ELSEVIER

Il Farmaco 54 (1999) 255–264

IL FARMACO

Synthesis and anti-HIV-1 activities of new pyrimido[5,4-*b*]indoles

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Received 23 October 1998; accepted 9 March 1999

Abstract

A set of new pyrimido[5,4-*b*]indole derivatives that are structurally related to some non-nucleoside HIV-1 reverse transcriptase inhibitors were synthesized and biologically evaluated for their activity as inhibitors of wild and mutant HIV-1 RT types in an 'in vitro' recombinant HIV-1 RT screening assay, as well as anti-infectives in HLT4lacZ-1_{IIIB} cells. Preliminary structure–activity relationships suggest that activity is promoted by simultaneous substitution in positions 2 and 4, especially when chains of alkyldiamine type are present, and by electron-releasing substituents (methoxy) in positions 7 and 8. The inactivity or the very low activity of title derivatives does not suggest interest in AIDS therapy.

Keywords: Pyrimido[5,4-*b*]indoles; HIV-1 RT inhibitors; HLT4lacZ-1_{IIIB} cells

1. Introduction

Once the HIV virus has entered into the host cell, the transcription process is set off, whereby, starting from viral RNA, the proviral DNA is synthesized which later will integrate into the genome of the cell. This activity is carried out by the reverse transcriptase enzyme, a characteristic enzyme of the retroviruses, and due to its uniqueness it is considered to be a fundamental target for the development of new anti-retroviral agents.

With the discovery of non-nucleoside agents such as HEPT [1] or TIBO [2] (Fig. 1), that are capable of inhibiting this enzyme, a new field has been initiated in anti-AIDS research, revealing a great variety of compounds such as bis(heteroaril)piperazines (BHAPs) [3], TSAO-T [4], or alkenyldiarylmethanes (ADAMs) [5]. Delavirdine (U-90152s), a BHAP non-nucleoside reverse transcriptase inhibitor, has recently been approved by the FDA (Fig. 1). Although these compounds belong to very different structural groups, their interaction with the enzyme in an allosteric site is a common mechanism [6].

The good oral bioavailability that the non-nucleoside inhibitors present and the lack of significant toxicities to the active doses have constituted a stimulus for scientific research in an attempt to find more potent and selective compounds.

However, it has been determined that the more specific an antiviral compound in its antiviral action, the faster it should be in the development of virus-drug resistance. Taking into account that the compounds described up to now are highly specific for the reverse transcriptase, the rapid development of resistances can be related to this specific character. Thus, through directed studies of mutagenesis, in which the mutation of a single aminoacid can be unequivocally associated with the appearance of resistant viruses, the role which the aminoacids of positions 100, 103, 106, 138, 181, 188 and 236 of the HIV-1 reverse transcriptase play in the appearance of resistance to compounds TIBO, HEPT, Nevirapine, Pyridinones, TSAO and BHAPs was confirmed [7–9] (Fig. 1).

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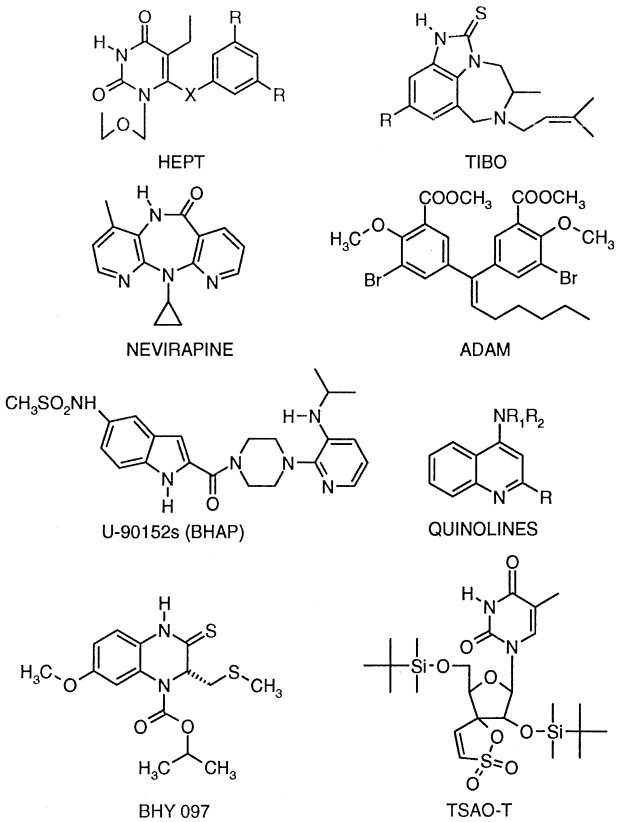


Fig. 1.

Certain mutations are related to the use of specific compounds; thus, the substitution of a proline in position 236 by a leucine (mutant type P236L) offers resistance to the BHAPs; other mutations, such as the substitution of a tirosine in position 181 by a cysteine (mutant type Y181C), appear after treatments with TIBO, HEPT, Nevirapine, Pyridinones and BHAPs, among others, observing, in addition, the existence of crossed resistances among the majority of the nucleoside inhibitors [10,11].

It was also observed that, within a determined type of non-nucleoside inhibitor, small modifications in their structures can increase and even restore the activity of the new resulting compounds against the resistant HIV-1 strains [12].

Taking into consideration all of the aforementioned, and within the research carried out on new anti-AIDS agents by our team [13–15], we present the synthesis and preliminary study of new pyrimido[5,4-*b*]indol-4-one (**I**) and pyrimido[5,4-*b*]indole (**II**) analogs as HIV-1

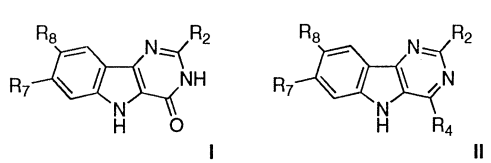


Fig. 2.

