Synthesis of New Pyridazine Derivatives as Potential Anti-HIV-1 Agents

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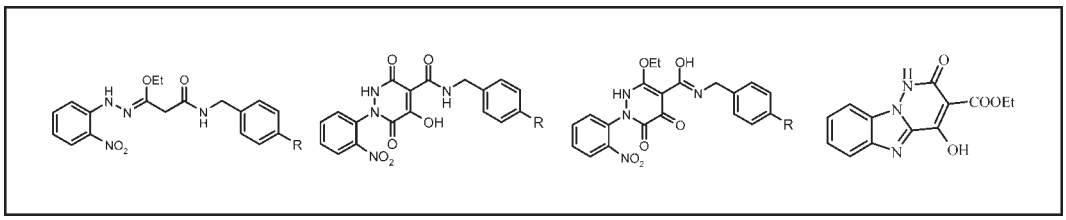

We herein report conventional and microwave-assisted methods for the synthesis of a series of compounds containing some bivalent ion chelating requirements useful to exert potential HIV-1 integrase (IN) enzyme inhibition activity. The biological screening highlighted that only derivative **5** proved to be a strand-transfer step inhibitor of the virus integration process at micromolar concentration.

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INTRODUCTION

Human immunodeficiency virus (HIV), the etiological agent of acquired immunodeficiency syndrome (AIDS) continues to be a major health threat worldwide. The main steps in the viral life cycle include viral entry, replication of the viral genome, and proteolytic processing of viral polyproteins. In addition, HIV replication depends on the integration of the proviral DNA into the genome of infected cells, which is catalyzed by HIV integrase (IN). All these steps are potential targets for antiviral drugs.

The IN-catalyzed insertion of viral DNA into the host cell chromosome is a multistep process that includes two catalytic reactions: 3′-Processing (3′P) and strand-transfer (ST) [1]. Moreover, it has been suggested that the integration mechanism involves bivalent metal ions (Mg^{2+} or Mn^{2+}) in the enzyme catalytic site [2,3].

After many years of research, several molecules were identified as IN inhibitors and among them β-diketo acids (DKAs) [4] and their analogs [5] represent one of the major leads in the identification of new antiviral agents. In particular, it has been reported that the DKA pharmacophoric motif could be able to sequester one or both metal ions necessary for IN-enzymatic activity [2].

In fact, the only one integrase strand-transfer inhibitor (INSTI) (Raltegravir) (Fig. 1) [6] so far approved by Food and Drug Administration (FDA), belongs to the DKAs analogs class, in which the diketo acid moiety, as ''ketoenole,'' has been included in a rigid system.

Taking into account these findings and considering the results of our previously reported studies [7–10], we planned the synthesis of new derivatives containing a pyridazine core as well as the chemical features able to inhibit IN activity as chelating agents for bivalent metal ions. The obtained compounds were characterized and tested to evaluate their antiviral activity and enzymatic inhibition.

RESULTS AND DISCUSSION

The synthesis of the first designed compound **4** was carried out, as reported in Scheme 1, starting from the commercially available nitrophenylhydrazine **1**.

At first it was converted into 3-ethoxy-3-[(2-nitrophenyl)hydrazono]propanoic acid ethyl ester (**2**) by reaction with 3-amino-3-ethoxy-acrylic acid ethyl ester. This is a novel synthetic approach with respect to a previously reported procedure [11] thus improving the yields and reducing reaction time. The obtained mixture of Z- and E-isomers (**2**), without further separation, was successively used for the cyclocondensation with oxalyl chloride to give the ethyl 5-hydroxy-1-(2-nitrophenyl)-3,6-dioxo-1,2,3,6-tetrahydropyridazine-4-carboxylate (**3**). The pyridazine

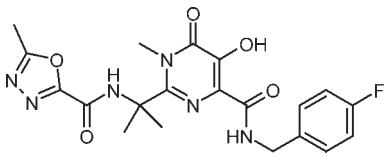


Figure 1. Chemical structure of Raltegravir.

derivative **3** was finally treated with platinum dioxide under hydrogen atmosphere to give the ethyl 4-hydroxy-2-oxo-1,2-dihydropyridazino[1,6-a]benzimidazole-3-carboxylate (**4**) resulting from the nitro-group reduction and the consequent ring closure.

