	Me	90	0.02	4,500
	H	H	>200	0.08	>2,500
	Me	H	>200	0.08	>2,500
	H	Me	>200	0.03	>6,666
	Me	Me	>200	0.006	>33,333
	H	H	>200	2.0	>100
	Me	H	>200	6.0	>33
	H	H	>200	1.7	>118
	Me	H	>200	0.6	>333

Fig. 6. Activity of amino-DABOs compared with those of some related thio-DABOs.

[α-(2,6-difluorophenyl)ethyl]-4-oxopyrimidine and 2-(*N*-methyl-*N*-ethylamino)-5-methyl-6-[α-(2,6-difluorophenyl)ethyl]-4-oxopyrimidine, showed activities against WT_{IIIB} and Y181C mutant comparable to those displayed by efavirenz as evidenced by the comparison of EC_{50} values (7 and 4 nM *versus* 4 nM for efavirenz) and fold resistance ratios (13 and 17 *versus* 6 for efavirenz). Our MTM derivative had low activity against Y181C mutant similar to the 5-isopropyl analogues described by Mao *et al.* (42).

Development of the amino-DABO series is still ongoing, with particular attention focused on SAR studies, molecular modeling and assays against resistant mutants to select more appropriate drugs for preclinical trials.

C-DABOs

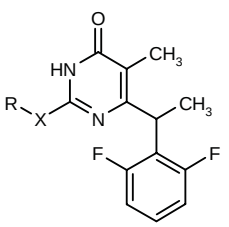
To prove that heteroatoms O (DABOs), S (thio-DABOs) and N (amino-DABOs), forming a bridge between the alkyl/cycloalkyl aliphatic chain and the pyrimidine ring, are an important structural feature relevant for antiviral activity, we decided to synthesize a novel class of DABOs (**29**) with the heteroatom replaced by a methylene group (52). Such derivatives, called *C*-DABOs (carbo-DABOs), in contrast to *O*-, *S*- and *N*-DABOs, lack the capability to form additional hydrogen bonds with aminoacids located in the non-nucleoside binding cleft of RT. The diminished interactions between ligand and enzyme lead to a significant decrease in activity, as seen from the comparison between the isopropyl-*C*-DABO (**29a**) and the bioisosteric dimethyl-*N*-DABO (**28k**) (Fig. 7).

2-Cyclopropyl- (**29b**) and 2-phenylmethyl-6-[(2,6-difluorophenyl)methyl]pyrimidine (**29c**) of the *C*-DABOs series were also endowed with low activity and selectivity. Actually, these results do not encourage further research on carbo-DABOs.

Synthesis of DABOs

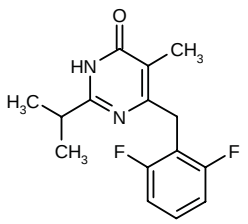
The synthesis of DABOs (28, 30, 32, 34, 36, 37, 43, 44, 53, 54) required as starting material the methyl esters of 2-alkyl-4-aryl-3-oxobutanoic acids. These esters were synthesized by several routes as depicted in Scheme 1.

Table VII: In vitro *activity of some restricted DABOs against the Y181C-resistant mutant.*

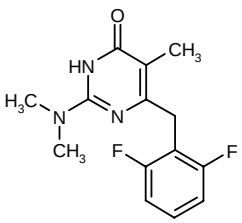


X	R	EC_{50} (nM) WT_{IIIB}	Y181C	
S	cyclopentyl	7	90	(13)[a]
S	*sec*-butyl	20	300	(15)
S	*iso*-propyl	50	300	(6)
S	CH_2SMe	40	90,000	(2250)
NH	*nor*-propyl	20	500	(25)
NMe	Me	8	800	(100)
NMe	Et	4	70	(17)
Morpholine	-	6	600	(100)
Nevirapine		370	>30,000	(>81)
Efavirenz		4	25	(6)

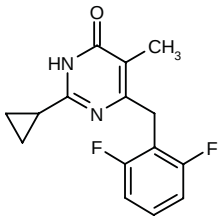
[a]Fold resistance: $Y181C/WT_{IIIB}$ ratio.