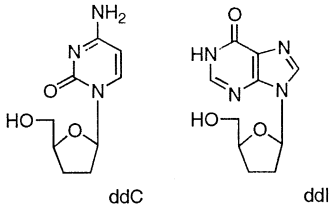


Fig. 3.

reverse transcriptase inhibitors (wild and mutant types) (Fig. 2).

In their structure, these compounds possess an indolic ring, characteristic of compounds of high activity as antiretrovirals (BHAPs, Fig. 1), fusioned with a pyrimidine ring, present in non-nucleoside analogs (TSAO-T, HEPT, Fig. 1) as well as in nucleoside analogs such as ddI or ddC (Fig. 3), up to now the most efficient, along with AZT, in the therapy of AIDS.

Taking as a reference these structures **I** and **II**, different modifications have been carried out in order to obtain data which permit the establishment of the opportune structure–activity relationships in this type of compound.

Derivatives **I** have substituents in position 2, while derivatives **II** in positions 2 and 4. These substituents have been selected using the structure–activity relationships cited in the bibliography as reference for the different groups of non-nucleoside compounds, especially in the case of the BHAPs [3,16] and the quinoline derivatives (Fig. 1) [17].

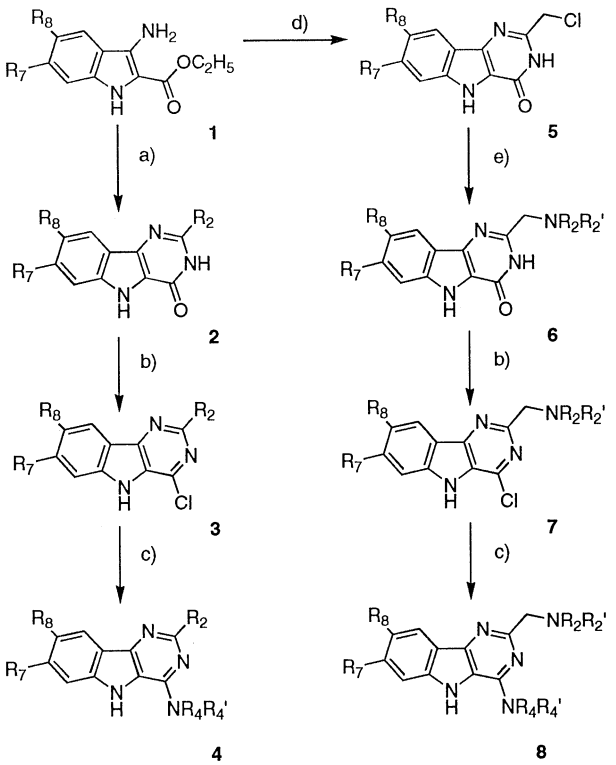
Thus, by analogy to the quinoline series, aliphatic amines and heterocyclic rings are selected as substituents. In the case of **2**, these substituents are connected directly to position 2 of the pyrimido-[5,4-*b*]indol-4-one ring. While maintaining these substituents in position 2, different primary alkyldiamines are selected and introduced in position 4, series **4**.

Bearing in mind the SAR data in the BHAP series, secondary aliphatic cyclic amines (morpholine, piperidine, phenylpiperazines,...) are selected as substituents in series **6**, where they are connected to position 2 by a methylene bridge. For series **8**, alkyldiamines and secondary aliphatic cyclic amines are introduced simultaneously in position 4.

The increase in activity observed in the BHAP series when electrodonor groups are introduced in the indole ring lead us to introduce the methoxy substituent in positions 7 and 8 of the pyrimido[5,4-*b*]indole ring.

2. Chemistry

The synthesis of the compounds described in the present work has been carried out according to Scheme 1. The starting products, ethyl 3-aminoindol-2-carboxylate (**1a**) and ethyl 3-amino-2-carboxylate-5,6-



Scheme 1. Reagents: (a) R_2CN/HCl (gas); (b) $POCl_3$; (c) $NHR_4R'_4$; (d) $ClCH_2CN$; (e) $NHR_2R_{2'}$. R_7, $R_8 = H$; CH_3O.

dimethoxyindole (**1b**), were synthesized with high yield using previously described methods [18,19]. The condensation of **1** with the appropriate nitriles, in a dry dioxane medium and under a flow of HCl (g), gave the corresponding 3,4-dihydro-5*H*-pyrimido[5,4-*b*]indol-4-ones (**2**, Table 1). The treatment of these compounds **2** with $POCl_3$ and dry dioxane at reflux temperature leads to 4-chloro-5*H*-pyrimido[5,4-*b*]indoles (**3**, Table 2). By reaction of these compounds with different primary or secondary amines, in the presence of sodium carbonate in refluxing ethanol or dioxane, compounds **4** were synthesized (Table 2). Application of the method of Shishoo et al. [20] for the obtainment of highly functional pyrimidine rings in a single step allows us to synthesize 2-chloromethyl-5*H*-3,4-dihydropyrimido[5,4-*b*]indol-4-one (**5**), by reaction of **1a** with chloroacetonitrile, with dry dioxane under a flow of HCl (g). The reaction of **5** with the appropriate secondary amines, with ethanol as the solvent and with sodium carbonate, leads to compounds **6** (Table 1). The oxygen in position 4 of compounds **6** can be substituted by chloro, as in the preceding case, through reaction with $POCl_3$, in refluxing dry dioxane, thereby obtaining derivatives **7** (Table 2). The reaction of the last compounds with the selected amines leads to the corresponding derivatives **8** (Table 2).