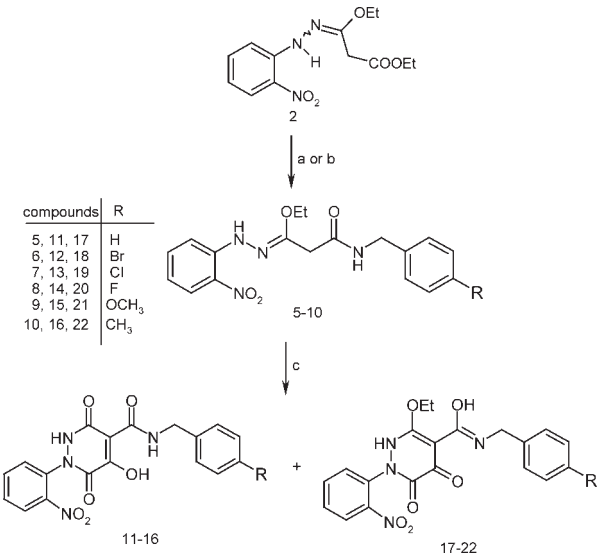
With the aim to prepare the amide derivatives **5–22**, we used the synthetic route described in Scheme 2. The (Z) isomer of intermediate **2**, obtained as red crystals from ethanol, was used as starting material, and was converted into the corresponding (*Z*)*N*-benzyl-3-ethoxy-3-[(2-nitrophenyl)hydrazono]propanamides **5–10**, by reaction with appropriate benzylamine. With the aim to optimize the synthetic procedure, we performed this step both at room temperature and under reflux (50°C). We found that even elevating the reaction temperature no significant reduction in reaction time occurred. Alternatively, when we used the microwave-assisted organic synthesis (MAOS) approach we observed a remarkable reduction of reaction time (1 h rather than 15 h). Moreover, this approach was used in free-solvent conditions (see experimental section). Then, the condensation with oxalyl chloride gave a mixture of two different series (**11–16** and **17–22** in a ratio of 2:1) of pyridazine derivatives depending on the tautomeric form of the amide precursors **5–10** as well as the hydrolytic processes occurred on their ethoxy functionality.

The *N*-benzyl-5-hydroxy-1-(2-nitrophenyl)-3,6-dioxo-1,2,3,6-tetrahydropyridazine-4-carboxamides **11–16** were directly recovered as crude precipitate from reaction mixture, while the evaporation of the resulting solution furnished the *N*-benzyl-3-ethoxy-1-(2-nitrophenyl)-5,6-dioxo-1,2,5,6-tetrahydropyridazine-4-carboximidic acids **17–22**.

All the synthesized compounds were tested for their IN enzymatic inhibitory effect both against the overall integration reaction and more specifically against the strand-transfer step. The biological results showed that compound **5** was able to inhibit the enzymatic activity at micromolar concentration (Table 1).

Moreover, the HIV-induced cytopathic effect (CPE) in human lymphocyte MT-4 cell culture was determined by the MT-4/MTT-assay. The screening data put in evidence that only the *N*-benzyl-3-ethoxy-3-[(2-nitrophenyl)hydrazono]propanamides (**5–10**) showed antiviral activity in cells test, but also toxicity at same concentration.

Scheme 2. Reagents and conditions: a) $ArCH_2NH_2$, CH_2Cl_2, r.t. 15 h; b) $ArCH_2NH_2$, two steps in the same conditions: 280 W, 50°C, 30 min; and c) oxalyl chloride, toluene, 0°C, 2 h.



EXPERIMENTAL

Chemistry. All microwave-assisted reactions were carried out in a CEM Focused Microwave Synthesis System, Model Discover working at the potency necessary for refluxing under atmospheric conditions. Melting points were determined on a BUCHI Melting Point B-545 apparatus and are uncorrected. Elemental analyses (C, H, N) were carried out on a Carlo Erba Model 1106 Elemental Analyzer and the results are within ±0.4% of the theoretical values. Merck silica gel 60 F_{254} plates were used for analytical tlc. ^{1}H NMR spectra were recorded in DMSO-d_6 on a Varian Gemini-300 spectrometer. Chemical shifts were expressed in δ (ppm).

Scheme 1. Reagents and conditions: a) 3-amino-3-ethoxy-acrylic acid ethyl ester, dry EtOH, N_2, rt, 2h; b) oxalyl chloride, toluene, 0°C, 5 h; and c) PtO_2, H_2, EtOH, rt, 5 h.