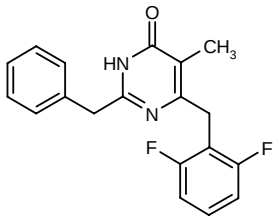
29a
CC_{50} >100 μM
EC_{50} = 0.3 μM
SI >333



28k
CC_{50} >100 μM
EC_{50} = 0.03 μM
SI >3,333



29b
CC_{50} >100 μM
EC_{50} = 1 μM
SI >100

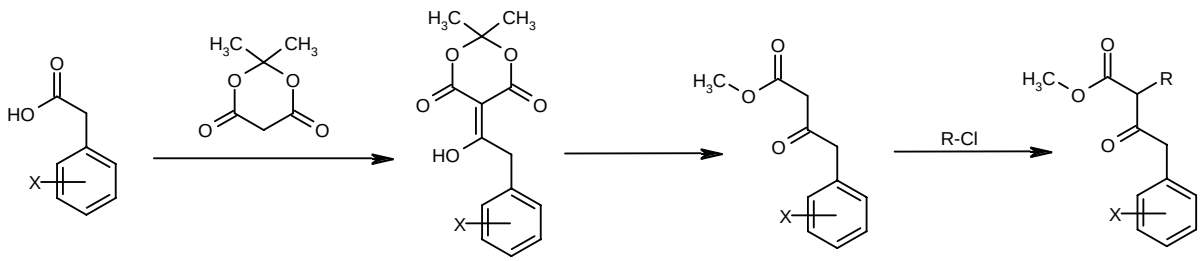


29c
CC_{50} ≥100 μM
EC_{50} = 0.6 μM
SI ≥167

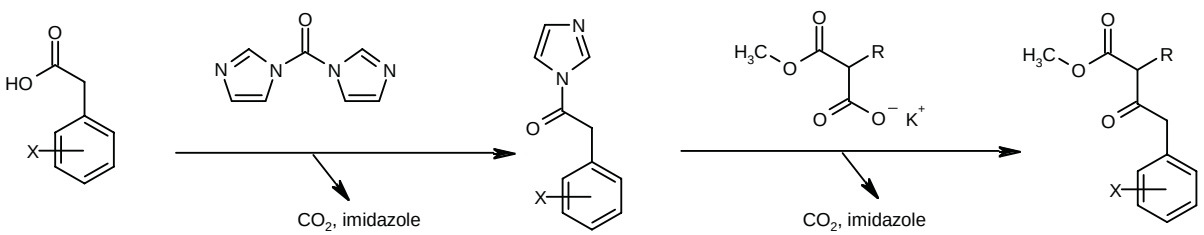
Fig. 7. Some carbo-DABO derivatives.