3. Biology

Initially, the enzyme inhibitory activity of each compound was evaluated in an in vitro recombinant HIV-1 RT wild type screening assay [21–23], at a concentration of 5–50 μM. The compounds whose inhibitory activities were lower than 50% at 50 μM were considered inactive. The active compounds were subsequently evaluated as inhibitors of the mutant HIV-1 RT Y181C enzyme type. Finally, these selected compounds were assayed as anti-infectives in HLT4lacZ-1 cells, and their potential toxicity was evaluated simultaneously [24,25]. The data corresponding to the active products are shown in Table 3. The compounds that do not appear in Table 3 were considered inactive.

4. Discussion

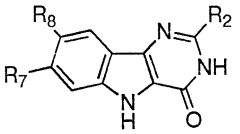
From the initial biological activity data obtained in the in vitro inhibition assay of HIV-1 RT wild type, the following observations can be proposed:

1. The presence of an oxo substituent in position 4 of the pyrimido[5,4-*b*]indole ring is unfavorable for activity. All the analogs of series **2** and **6** studied were inactive in this assay.
2. The presence of a methylene bridge connecting amine substituents in position 2 provokes a notable decrease in activity. The analogs of series **6** and **8** are also inactive.
3. The simultaneous substitution in positions 2 and 4 is favorable for activity, especially when chains of alkyldiamine type are present. In this way, the active products are included in series **4** (Table 3).
4. The introduction of the methoxyl groups at positions 7 and 8 is beneficial; in general, an increase in activity is observed.

In this way, compounds **4e** ($IC_{50} = 37.0$ μM), **4i** ($IC_{50} = 29.2$ μM), **4j** ($IC_{50} = 37.8$ μM), **4m** ($IC_{50} = 69.4$ μM) and **4t** ($IC_{50} = 34.1$ μM) were selected; they are now being studied as inhibitors of the mutant HIV-1 RT Y181C enzyme. The derivatives **4e**, **4i** and **4j** show an activity which is similar to that found against the wild type; no significant differences were found among the IC_{50} values for the mutant and wild types. In the case of the derivatives **4m** and **4t**, a notable decrease in activity against the mutant type is observed.

As an initial approximation, it could be said that the presence of an electrodonor group in positions 7 and 8 and the presence of either a system which is rich in π electrons (furane ring) or an electrodonor group (dimethylamine) in position 2, is favorable for activity against the mutant type. On the contrary, the absence of methoxy substituents in the indole ring and the presence of a heteroaromatic ring deficient in π electrons (pyridine ring) in position 2 leads to compounds

Table 1
Physical constants of 3,4-dihydro-5*H*-pyrimido[5,4-*b*]indol-4-one analogs (**2**, **5**, and **6**)



Comp.	R_2	R_7	R_8	M.p. (°C)	Yield (%) [a]	Recrystallization solvent [b]	Formula [c]
2a	2′-furyl	H	H	270–273	42	A	$C_{14}H_9N_3O_2$
2b	2′-furyl	CH_3O	CH_3O	278–280	60	B	$C_{13}H_{13}N_3O_4$
2c	$N(CH_3)_2$	H	H	290–293	63	C	$C_{12}H_{12}N_4O$
2d	$N(CH_3)_2$	CH_3O	CH_3O	296–298	45	B	$C_{14}H_{16}N_4O_3$
2e	2′-pyridyl	H	H	295–299	51	B	$C_{15}H_{10}N_4O$
2f	2′-pyridyl	CH_3O	CH_3O	>300	58	A	$C_{17}H_{14}N_4O_3$
2g	3′-pyridyl	H	H	>300	18	D	$C_{15}H_{10}N_4O$
2h	3′-pyridyl	CH_3O	CH_3O	>300	50	A	$C_{17}H_{14}N_4O_3$
5	CH_2Cl	H	H	282 (dec.)	43	E	$C_{11}H_{18}ClN_3O$
6a	$CH_2N(C_2H_5)_2$	H	H	196–197	42	A	$C_{15}H_{18}N_4O$
6b	methyl-*N*-morpholinyl	H	H	237–239	43	A	$C_{15}H_{16}N_4O_2$
6c	methyl-*N*-piperidinyl	H	H	235 (dec.)	47	A	$C_{16}H_{18}N_4O$
6d	methyl-*N*-[4-(2′-methoxyphenyl)]piperazinyl	H	H	226–227	41	A	$C_{22}H_{23}N_5O_2$
6e	methyl-*N*-[4-(2′-ethoxyphenyl)]piperazinyl	H	H	223–225	65	A	$C_{23}H_{25}N_5O_2$
6f	methyl-*N*-[4-(2′-pyridyl)]piperazinyl	H	H	254–256	62	A	$C_{20}H_{20}N_6O$
6g	methyl-*N*-[4-(4′-chlorophenyl)]piperazinyl	H	H	252–253	32	F	$C_{21}H_{20}ClN_5O$
6h	methyl-*N*-[4-(2′-furoyl)]piperazinyl	H	H	142–144	17	B	$C_{20}H_{19}N_5O_3$
6i	methyl-*N*-[4-(3′,4′-methylendioxophenyl)] piperazinyl	H	H	254–256	20	A	$C_{23}H_{23}N_5O_3$

[a] Value of the final transformation is expressed.
[b] Recrystallization solvent: A, 2-PrOH; B, EtOH/H_2O; C, MeOH/H_2O; D, toluene; E, 2-PrOH/dioxane; F, dioxane.
[c] All compounds were analyzed for C, H, N and results were in agreement to within $\pm 0.4\%$ of the theoretical values.

that, although they possess activity against the wild type, they lose it against the mutant type.