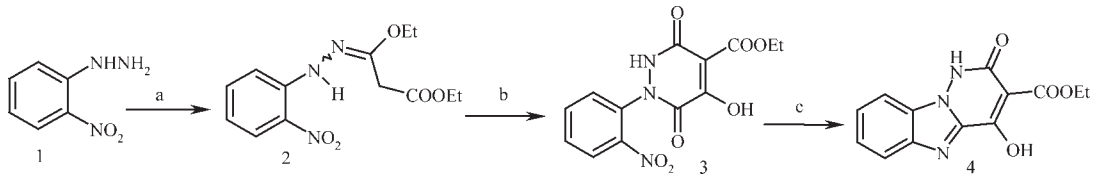


Table 1

Inhibition of HIV-1 integrase enzymatic activity, replication of HIV-1 (IIIB), and cytotoxicity in MT-4 cells.

Compound	IN enzymatic activity		Activity in MT-4 cells		
	Over-all[a] IC_{50} (μ*M*)	ST[b] IC_{50} (μ*M*)	HIV-1[c] EC_{50} (μ*M*)	Cytotoxicity[d] CC_{50} (μ*M*)	SI[e]
5	15.32	37.39	0.82	0.82	1
6	>250	>250	53.28	53.28	1
7	>250	>250	15.91	15.91	1
8	>250	>250	5.17	5.17	1
9	>250	>250	>30.68	>30.68	1
10	>250	>250	18.16	18.16	1
CHI 1043[g]	0.08	0.14	0.59	41.1	70

[a] Concentration required to inhibit the *in vitro* overall integrase activity by 50%.
[b] Concentration required to inhibit the *in vitro* strand transfer step by 50%.
[c] Effective concentration required to reduce HIV-1-induced cytopathic effect by 50% in MT-4 cells.
[d] Cytotoxic concentration to reduce MT-4 cell viability by 50%.
[e] Selectivity index: ratio CC_{50} /EC_{50}.

Synthesis of (*Z-E*)3-ethoxy-3-[(2-nitrophenyl)hydrazono]-propanoic acid ethyl ester (2). 2-Nitrophenylhydrazine (0.001 mol, 137.14 mg) (**1**) and 3-amino-3-ethoxy-acrylic acid ethyl ester hydrochloride (0.001 mol, 195.65 mg) were suspended in ethanol (3 mL), under N_2 atmosphere, and stirred at room temperature for 2 h. The precipitate was filtered off and the solution concentrated *in vacuo*. Yield 69%. Spectral measumerents (^{1}H NMR) and experimental data were in accordance with reported data in the literature [11].

Synthesis of ethyl 5-hydroxy-1-(2-nitrophenyl)-3,6-dioxo-1,2,3,6-tetrahydropyridazine-4-carboxylate (3). Oxalyl chloride (0.001 mol, 126.93 mg) was added dropwise at 0°C to a solution of (*Z-E*)3-ethoxy-3-[(2-nitrophenyl)hydrazono]propanoic acid ethyl ester (**2**) (0.001 mol, 295.30 mg) in toluene (6 mL). The mixture was stirred for 5 h and allowed to reach room temperature. Yellow crystals were formed, the precipitate was filtered off, and crystallized from diethyl ether. Mp 260–262°C yield 81%; ^{1}H NMR: δ 1.15 (t, 3H, J = 7.1, CH_3), 3.40 (q, 2H, J = 7.1, CH_2), 6.78–8.12 (m, 4H, ArH), 9.40 (bs, 1H, NH). Anal. Calcd. for $C_{13}H_{11}N_3O_7$: C, 48.61; H, 3.45; N, 13.08. Found: C, 48.54; H, 3.38; N, 13.24.

Synthesis of ethyl 4-hydroxy-2-oxo-1,2-dihydropyridazino[1,6-a]benzimidazole-3-carboxylate (4). Compound **3** (0.001 mol, 321.25 mg) was dissolved in dry ethanol (50 mL) in a flask suitable for a normal pressure hydrogenation. Platinum dioxide (0.002 mol, 32 mg) was added and the compound **3** was hydrogenated under ambient pressure. The mixture was stirred for 5 h. The catalyst was filtered off on diatomaceous earth and the solvent removed under reduced pressure. The residue was powdered by treatment with diethyl ether and crystallized from ethanol. Mp 230–232°C, yield 26%; ^{1}H NMR: δ 1.22 (t, 3H, J = 7.1, CH_3), 4.16 (q, 2H, J = 7.1, CH_2), 6.55–7.97 (m, 4H, ArH), 10.43 (bs, 1H, NH). Anal. Calcd. for $C_{13}H_{11}N_3O_4$: C, 57.14; H, 4.06; N, 15.38. Found: C, 57.20; H, 4.12; N, 15.30.