Scheme 1: Synthesis of 2-alkyl-4-aryl-3-oxobutanoic acid methyl esters

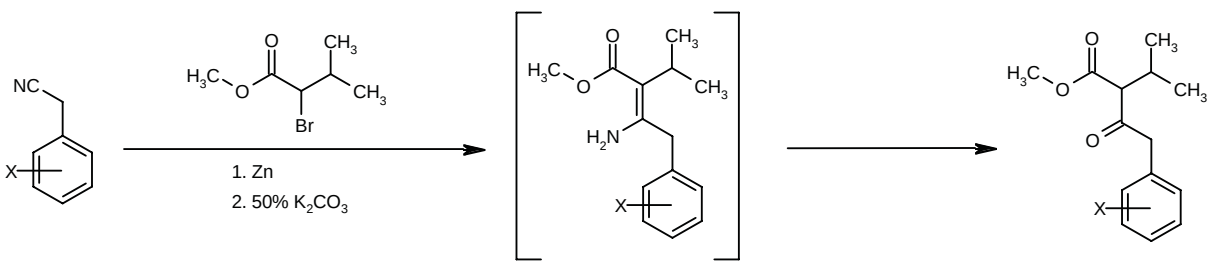
Method A



Method B



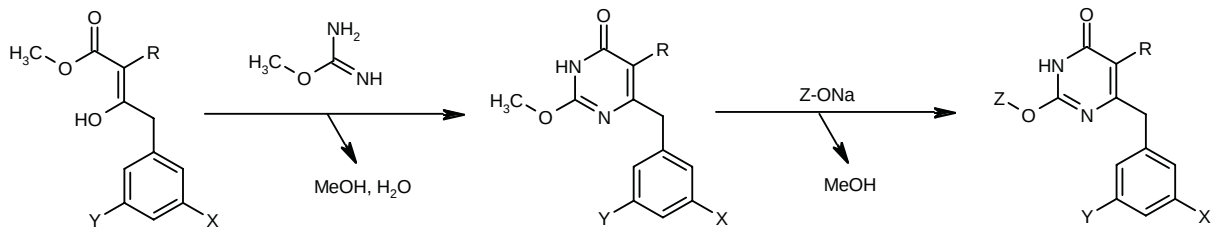
Method C



X = H; *o,m,p*-Me; *o,m,*p-Cl; *o,m,p*-F; 3,5-Me_2; 2,6-Cl_2; 2,6-F_2

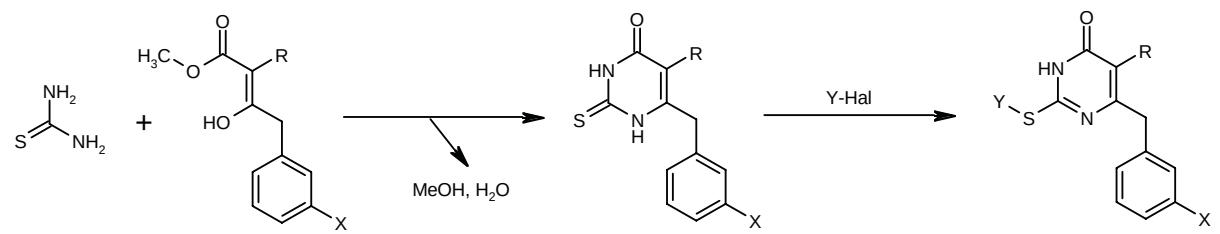
R = H, Me, Et

Scheme 2: Synthesis of DABOs

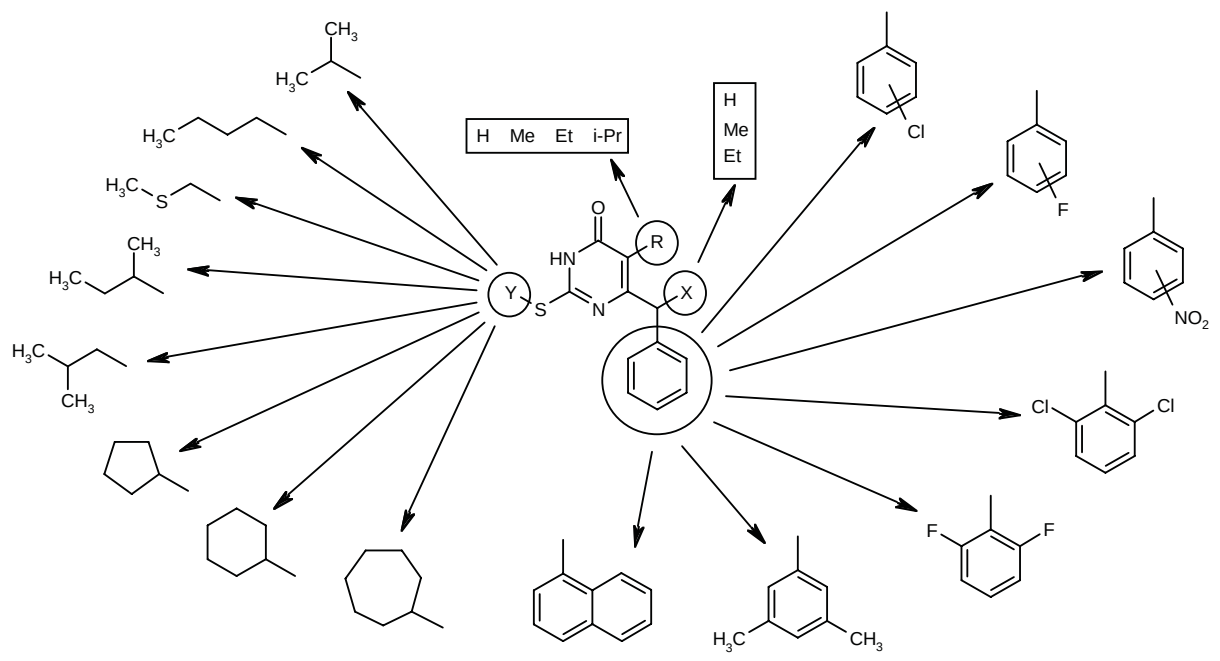


X, Y = H, Me; Z = alkyl, cycloalkyl; R = H, Me, Et

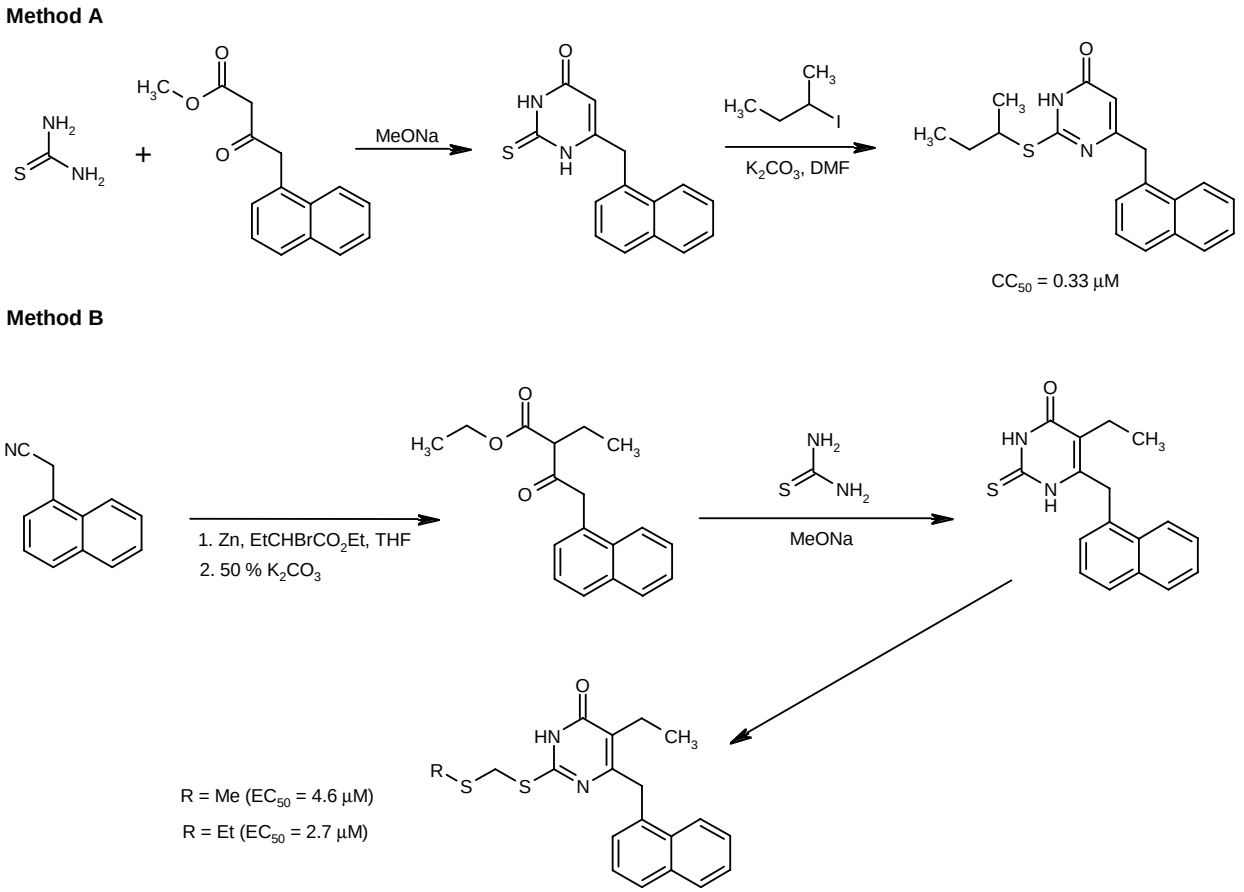
Scheme 3: Synthesis of thio-DABOs with a map of chemical modulations



R, X, = H, Me; Y = alkyl, cycloalkyl



Scheme 4: Synthesis of DATNOs



Reaction of appropriate arylacetyl chlorides with 2,2-dimethyl-1,3-dioxane-4,6-dione (Meldrum's acid) in the presence of anhydrous pyridine followed by methanolysis of the resulting intermediates afforded methyl 4-arylacetyl acetates, which were then alkylated by treatment with an equimolar amount of methyl iodide in the presence of sodium methoxide (Method A).