The interpretation of the results obtained in the assay for determining the anti-infective activity of these compounds is difficult, due to the high cytotoxicity that they show: unfortunately, the anti-infective effective doses are found to be very near the cytotoxic doses. As the described compounds show low potency against HIV-1 infected cells, no molecular modeling studies have been performed, so we do not know whether the butterfly-like model matches with these compounds [26].

The biological data obtained confirm the interest that these new derivatives could have in the field of anti-AIDS therapy; however, due to the high cytotoxicity shown by these derivatives, new structural modifications are now being proposed in this type of nucleus. These modifications could permit the increase of inhibitory activity on the HIV-1 RT mutant types, decreasing the toxic effects until an adequate safety margin is reached.

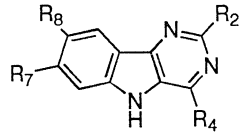
5. Experimental protocols

5.1. Chemistry

General laboratory chemicals were purchased from Merck, Sigma, Janssen, and Scharlau.

All the new compounds were characterized by elemental analysis, IR and ^{1}H NMR. The IR spectra are recorded on a Perkin–Elmer FT 681 using KBr pellets. The ^{1}H NMR spectra are obtained on a Bruker AC-200E (200 MHz) instrument with Me_4Si as the internal standard, and at a concentration of approximately 0.1 g/ml. The mass spectra were obtained on a Hewlett–Packard HP-5890 (GC/HPLC/DIP) instrument. All spectra were consistent with assigned structures. Thin layer chromatography was performed on aluminum sheets precoated with silica gel (HF 254, Merck). The developed chromatograms were viewed under UV light or iodine revelation. Melting points were determined on a Mettler FP82 hot stage apparatus equipped with a FP800/FP80 processor and an Olympus

Table 2
Physical constants of 5*H*-pyrimido[5,4-*b*]indole analogs (**3**, **4**, **7** and **8**)



Comp.	R_2	R_4	R_7	R_8	M.p. (°C)	Yield (%) [a]	Recrystallization solvent [b]	Formula [c]
3a	2′-furyl	Cl	H	H	217–220	51	A	$C_{14}H_8ClN_3O$
3b	2′-furyl	Cl	CH_3O	CH_3O	221–223	39	B	$C_{16}H_{12}ClN_3O_3$
3c	$N(CH_3)_2$	Cl	H	H	197–199	31	C	$C_{12}H_{11}ClN_4$·HCl
3d	$N(CH_3)_2$	Cl	CH_3O	CH_3O	215–219	33	D	$C_{14}H_{15}ClN_4O_2$·HCl [d]
3e	2′-pyridyl	Cl	H	H	130–132	58	E	$C_{15}H_{19}ClN_4$
3f	2′-pyridyl	Cl	CH_3O	CH_3O	294–295	45	D	$C_{17}H_{13}ClN_4O_2$·HCl
3g	3′-pyridyl	Cl	H	H	>300 (dec.)	16	D	$C_{15}H_9ClN_4$
3h	3′-pyridyl	Cl	CH_3O	CH_3O	>300	28	F	$C_{17}H_{13}ClN_4O_2$
4a	2′-furyl	$NH(CH_2)_2N(CH_3)_2$	H	H	220–225	14	E	$C_{18}H_{19}N_5O$
4b	2′-furyl	$NH(CH_2)_3N(CH_3)_2$	H	H	92–95	6	E	$C_{19}H_{21}N_5O$
4c	2′-furyl	$NH(CH_2)_2N(C_2H_5)_2$	H	H	187–189	18	E	$C_{20}H_{23}N_5O$
4e	2′-furyl	$NH(CH_2)_2N(CH_3)_2$	CH_3O	CH_3O	235–239	10	E	$C_{20}H_{23}N_5O_3$
4f	2′-furyl	$NH(CH_2)_3N(CH_3)_2$	CH_3O	CH_3O	111–113	8	E	$C_{21}H_{25}N_5O_3$
4g	2′-furyl	$NH(CH_2)_2N(C_2H_5)_2$	CH_3O	CH_3O	170–172	4	G	$C_{22}H_{27}N_5O_3$
4h	$N(CH_3)_2$	$NH(CH_2)_2N(CH_3)_2$	CH_3O	CH_3O	205–206 (dec.)	6	G	$C_{18}H_{26}N_6O_2$ [e]
4i	$N(CH_3)_2$	$NH(CH_2)_3N(CH_3)_2$	CH_3O	CH_3O	148–151	12	G	$C_{19}H_{28}N_6O_2$
4j	$N(CH_3)_2$	$NH(CH_2)_2N(C_2H_5)_2$	CH_3O	CH_3O	141–144	19	G	$C_{20}H_{30}N_6O_2$ [f]
4k	2′-pyridyl	*N*-morpholinyl	H	H	>300	64	E	$C_{19}H_{17}N_5O$
4l	2′-pyridyl	*N*-[4-(2′-ethoxyphenyl)]piperazinyl	H	H	222–225	29	E	$C_{27}H_{26}N_6O$
4m	2′-pyridyl	$NH(CH_2)_2N(CH_3)_2$	H	H	242–245	22	E	$C_{19}H_{19}N_6$
4n	2′-pyridyl	$NH(CH_2)_3N(CH_3)_2$	H	H	223–227	12	H	$C_{20}H_{22}N_6$
4o	2′-pyridyl	$N(CH_3)(CH_2)_2N(CH_3)_2$	H	H	116–117	23	E	$C_{20}H_{22}N_6$
4p	2′-pyridyl	$NH(CH_2)_2N(C_2H_5)_2$	H	H	207–209	8	I	$C_{21}H_{24}N_6$
4q	2′-pyridyl	$NH(CH_2)_2N(CH_3)_2$	CH_3O	CH_3O	88–91	7	G	$C_{21}H_{24}N_6O_2$
4r	2′-pyridyl	$NH(CH_2)_3N(CH_3)_2$	CH_3O	CH_3O	133–135	6	G	$C_{22}H_{26}N_6O_2$
4s	2′-pyridyl	$NH(CH_2)_2N(C_2H_5)_2$	CH_3O	CH_3O	138–142	6	G	$C_{23}H_{28}N_6O_2$
4t	3′-pyridyl	$NH(CH_2)_2N(CH_3)_2$	H	H	241–243	24	E	$C_{19}H_{20}N_6$
4u	3′-pyridyl	$NH(CH_2)_3N(CH_3)_2$	H	H	192–195	7	G	$C_{20}H_{22}N_6$
4v	3′-pyridyl	$NH(CH_2)_2N(C_2H_5)_2$	H	H	202–204	18	J	$C_{21}H_{24}N_6$
4w	3′-pyridyl	$NH(CH_2)_2N(CH_3)_2$	CH_3O	CH_3O	162–166	7	G	$C_{21}H_{24}N_6O_2$
4x	3′-pyridyl	$NH(CH_2)_3N(CH_3)_2$	CH_3O	CH_3O	215–218	3	G	$C_{22}H_{26}N_6O_2$
4y	3′-pyridyl	$NH(CH_2)_2N(C_2H_5)_2$	CH_3O	CH_3O	162–164	19	G	$C_{23}H_{28}H_6O_2$