General procedure for the synthesis of (*Z*)*N*-benzyl-3-ethoxy-3-[(2-nitrophenyl)hydrazono]propanamides (5–10)

Conventional method. (*Z*)3-Ethoxy-3-[(2-nitrophenyl)hydrazono]propanoic acid ethyl ester (**2**) (0.001 mol, 295.30 mg) was dissolved in methylene chloride (2 mL), under N_2 atmosphere, and the solution stirred at room temperature for 10 min. The appropriate benzylamine (0.010 mol) was added and the resulting solution was stirred for 15 h. Precipitate was filtered and crystallized from ethanol.

Microwave-assisted synthesis. The appropriate benzylamine (0.010 mol) was added to the (*Z*)3-ethoxy-3-[(2-nitrophenyl)-hydrazono]propanoic acid ethyl ester (**2**) (0.001 mol, 295.30 mg), under N_2 atmosphere, and the resulting mixture was placed in a cylindrical quartz tube (Ø 2 cm). The reaction mixture was then stirred and irradiated in a microwave oven for two subsequent periods in the same conditions: 280 W, 30 min, and 50°C. After cooling the work-up was carried out as described for the conventional method.

(Z)N-Benzyl-3-ethoxy-3-[(2-nitrophenyl)hydrazono]propanamide (5). Mp 172–174°C, yield 100%; ^{1}H NMR: δ 1.30 (t, 3H, J = 7.1, CH_3), 3.55 (s, 2H, CH_2), 4.23 (q, 2H, J = 7.1, CH_2), 4.31 (d, 2H, J = 5.99, CH_2), 6.76–8.08 (m, 9H, ArH), 8.68 (t, 1H, J = 5.99, NH), 10.93 (bs, 1H, NH). Anal. Calcd. for $C_{18}H_{20}N_4O_4$: C, 60.67; H, 5.66; N, 15.72. Found: C, 60.60; H, 5.59; N, 15.78.

(Z)N-(4-Bromobenzyl)-3-ethoxy-3-[(2-nitrophenyl)hydrazono]propanamide (6). Mp 175–177° C, yield 99%; ^{1}H NMR: δ 1.30 (t, 3H, J = 6.9, CH_3), 3.54 (s, 2H, CH_2), 4.22 (q, 2H, J = 6.9, CH_2), 4.27 (d, 2H, J = 5.8, CH_2), 6.76–8.08 (m, 8H, ArH), 8.71 (t, 1H, J = 5.8, NH), 10.92 (bs, 1H, NH). Anal. Calcd. for $C_{18}H_{19}BrN_4O_4$: C, 49.67; H, 4.40; N, 12.87. Found: C, 49.62; H, 4.43; N, 12.83.

(Z)N-(4-Chlorobenzyl)-3-ethoxy-3-[(2-nitrophenyl)hydrazono]propanamide (7). Mp 171–173°C, yield 100%; ^{1}H NMR: δ 1.30 (t, 3H, J = 6.9, CH_3), 3.69 (s, 2H, CH_2), 4.22 (q, 2H, J = 6.9, CH_2), 4.29 (d, 2H, J = 5.8, CH_2), 6.76–8.08 (m, 8H, ArH), 8.72 (t, 1H, J = 5.8, NH), 10.93 (bs, 1H, NH). $C_{12}H_{12}O_4$: C, 65.45; H, 5.49; C, 65.31; H, 5.63. Anal. Calcd. for $C_{18}H_{19}ClN_4O_4$: C, 55.32; H, 4.90; N, 14.34. Found: C, 55.28; H, 4.95; N, 14.31.

(Z)N-(4-Fluorobenzyl)-3-ethoxy-3-[(2-nitrophenyl)hydrazono]propanamide (8). Mp 180–182°C, yield 93%; ^{1}H NMR: δ 1.30 (t, 3H, J = 7.1, CH_3), 3.69 (s, 2H, CH_2), 4.22 (q, 2H, J = 7.1, CH_2), 4.29 (d, 2H, J = 5.8, CH_2), 6.78–8.08 (m, 8H, ArH), 8.68 (t, 1H, J = 5.8, NH), 10.93 (bs, 1H, NH). Anal. Calcd. for $C_{18}H_{19}FN_4O_4$: C, 57.75; H, 5.12; N, 14.97. Found: C, 57.71; H, 5.08; N, 14.93.