Alternatively, 4-arylacetic acids were reacted with 1,1'-carbonyldiimidazole to afford 1-(4-arylacetyl)-1*H*-imidazolides, which acylated the potassium salt of the monomethyl ester of alkylmalonic acid in the presence of the magnesium dichloride-triethylamine reagent system (Clay's procedure) to afford the required methyl esters of 2-alkyl-4-aryl-3-oxobutanoic acids (Method B).

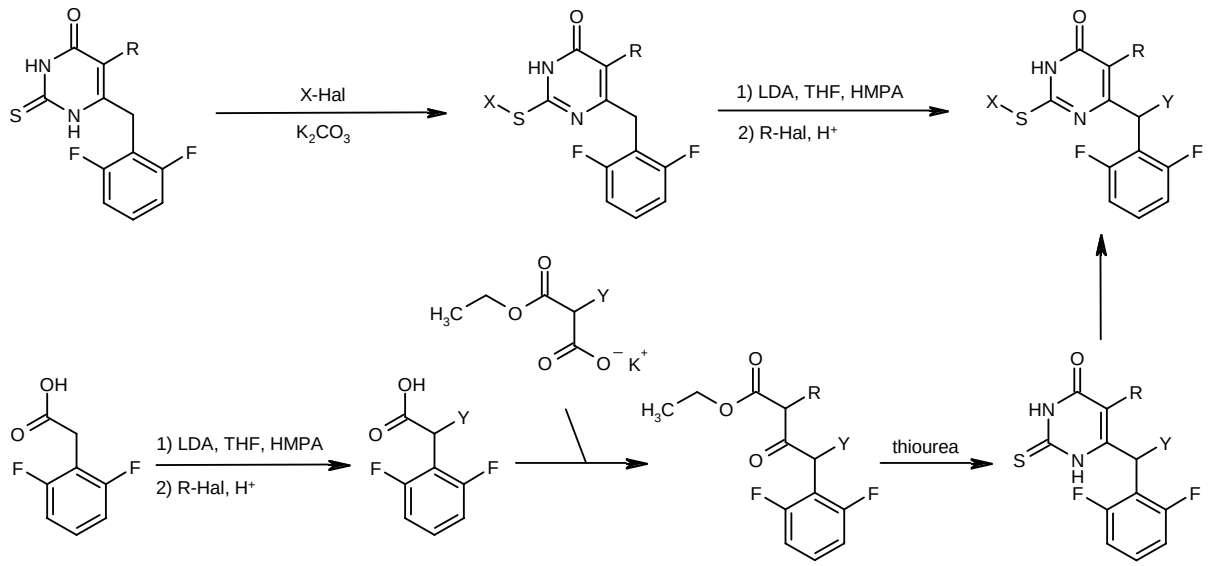
In some cases, *e.g.* for the synthesis of 2-*iso*-propyl-4-aryl-3-oxobutanoic acid methyl esters, arylacetonitriles were used as starting materials. Reaction of nitriles with ethyl-2-bromo-3-methylbutanoate in the presence of activated zinc dust followed by alkaline hydrolysis gave the expected ester (Method C).

Condensation of 2-alkyl-4-aryl-3-oxobutanoic acid methyl esters with *O*-methyl isourea led to the formation of 5-alkyl-6-arylmethyl-3,4-dihydro-2-methoxy-4-oxopyrimidines. Subsequent displacement of the methoxy group linked at C-2 of the pyrimidine ring by treatment with alkoxy or cycloalkoxy potassium salt furnished 2-alkoxy-5-alkyl-6-arylmethyl-3,4-dihydro-4-oxopyrimidines (DABOs) (Scheme 2).

When 2-alkyl-4-aryl-3-oxobutanoic acid methyl esters were reacted with thiourea, in the presence of sodium methoxide, condensation occurred with formation of 5-alkyl-3,4-dihydro-6-arylmethyl-4-oxo-2-thioxopyrimidines. Reaction of the latter compounds with the appropriate alkyl or cycloalkyl halide in alkaline medium afforded the required 2-(alkylthio)-5-alkyl-6-arylmethyl-3,4-dihydro-4-oxopyrimidines (thio-DABOs). By this procedure a variety of derivatives including chloro-, fluoro- and nitro-phenylmethyl moieties, and *sec*-butyl-, cyclopentyl- and cyclohexyl-thio substituents at the C-2 position of 5-alkyl-4-oxopyrimidines were synthesized for SAR studies (Scheme 3).