Table 2 (Continued)

Comp.	R_2	R_4	R_7	R_8	M.p. (°C)	Yield (%) [a]	Recrystallization solvent [b]	Formula [c]
7a	methyl-*N*-morpholinyl	Cl	H	H	225 (dec.)	58	D	$C_{15}H_{15}ClN_4O{\cdot}HCl$
7b	methyl-*N*-[4-(2′-methoxyphenyl)]piperazinyl	Cl	H	H	98–99	35	E	$C_{22}H_{22}ClN_5O$
7c	methyl-*N*-[4-(2′-ethoxyphenyl)]piperazinyl	Cl	H	H	245–247	19	D	$C_{23}H_{24}ClN_5O{\cdot}HCl$
8a	methyl-*N*-morpholinyl	$NH(CH_2)_2N(CH_3)_2$	H	H	230–232	10	E	$C_{19}H_{26}N_6O$
8b	methyl-*N*-morpholinyl	$NH(CH_2)_3N(CH_3)_2$	H	H	61–64	13	E	$C_{20}H_{28}N_6O$
8c	methyl-*N*-morpholinyl	$NH(CH_2)_2N(C_2H_5)_2$	H	H	201–203	43	E	$C_{21}H_{30}N_6O$
8d	methyl-*N*-morpholinyl	*N*-morpholinyl	H	H	257–258	48	E	$C_{19}H_{23}N_5O_2$
8e	methyl-*N*-morpholinyl	*N*-[4-(2′-furoyl)]piperazinyl	H	H	192–195	29	E	$C_{24}H_{26}N_6O_3$
8f	methyl-*N*-morpholinyl	*N*-[4-(2′-ethoxyphenyl)]piperazinyl	H	H	268–269	23	E	$C_{27}H_{32}N_6O_2$
8g	methyl-*N*-[4-(2′-ethoxyphenyl)]piperazinyl	$NH(CH_2)_2N(CH_3)_2$	H	H	100–102	30	E	$C_{27}H_{35}N_7O$
8h	methyl-*N*-[4-(2′-ethoxyphenyl)]piperazinyl	$NH(CH_2)_3N(CH_3)_2$	H	H	212–216	22	E	$C_{28}H_{37}N_7O$
8i	methyl-*N*-[4-(2′-ethoxyphenyl)]piperazinyl	$NH(CH_2)_2N(C_2H_5)_2$	H	H	86–89	17	E	$C_{29}H_{39}N_7O$

[a] Value of the final transformation is expressed.
[b] Recrystallization solvent: A, 2-PrOH; B, CH_2Cl_2; C, EtOH/hexanes; D, EtOH; E, $EtOH/H_2O$; F, MeOH; G, AcOEt/hexanes; H, isolated from reaction mixture; I, $MeOH/H_2O$; J, AcOEt.
[c] All compounds were analyzed for C, H, N and results were in agreement to within ± 0.4% of the theoretical values except in compounds **3d**, **4h** and **4j**.
[d] **3d** *Anal.* ($C_{14}H_{15}ClN_4O_2{\cdot}HCl$) H, N, C: Calc. 57.39; Found 57.97%.
[e] **4h** *Anal.* ($C_{18}H_{26}N_6O_2$) C, H, N: Calc. 23.46; Found 23.90%.
[f] **4j** *Anal.* ($C_{20}H_{30}N_6O_2$) H, N, C: Calc. 62.15; Found 61.74%.

8091 microscope provided with a video system and were uncorrected.

Elemental analyses of vacuum-dried samples were obtained on a Carlo Erba 1106 elemental analyzer (over P_2O_5 at 1–2 mmHg, 24 h at 60–80°C). Results are within 0.4% of theoretical values unless otherwise indicated.

*5.1.1. Ethyl 3-aminoindol-2-carboxylate (**1a**)*

Previously reported [15].

*5.1.2. Ethyl 3-amino-2-carboxylate-5,6-dimethoxyindole (**1b**)*

Previously reported [14,15].