(Z)N-(4-Methoxybenzyl)-3-ethoxy-3-[(2-nitrophenyl)hydrazono]propanamide (9). Mp 171–173°C, yield 84%; ^{1}H NMR:

δ 1.30 (t, 3H, J = 7.3, CH_3), 3.52 (s, 2H, CH_2), 3.72 (s, 3H, CH_3O), 4.21 (q, 2H, J = 7.3, CH_2), 4.23 (d, 2H, J = 5.7, CH_2), 6.75–8.07 (m, 8H, ArH), 8.58 (t, 1H, J = 5.7, NH), 10.92 (bs, 1H, NH). Anal. Calcd. for $C_{19}H_{22}N_4O_5$: C, 59.06; H, 5.74; N, 14.50. Found: C, 59.02; H, 5.69; N, 14.55.

(Z)N-(4-Methylbenzyl)-3-ethoxy-3-[(2-nitrophenyl)hydrazono]propanamide (10). Mp 179–181°C, yield 90%; ^{1}H NMR: δ 1.30 (t, 3H, J = 7.1, CH_3), 2.27 (s, 3H, CH_3), 3.53 (s, 2H, CH_2), 4.22 (q, 2H, J = 7.1, CH_2), 4.26 (d, 2H, J = 5.7, CH_2), 6.76–8.08 (m, 8H, ArH), 8.63 (t, 1H, J = 5.7, NH), 10.93 (bs, 1H, NH). Anal. Calcd. for $C_{19}H_{22}N_4O_4$: C, 61.61; H, 5.99; N, 15.13. Found: C, 61.58; H, 5.95; N, 15.16.

General procedures for the synthesis of N-benzyl-5-hydroxy-1-(2-nitrophenyl)-3,6-dioxo-1,2,3,6-tetrahydropyridazine-4-carboxamides (11–16) and N-benzyl-3-ethoxy-1-(2-nitrophenyl)-5,6-dioxo-1,2,5,6-tetrahydropyridazine-4-carboximidic acids (17–22). The appropriate (*Z*)*N*-benzyl-3-ethoxy-3-[(2-nitrophenyl)hydrazono]propanamide (**5–10**) (0.001 mol) was dissolved in dry toluene (6.5 mL). Oxalyl chloride (0.001 mol, 126.93 mg) was added dropwise at 0°C and the reaction mixture stirred for 2 h. The obtained precipitate was filtered and crystallized from ethanol/methanol (1:1, v/v) to give derivatives **11–16**. The solution was concentrated under reduced pressure and the residue treated with diethyl ether to afford derivatives **17–22**.

N-Benzyl-5-hydroxy-1-(2-nitrophenyl)-3,6-dioxo-1,2,3,6-tetrahydropyridazine-4-carboxamide (11). Mp 164–166°C, yield 56%; ^{1}H NMR: δ 4.41 (s, 2H, CH_2), 6.87–8.12 (m, 9H, ArH), 9.44 (bs, 1H, NH). Anal. Calcd. for $C_{18}H_{14}N_4O_6$: C, 56.55; H, 3.69; N, 14.65. Found: C, 56.49; H, 3.65; N, 14.68.

N-(4-Bromobenzyl)-5-hydroxy-1-(2-nitrophenyl)-3,6-dioxo-1,2,3,6-tetrahydropyridazine-4-carboxamide (12). Mp 171–173°C, yield 64%; ^{1}H NMR: δ 4.36 (s, 2H, CH_2), 6.84–8.11 (m, 8H, ArH), 9.41 (bs, 1H, NH). Anal. Calcd. for $C_{18}H_{13}BrN_4O_6$: C, 46.87; H, 2.84; N, 12.15. Found: C, 46.61; H, 2.55; N, 12.82.

N-(4-Chlorobenzyl)-5-hydroxy-1-(2-nitrophenyl)-3,6-dioxo-1,2,3,6-tetrahydropyridazine-4-carboxamide (13). Mp 182–184°C, yield 48%; ^{1}H NMR: δ 4.36 (s, 2H, CH_2), 6.82–8.09 (m, 8H, ArH), 9.41 (bs, 1H, NH). Anal. Calcd. for $C_{18}H_{13}ClN_4O_6$: C, 51.87; H, 3.14; N, 13.44. Found: C, 51.63; H, 3.38; N, 13.21.

