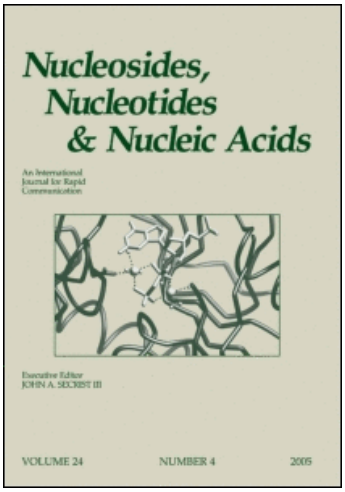

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A NEW CLASS OF ACYCLIC NUCLEOSIDE PHOSPHONATES: SYNTHESIS AND BIOLOGICAL ACTIVITY OF 9-{[(PHOSPHONOMETHYL)AZIRIDIN-1-YL]METHYL}GUANINE (PMAMG) AND ANALOGUES

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ABSTRACT

A new class of acyclic nucleoside phosphonates PMAMG, PMAMA, PMAMC, and PMAMT (compounds **1**, **2**, **3** and **4**) have been synthesized and tested in vitro against a wide variety of viruses, fungi and bacteria. PMAMG (**1**) was synthesized by the alkylation reaction of acetylguanine with the phosphonate side-chain, diisopropyl {[2-(bromomethyl)aziridin-1-yl]methyl}phosphonate (**9**), followed by deesterification reaction in the presence of TMSBr. In similar way, PMAMA, PMAMC, and PMAMT were prepared.

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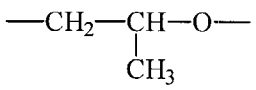
INTRODUCTION

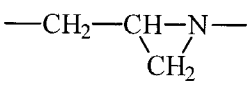
The acyclic nucleoside phosphonates (ANPs) are broad-spectrum antiviral agents with potent and selective antiviral activity in vitro and in vivo. ANPs have been proved to be effective against different DNA viruses and retroviruses including HIV.[1,2] These compounds were designed to circumvent the first phosphorylation step that is necessary for the activation of the nucleoside analogues,[3] once inside the cells through an endocytosis-like process, they are converted into active diphosphorylated metabolites which react with the viral DNA polymerase or act as chain terminators in the HIV reverse transcriptase (RT) reaction.[4] Numerous ANPs were synthesized and several structure-activity relationship (SAR) studies have been reported. (*S*)-9-[3-Hydroxy-2-(phosphonomethoxy)propyl]adenine, [(*S*)-HPMPA], an acyclic nucleotide analogue reported by Holy and De Clercq,[5] is a representative of this class which possesses broad spectrum antiviral activity. (*S*)-1-(3-Hydroxy-2-phosphonomethoxypropyl)cytosine (HPMPC, cidofovir) has been approved for the treatment of CMV-retinitis in AIDS patients;[6] 9-(2-Phosphonomethoxyethyl)adenine (PMEA, adefovir) and its oral prodrug form [*bis*(pivaloyloxymethyl)-PMEA] (POM-PMEA, adefovir dipivoxil) is currently being evaluated in patients infected with hepatitis B virus.[6] 9-(*R*)-[2-Phosphonomethoxypropyl]adenine (PMPA) has selective activity against a wide range of hepadnaviruses and retroviruses including the human immunodeficiency virus.[1] and the isopropyloxy carbonyloxymethyl ester prodrug of PMPA (Tenofovir disproxil) is approved now for the treatment of HIV. Many other compounds were synthesized and tested in vitro for antiviral activity, such as (*R*)-PMPG,[7] (*R*)-8-aza-PMPG,[8] and the methylidene derivative[9] of (*R*)-PMPG, to study the SAR of (*R*)-PMPG by modifications involving the purine moiety and/or the phosphonate side-chain.

In our continued efforts to study the SAR of (*R*)-PMPG, and PMPA, we here describe the synthesis of PMAMG, and PMAMA (compounds **1**, and **2**). They were obtained by modifications involving the phosphonate side-chain by the introduction of a nitrogen atom at the oxygen position on the phosphonate side-chain of (*R*)-PMPG, and PMPA in which the propyloxy group (portion **A**) in (*R*)-PMPG and PMPA was replaced by aziridinylmethyl group (portion **B**), its bioisoster closed system (Fig. 1). The formation of a three member ring (aza-cyclopropane) which therefore will confer rigidity to the phosphonate side-chain, could affect preferentially the interaction with the target enzyme(s).

Thereby, PMAMC and PMAMT (compounds **3** and **4**) analogues of HPMPC were prepared for the same rational.