*5.1.3. General procedure for the synthesis of 3,4-dihydropyrimido[5,4-b]indol-4-ones, 2-substituted (**2**)*

A flow of HCl (g) was passed through a mixture of **1** (50 mmol), the appropriate nitrile (120 mmol) and dry dioxane (60 ml), with magnetic stirring and maintained at 0°C for 9–12 h. The mixture was then set aside for 12 h at room temperature (r.t.). The mixture was poured over ice (50 g) and then neutralized with 10% NH_4OH. The solid that precipitated was isolated by filtration, dried and purified. In this way, compounds **2a**, **2c**, **2e** and **2g** were synthesized from **1a**, and compounds **2b**, **2d**, **2f** and **2h** were synthesized from **1b** (Table 1).

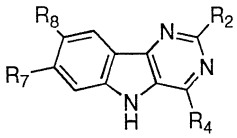
*5.1.4. General procedure for the synthesis of 4-chloropyrimido[5,4-b]indoles, 2-substituted (**3**)*

A mixture of **2** (24 mmol) and $POCl_3$ (50 ml) was refluxed with magnetic stirring for 8–10 h. The excess reagent was eliminated by vacuum evaporation. 10% NH_4OH was added over the residue, until neutralization was attained. The solid obtained was isolated by filtration, washed with abundant H_2O, dried and recrystallized. If the compound is described as hydrochloride (**3c**, **3d** and **3f**), this was obtained by dissolving the solid isolated before recrystallization in AcOEt (100 ml). The solution was dried over Na_2SO_4 and filtered. 35% HCl (3 ml) was added over the solution with stirring and the solid obtained was filtered, dried and recrystallized (Table 2).

*5.1.5. General procedure for the synthesis of 4-aminopyrimido[5,4-b]indoles, 2-substituted (**4**)*

A mixture of **3** (4 mmol), the corresponding amine (15 mmol), sodium carbonate (7 mmol) and the appropriate solvent (dioxane, 40–50 ml; ethanol, 40 ml) was refluxed with constant stirring for 20–30 h. The mixture was then poured over ice-cold water (200 ml). The solid obtained was washed with abundant H_2O, dried and recrystallized. In this way, compounds **4a–c** were synthesized from **3a**, compounds **4e–g** from **3b**, com-

Table 3
HIV-1 RT inhibitory activity of selected 5*H*-pyrimido[5,4-*b*]indoles



Comp.	R_2	R_4	R_7	R_8	% Inhibition (wild type) [a]	IC_{50} (μM) [b]	% Inhibition (Y181C type) [a]	IC_{50} (μM) [b]
4e	2′-furyl	$NH(CH_2)_2N(CH_3)_2$	CH_3O	CH_3O	83.1 ± 6.0	37.0	74.0 ± 7.5	35.9
4i	$N(CH_3)_2$	$NH(CH_2)_3N(CH_3)_2$	CH_3O	CH_3O	78.5 ± 5.9	29.2	78.5 ± 5.9	32.2
4j	$N(CH_3)_2$	$NH(CH_2)_2N(C_2H_5)_2$	CH_3O	CH_3O	76.5 ± 2.3	37.8	76.5 ± 2.3	38.7
4m	2′-pyridyl	$NH(CH_2)_2N(CH_3)_2$	H	H	66.4 ± 6.8 [c]	69.4	28.4 ± 2.4	–
4t	3′-pyridyl	$NH(CH_2)_2N(CH_3)_2$	H	H	65.4 ± 6.4	34.1	20.6 ± 6.0	–
	U-90152s					0.40		4.5

[a] % inhibition at 50 μM ($n = 3$–5).
[b] Concentration–activity curves are carried out with four or more concentrations of test compounds; IC_{50} values are calculated from log curve.
[c] % inhibition at 100 μM ($n = 3$–5).

pounds **4h–j** from **3d**, compounds **4k–p** from **3e**, compounds **4q–s** from **3f**, compounds **4t–v** from **3g** and compounds **4w–y** from **3h** (Table 2).

*5.1.6. 7,8-Dimethoxy-4-N-[2-(N,N-dimethylamino)]-ethylamino-2-(2′-furyl)pyrimido[5,4-b]indole (**4e**)*

From **3b** and 2-(*N*,*N*-dimethylamino)ethylamine. As white powder. ^{1}H NMR (DMSO-d_6) δ: 2.25 (s, 6H, CH_3N); 2.56 (t, 2H, CH_2N); 3.75 (t, 2H, CH_2N); 3.89 (s, 3H, CH_3O); 3.90 (s, 3H, CH_3O); 6.62 (d, 1H, $H_{5'}$; $J = 2.4$ Hz); 7.08–7.09 (m, 2H, H_6, NH); 7.16 (s, 1H, $H_{4'}$); 7.50 (s, 1H, H_9); 7.79 (s, 1H, $H_{3'}$); 10.97 (s, 1H, NH). MS-DIP (70 eV) *m*/*e*: 381 (M^+). *Anal.* ($C_{20}H_{23}N_5O_3$) C, H, N.

*5.1.7. 7,8-Dimethoxy-2-(N,N-dimethylamino)-4-N-[3-(N,N-dimethylamino)]propylaminopyrimido[5,4-b]-indole (**4i**)*

From **3d** and 2-(*N*,*N*-dimethylamino)propylamine. As white powder. ^{1}H NMR (DMSO-d_6) δ: 1.72–1.78 (m, 2H, CH_2); 2.13 (s, 6H, CH_3N); 2.30 (t, 2H, CH_2N); 3.11 (s, 6H, CH_3N); 3.50 (q, 2H, CH_2N); 3.80 (s, 3H, CH_3O); 3.83 (s, 3H, CH_3O); 6.83 (s, 1H, NH); 7.02 (s, 1H, H_6); 7.26 (s, 1H, H_9); 10.05 (s, 1H, NH). MS-DIP (70 eV) *m*/*e*: 372 (M^+). *Anal.* ($C_{19}H_{28}N_6O_2$) C, H, N.