N-(4-Fluorobenzyl)-1-(2-nitrophenyl)-3,6-dioxo-1,2,3,6-tetrahydropyridazine-4-carboxamide (14). Mp 172–174°C, yield 59%; ^{1}H NMR: δ 4.37 (s, 2H, CH_2), 6.86–8.09 (m, 8H, ArH), 9.42 (bs, 1H, NH). Anal. Calcd. for $C_{18}H_{13}FN_4O_6$: C, 54.01; H, 3.27; N, 14.00. Found: C, 54.06; H, 3.31; N, 14.05.

N-(4-Methoxylbenzyl)-5-hydroxy-1-(2-nitrophenyl)-3,6-dioxo-1,2,3,6-tetrahydropyridazine-4-carboxamide (15). Mp 184–186°C, yield 37%; ^{1}H NMR: δ 3.72 (s, 3H, CH_3O), 4.33 (s, 2H, CH_2), 6.87–8.13 (m, 8H, ArH), 9.43 (bs, 1H, NH). Anal. Calcd. for $C_{19}H_{16}N_4O_7$: C, 55.34; H, 3.91; N, 13.59%. Found: C, 55.31; H, 3.96; N, 13.52%.

N-(4-Methylbenzyl)-5-hydroxy-1-(2-nitrophenyl)-3,6-dioxo-1,2,3,6-tetrahydropyridazine-4-carboxamide (16). Mp 180–182°C, yield 44%; ^{1}H NMR: δ 2.27 (s, 3H, CH_3), 4.36 (s, 2H, CH_2), 6.87–8.12 (m, 8H, ArH), 9.43 (bs, 1H, NH). Anal. Calcd. for $C_{19}H_{16}N_4O_6$: C, 57.58; H, 4.07; N, 14.14. Found: C, 57.53; H, 4.02; N, 14.19.

N-Benzyl-3-ethoxy-1-(2-nitrophenyl)-5,6-dioxo-1,2,5,6-tetrahydropyridazine-4-carboximidic acid (17). Mp 190–192°C, yield 21%; ^{1}H NMR: δ 1.33 (t, 3H, J = 6.8, CH_3), 4.20 (q, 2H, J = 6.8, CH_2), 4.54 (s, 2H, CH_2), 6.90–8.16 (m, 9H, ArH), 11.19 (bs, 1H, NH). Anal. Calcd. for $C_{20}H_{18}N_4O_6$: C, 58.54; H, 4.42; N, 13.65. Found: C, 58.51; H, 4.47; N, 13.61.

N-(4-Bromobenzyl)-3-ethoxy-1-(2-nitrophenyl)-5,6-dioxo-1,2,5,6-tetrahydropyridazine-4-carboximidic acid (18). Mp 184–186°C, yield 23%; ^{1}H NMR: δ 1.35 (t, 3H, J = 6.8, CH_3), 4.24 (q, 2H, J = 6.8, CH_2), 4.52 (s, 2H, CH_2), 6.96–8.19 (m, 8H, ArH), 11.23 (bs, 1H, NH). Anal. Calcd. for $C_{20}H_{17}BrN_4O_6$: C, 49.10; H, 3.50; N, 11.45. Found: C, 49.16; H, 3.57; N, 11.50.

N-(4-Chlorobenzyl)-3-ethoxy-1-(2-nitrophenyl)-5,6-dioxo-1,2,5,6-tetrahydropyridazine-4-carboximidic acid (19). Mp 184–186°C, yield 25%; ^{1}H NMR: δ 1.34 (t, 3H, J = 6.8, CH_3), 4.19 (q, 2H, J = 6.8, CH_2), 4.19 (s, 2H, CH_2), 6.99–8.16 (m, 8H, ArH), 11.26 (bs, 1H, NH). Anal. Calcd. for $C_{20}H_{17}ClN_4O_6$: C, 54.00; H, 3.85; N, 12.59. Found: C, 54.05; H, 3.89; N, 12.53.