Condensation of 4-(1-naphthylmethyl)-3-oxobutanoic acid methyl ester with thiourea followed by alkylation of the thioxo group led to the formation of DATNOs (Method A). Using 1-naphthyl acetonitrile as starting material, Danel *et al.* (Method B) synthesized some 5-ethyl analogues of DATNO bearing an alkylthiomethylthio moiety at the C-2 position of the pyrimidine ring (Scheme 4).

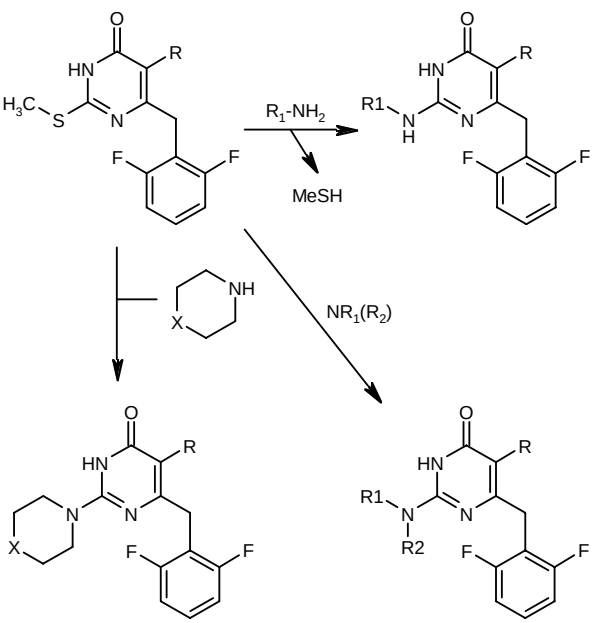
Scheme 5: Synthesis of difluorothio-DABOs and related α-(2,6-difluorophenyl)alkyl analogues



R, Y = H, Me, Et; X = Me, *i*-Pr, *n*-Bu, *i*-Bu; cyclopentyl, cyclohexyl

Scheme 6: Synthesis of amino-DABOs and carbo-DABOs

A. Amino-DABOs

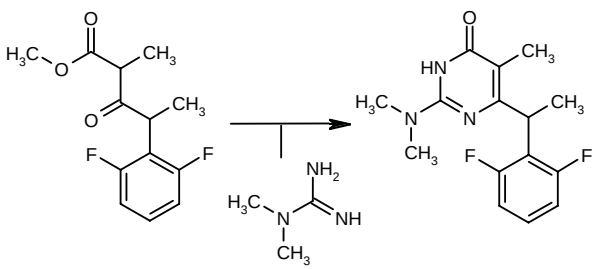


R = H, Me, Et

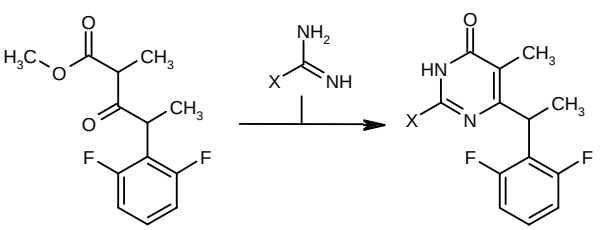
R1,R2 = Me, Et, *n*-Pr, *i*-Pr

X = O, S, CH_2, NH, NR

B. Virucidal amino-DABO MC-1220



C. Carbo-DABOs



X = *i*-Pr, cyclopropyl, phenyl

Alkylation with the appropriate alkyl halides of 5-alkyl-3,4-dihydro-6-(2,6-difluorophenyl)methyl-4-oxo-2-thioxopyrimidines afforded 2-alkylthio-5-alkyl-3,4-dihydro-6-(2,6-difluorophenyl)methyl-4-oxopyrimidines (F_2-*S*-DABOs). The related constrained α-alkyl benzyl analogues (Scheme 5) were prepared by alkylation of 5-alkyl-3,4-dihydro-6-[α-(2,6-difluorophenyl)alkyl]-4-oxo-2-thioxopyrimidines. These were obtained starting from 2,6-difluoroacetic acid as depicted in Scheme 5. Alternatively, 2-alkylthio-5-alkyl-3,4-dihydro-6-[α-(2,6-difluorophenyl)alkyl]-4-oxopyrimidines were obtained from 2-alkylthio-5-alkyl-3,4-dihydro-6-(2,6-difluorophenyl)methyl-4-oxopyrimidines by lithiation at benzylic carbon and subsequent alkylation with the related alkyl halide.