*5.1.8. 4-N-[2-(N,N-Diethylamino)]ethylamino-7,8-dimethoxy-2-(N,N-dimethylamino)pyrimido[5,4-b]indole (**4j**)*

From **3d** and 2-(*N*,*N*-diethylamino)ethylamine. As brown powder. ^{1}H NMR (DMSO-d_6) δ: 1.04 (t, 6H, CH_3); 2.73–2.82 (m, 6H, CH_2); 3.38–3.56 (m, 6H, CH_2); 3.65 (q, 2H, CH_2); 3.82 (s, 3H, CH_3O); 3.85 (s, 3H, CH_3O); 7.01 (s, 1H, H_6); 7.11 (s, 1H, NH); 7.29 (s, 1H, H_9); 10.35 (s, 1H, NH). MS-DIP (70 eV) *m*/*e*: 386 (M^+). *Anal.* ($C_{20}H_{30}N_6O_2$) H, N; C: Calc. 62.15; Found: 61.74%.

*5.1.9. 4-N-[2-(N,N-Dimethylamino)]ethyl-amino-2-(2′-pyridyl)pyrimido[5,4-b]indole (**4m**)*

From **3e** and 2-(*N*,*N*-dimethylamino)ethylamine. As white needles. ^{1}H NMR (DMSO-d_6) δ: 2.25 (s, 6H, CH_3N); 2.59 (t, 2H, CH_2N); 3.80 (q, 2H, CH_2N); 7.22–7.30 (m, 2H, H_7, NH); 7.42 (t, 1H, $H_{4'}$; $J = 6.3$ Hz); 7.52 (t, 1H, H_8; $J = 7.5$ Hz); 7.67 (d, 1H, H_6; $J = 8.2$ Hz); 7.92 (t, 1H, $H_{5'}$; $J = 7.7$ Hz); 8.16 (d, 1H, H_9; $J = 7.8$ Hz); 8.45 (d, 1H, $H_{6'}$; $J = 7.8$ Hz); 8.72 (d, 1H, $H_{3'}$; $J = 4.4$ Hz); 11.40 (s, 1H, NH). MS-DIP (70 eV) *m*/*e*: 274; 261; 78. *Anal.* ($C_{19}H_{20}N_6$) C, H, N.

*5.1.10. 4-N-[2-(N,N-Dimethylamino)]ethylamino-2-(3′-pyridyl)pyrimido[5,4-b]indole (**4t**)*

From **3g** and 2-(*N*,*N*-dimethylamino)ethylamine. As yellow powder. ^{1}H NMR (DMSO-d_6) δ: 2.29 (s, 6H, CH_3N); 2.63 (t, 2H, CH_2N); 3.83 (q, 2H, CH_2N); 7.24–7.32 (m, 2H, H_7, NH); 7.50–7.56 (m, 2H, H_8, $H_{5'}$); 7.68 (d, 1H, H_6; $J = 8.1$ Hz); 8.18 (d, 1H, H_9; $J = 7.7$ Hz); 8.63 (d, 1H, $H_{4'}$; $J = 3.8$ Hz); 8.77 (d, 1H, $H_{6'}$; $J = 7.8$ Hz); 9.64 (s, 1H, $H_{2'}$); 11.36 (s, 1H, NH). MS-DIP (70 eV) *m*/*e*: 332 (M^+). *Anal.* ($C_{19}H_{20}N_6$) C, H, N.

*5.1.11. 2-Chloromethyl-3,4-dihydropyrimido[5,4-b]-indole (**5**)*

A flow of HCl (g) was passed through a mixture of **1a** (13.25 g, 16 mmol), chloroacetonitrile (13.20 g, 42 mmol) and dry dioxane (200 ml), maintained with vigorous and constant stirring for 8 h. Next, the mixture was poured over ice (100 g) and basified with 10% NH_4OH. The precipitate obtained was filtered, washed with abundant H_2O, dried and purified as white powder (Table 1).

5.1.12. General procedure for the synthesis of 2-(amino)methyl-3,4-dihydropyrimido[5,4-b]indol-4-ones (6)

A mixture of **5** (2.5 g, 11 mmol), the appropriate amine (25 mmol), sodium carbonate (15 mmol) and EtOH (60 ml) was refluxed with magnetic stirring for 3–8 h. The mixture was poured over cold H_2O (200 ml). When a solid appeared, it was filtered, washed with abundant H_2O, dried and purified. When an oily residue appeared, it was extracted with ethyl acetate (3×25 ml). The organic extracts were dried with Na_2SO_4 and the solvent was eliminated under reduced pressure. The solid obtained was dried and recrystallized. In this way compounds **6a–i** were obtained (Table 1).

5.1.13. General procedure for the synthesis of 2-(amino)methyl-4-chloropyrimido[5,4-b]indole (7)

A mixture of **6** (6.6 mmol), $POCl_3$ (15 ml) and dry dioxane (20 ml) was refluxed with stirring for 18 h. The solvent and excess reagent were eliminated under reduced pressure. Dioxane (10 ml) was poured over the residue and the mixture was stirred. The solid obtained was filtered, dried and purified. If the compound is described as hydrochloride, this was obtained by dissolving the solid isolated in AcOEt (50 ml). The solution was dried over Na_2SO_4 and filtered. 35% HCl (2 ml) was added over the solution with stirring and the solid obtained was filtered, dried and recrystallized. In this way compound **7a** (hydrochloride) was synthesized from **6b**, compound **7b** from **6d** and compound **7c** from **6e** (Table 2).