N-(4-Fluorobenzyl)-3-ethoxy-1-(2-nitrophenyl)-5,6-dioxo-1,2,5,6-tetrahydropyridazine-4-carboximidic acid (20). Mp 186–188°C, yield 23%; ^{1}H NMR: δ 1.34 (t, 3H, J = 6.8, CH_3), 4.19 (q, 2H, J = 6.8, CH_2), 4.67 (s, 2H, CH_2), 6.97–8.17 (m, 8H, ArH), 11.27 (bs, 1H, NH). Anal. Calcd. for $C_{20}H_{17}FN_4O_6$: C, 56.08; H, 4.00; N, 13.08. Found: C, 56.03; H, 4.05; N, 13.03.

N-(4-Methoxylbenzyl)-3-ethoxy-1-(2-nitrophenyl)-5,6-dioxo-1,2,5,6-tetrahydropyridazine-4-carboximidic acid (21). Mp 184–186°C, yield 18%; ^{1}H NMR: δ 1.33 (t, 3H, J = 6.8, CH_3), 3.72 (s, 3H, CH_3O), 4.19 (q, 2H, J = 6.8, CH_2), 4.61 (s, 2H, CH_2), 6.88–8.16 (m, 8H, ArH), 11.26 (bs, 1H, NH). Anal. Calcd. for $C_{21}H_{20}N_4O_7$: C, 57.27; H, 4.58; N, 12.72. Found: C, 57.22; H, 4.53; N, 12.66.

N-(4-Methylbenzyl)-3-ethoxy-1-(2-nitrophenyl)-5,6-dioxo-1,2,5,6-tetrahydropyridazine-4-carboximidic acid (22). Mp 185–187°C, yield 17%; ^{1}H NMR: δ 1.33 (t, 3H, J = 6.8, CH_3), 2.27 (s, 3H, CH_3), 4.19 (q, 2H, J = 6.8, CH_2), 4.63 (s, 2H, CH_2), 6.97–8.16 (m, 8H, ArH), 11.26 (bs, 1H, NH). Anal. Calcd. for $C_{21}H_{20}N_4O_6$: C, 59.43; H, 4.75; N, 13.20. Found: C, 59.47; H, 4.71; N, 13.25.

Bioassays ***Overall integrase assay using an enzyme-linked immunosorbent assay and strand-transfer inhibition.*** We used enzyme-linked immunosorbent assays to determine the susceptibility of the HIV-1 integrase enzyme towards different compounds. These assays use an oligonucleotide substrate of which one oligonucleotide (5′-ACTGCTAGAGATTTTCCACACT GACTAAAAGGGTC-3′) is labeled with biotin at the 3′ end and the other oligonucleotide is labeled with digoxigenin at the 5′ end. For the overall integration assay the second 5′-digoxigenin labeled oligonucleotide is (5′-GACCCTTTTAGT CAGTGTGGAAAATCTCTAGCAGT-3′). For the strand transfer assay the second oligonucleotide lacks GT at the 3′ end. The integrase enzyme was diluted in 750 m*M* NaCl, 10 m*M* Tris pH 7.6, 10% glycerol, and 1 m*M* β-mercapto ethanol. To perform the reaction 4 μL diluted integrase (corresponding to a concentration of 1.6 μ*M*) and 4 μL of annealed oligonucleotides (7 n*M*) were added in a final reaction volume of 40 μL containing 10 m*M* $MgCl_2$, 5 m*M* DTT, 20 m*M* HEPES pH 7.5, 5% PEG and 15% DMSO. The reaction was carried out at 37°C for 1 h. Reaction products were denatured with 30 m*M* NaOH and detected by an immunosorbent assay on avidin-coated plates [12].

In vitro anti-HIV and drug susceptibility assays. The inhibitory effect of antiviral drugs on the HIV-induced cytopathic effect (CPE) in human lymphocyte MT-4 cell culture was determined by the MT-4/MTT-assay [13]. This assay is based on the reduction of the yellow colored 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by mitochondrial dehydrogenase of metabolically active cells to a blue formazan derivative, which can be measured spectrophotometrically. The 50% cell culture infective dose (CCID50) of the HIV(IIIB) strain was determined by titration of the virus stock using MT-4 cells. For the drug-susceptibility assays MT-4 cells were infected with 100–300 CCID50 of the virus stock in the presence of fivefold serial dilutions of the antiviral drugs. The concentration of various compounds achieving 50% protection against the CPE of the different HIV strains, which is defined as the EC_{50}, was determined. In parallel the 50% cytotoxic concentration (CC_{50}) was determined.

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