Displacement of the methylthio group by treatment of C-2 MeS-DABOs with appropriate amines furnished amino-DABOs, a novel class of DABOs belonging to the isocytosine series, characterized by alkylamino, dialkylamino, cycloalkyl or heterocycloalkyl moieties at position 2 of the pyrimidine ring (Scheme 6).

Carbo-DABOs, the last pursued series, were synthesized by reacting 2-alkyl-4-aryl-3-oxobutanoic acid methyl esters with the corresponding amidines (Scheme 6).

Virucidal action of DABOs

The heterosexual transmission of HIV requires measures capable of blocking the infective action of retrovirus during coitus. An alternative to condoms are drugs that act as a microbicide following topical application.

A promising candidate among all of the derivatives tested is the thiocarboxanilide UC-781. This compound blocks the early steps of HIV replication and is actually being developed as an effective vaginal microbicide (55). Other NNRT agents, such as MKC-442, α-APA, nevirapine, efavirenz and, among members of the DABOs family, MC-1047 and MC-1220 derivatives, were identified as microbicidal agents (Fig. 8).

A long-term assay for differentiating drugs which irreversibly (virucidal action) inhibit HIV-1 replication from those which act reversibly (virustatic action) has been reported recently by Pani *et al.* (56) during selection of effective and potent microbicides. To validate this assay some NRTIs (ddI, ddC, D4T and 3TC) and the above mentioned NNRTIs were tested.

Under chronic treatment the NRTI simply delayed the viral breakthrough with respect to untreated infected controls, whereas the NNRTIs were able to suppress HIV-1 replication for the entire experimental period of 40 days. Moreover, when drug treatment with NNRTIs was limited to the first 4 hours postinfection, nevirapine and efavirenz proved to be virustatic (the viral breakthrough ensued rapidly after their removal from culture medium); on the contrary, MC-1220 at a concentration of 3.5 μM was able to suppress HIV-1 replication in cultures acutely infected with very high multiplicity of infection (5 $CCID_{50}$/cell), thus demonstrating its potent virucidal activity. Further experiments are still ongoing to define the real potential of MC-1220 as a virucidal agent for future clinical therapy.

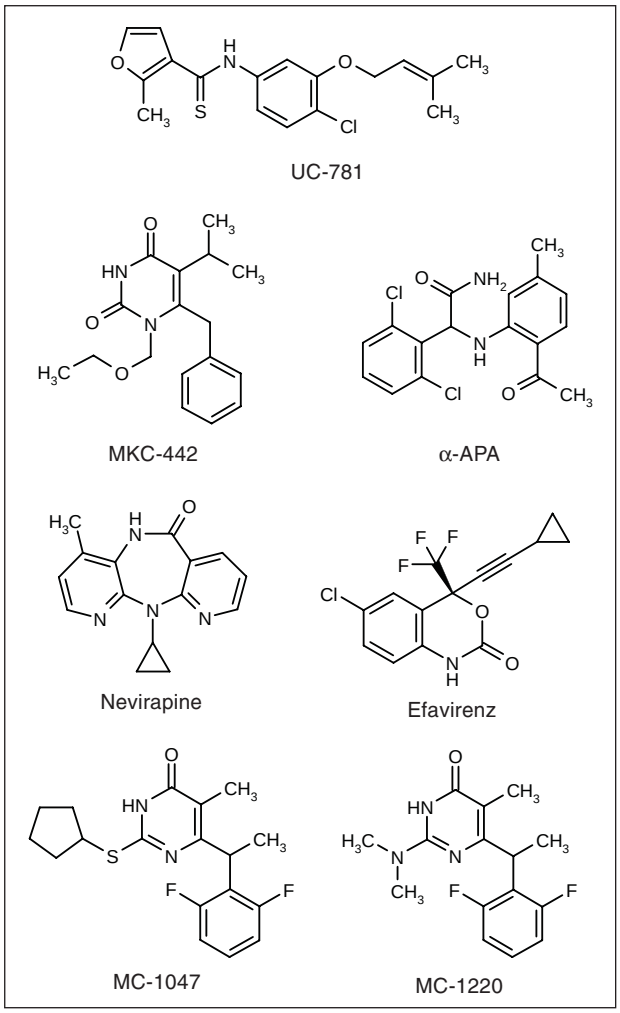


Fig. 8. NNRT virucidal agents.

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