5.1.14. General procedure for the 4-amino-2-(amino)methylpyrimido[5,4-b]indoles (8)

A mixture of **7** (3.3 mmol), the appropriate amine (15 mmol), sodium carbonate (6.67 mmol) and dioxane (40 ml) was refluxed with stirring for 20–30 h. The mixture was poured over ice-cold H_2O (200 ml). The solid obtained was filtered, washed with abundant H_2O, dried and recrystallized. In this way, compounds **8a–f** were synthesized from **7a** and compounds **8g–i** were synthesized from **7c** (Table 2).

5.2. Pharmacology

Poly-rA, oligo(dT)$_{10}$ and deoxythymidine triphosphate (dTTP) were obtained from Pharmacia LKB Biotechnology Inc. [^{35}S]-dTTPa was purchased from Amersham and DL-dithiothreitol was purchased from Sigma. Nonidet P-40 was obtained from Boehringer–Mannheim. The scintillation mixture used was Biogreen-11 which was obtained from Scharlau. Glass microfiber filters (Filtermats) were purchased from Skatron Instruments.

U-90152s was kindly provided by the Upjohn Company, Kalamazoo, USA. Recombinant HIV-1 reverse transcriptase (p66), wild and mutant types, purified according to previously reported methods [17–19], were also kindly provided by the Upjohn Company. Molt 3/HIV-1$_{IIIB}$ infected cells were kindly provided by Professor F. Barin and Dr B. Janvier from Université François Rabelais, Tours France; HLT4lacZ-1 cells were kindly provided by Dr S. Saragosti from Hôpital Cochin, Paris, France.

5.2.1. Reverse transcriptase assay

Enzyme activity was measured in a total volume of 50 ml using a standard reaction mixture containing 50 mM Tris–HCl (pH 8.3), 20 mM DL-dithiothreitol, 60 mM NaCl, 0.05% Nonidet P-40, 10 mM $MgCl_2$, 10 μg/ml poly-rA, 5 μg/ml oligo(dt)$_{10}$ and 10 μM [^{35}S]-dTTPa (0.2 Ci/mmol). The mixture was pre-incubated at 37°C for 2 min and the reaction was initiated by adding the enzyme. Reaction was stopped after 10 min with 50 μl of ice-cold 10% trichloroacetic acid. The insoluble material was collected on glass microfiber filters with a Skatron cell harvester and extensively washed with 5% trichloroacetic acid. Filters were dried in a microwave oven for 2 min, transferred to scintillation vials with 3 ml of cocktail and counted in a liquid scintillation counter. All compounds tested were solubilized in pure DMSO, diluted in water and assayed, maintaining the final concentration of DMSO at 0.5% (v/v).

5.2.2. Cell cultures

HIV-1$_{IIIB}$ chronically infected Molt-3 cells (Molt-3/HIV-1$_{IIIB}$) were cultured at 37°C with 5% CO_2 in RPMI 1640 (Gibco) supplemented with 20% FCS (foetal calf serum, Flow), 1% penicillin/streptomycin (Gibco), and 1% anti-PPLO (Gibco).

HT4lacZ-1 cells were cultured in DMEM (Dulbecco's modification of Eagle medium, ICN Flow) supplemented with 2 mM L-glutamine (Gibco), 10% FCS (Flow) and 1% penicillin/streptomycin (Gibco).

Titration of the virus was carried out by means of syncytium formation assay, co-cultivating serial dilutions from viral stocks with uninfected HLT4lacZ-1. The concentration of the virus which caused 100–150 syncytia per well was used for the infection inhibition assays.

5.2.3. Syncytia formation assay

The characteristics and use of HLT4lacZ-1 cells have been described previously [20]. We have adapted their use to perform a quantitative syncytia assay in 96-well plates in order to quantify the inhibitory activity of the synthesized products.

Briefly, 10 000 cells (200 μl)/well were plated the day before the assay. On the following day, the medium was removed and 100 μl of product solution and 100 μl of diluted virus were added. Controls with no product

were also made. On the third day of post-infection, the medium was removed and the cells were fixed for 5 min at r.t. with 200 ml of a PBS solution containing 1% formaldehyde (Merck) and 0.2% glutaraldehyde (Merck). After two washes with 0.9% NaCl (Merck), the cells were incubated for 1 h at 37°C with 200 μl of a reaction mixture containing X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, Boehringer–Mannheim) (400 μg/ml), 4 mM potassium ferrocyanide (Merck), and 2 nM $MgCl_2$ (Merck) in PBS. After two washes, 100 μl of 0.9% NaCl were added per well. The plates were examined under the microscope and only syncytia with three or more blue nuclei were counted in the entire well.

5.2.4. Cell toxicity

Viability of HLT4lacZ-1 cells was evaluated in the presence of the synthesized products using a modified cell lytic assay described previously [21]. It was performed in parallel with the syncytia formation assay.

A total of 200 μl of medium containing different concentrations of the products or medium (control wells) were added to 10 000 HLT4lacZ-1 cells plated the day before. On the third day, the wells were washed three times with PBs, and cell lysis was detected by staining the plate for 10 min at r.t., with 20 μl/well of a methanol/water (1:4 v/v) solution containing 0.5% crystal violet (Merck). Controls of wells have been prepared by staining the plates without cells (blank wells). Three washes were carried out by immersion of the plates in PBS, changing the PBS of the container each time. The plates were wiped and 100 μl/well of 0.1% SDS (sodium dodecyl sulfate, Sigma) were added. After complete desegregation of the cell membranes, the O.D. at 540 nm was read in a Titertek Multiskan II autoreader (Flow) and the percentage of viability (% V) was calculated.

Acknowledgements

This work was supported by the Upjohn Company within the National Plan of Scientific and Technological Investigation of Spain. The Gobierno Foral de Navarra (Spain) granted a scholarship to Isidro Merino.

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