A



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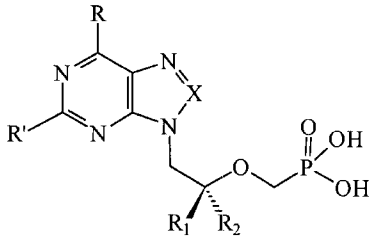
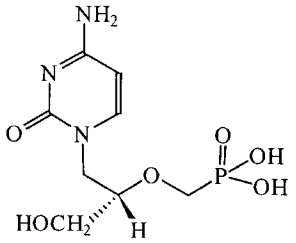
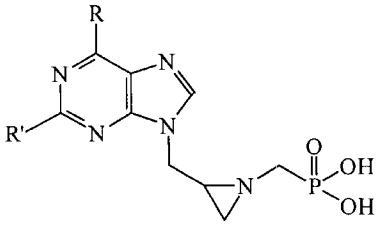
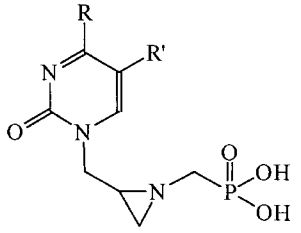




PMEA R', R_1, R_2 = H, R = NH_2, X = CH
PMPA R',R_1 = H, R_2 = CH_3, R = NH_2, X = CH
(*R*)-PMPG R' = NH_2, R_1 = H, R_2 = CH_3, R = OH, X = CH
(*R*)-8-aza-PMPG R' = NH_2, R_1 = H, R_2 = CH_3, R = OH, X = N
(*S*)-HPMPA R' = H, R_1 = H, R_2 = CH_2OH, R = NH_2, X = CH

HPMPC





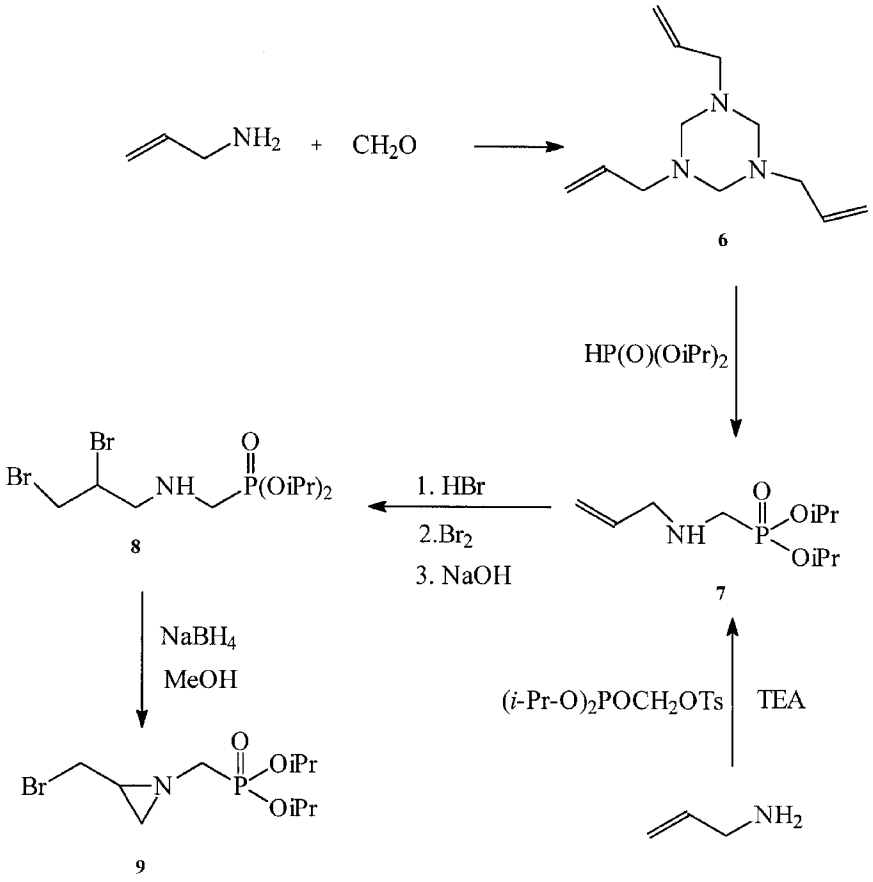
PMAMG R = OH, R' = NH_2 (**1**)
PMAMA R = NH_2, R' = H (**2**)

PMAMC R = NH_2, R' = H (**3**)
PMAMT R = OH, R' = CH_3 (**4**)

Figure 1.

CHEMISTRY

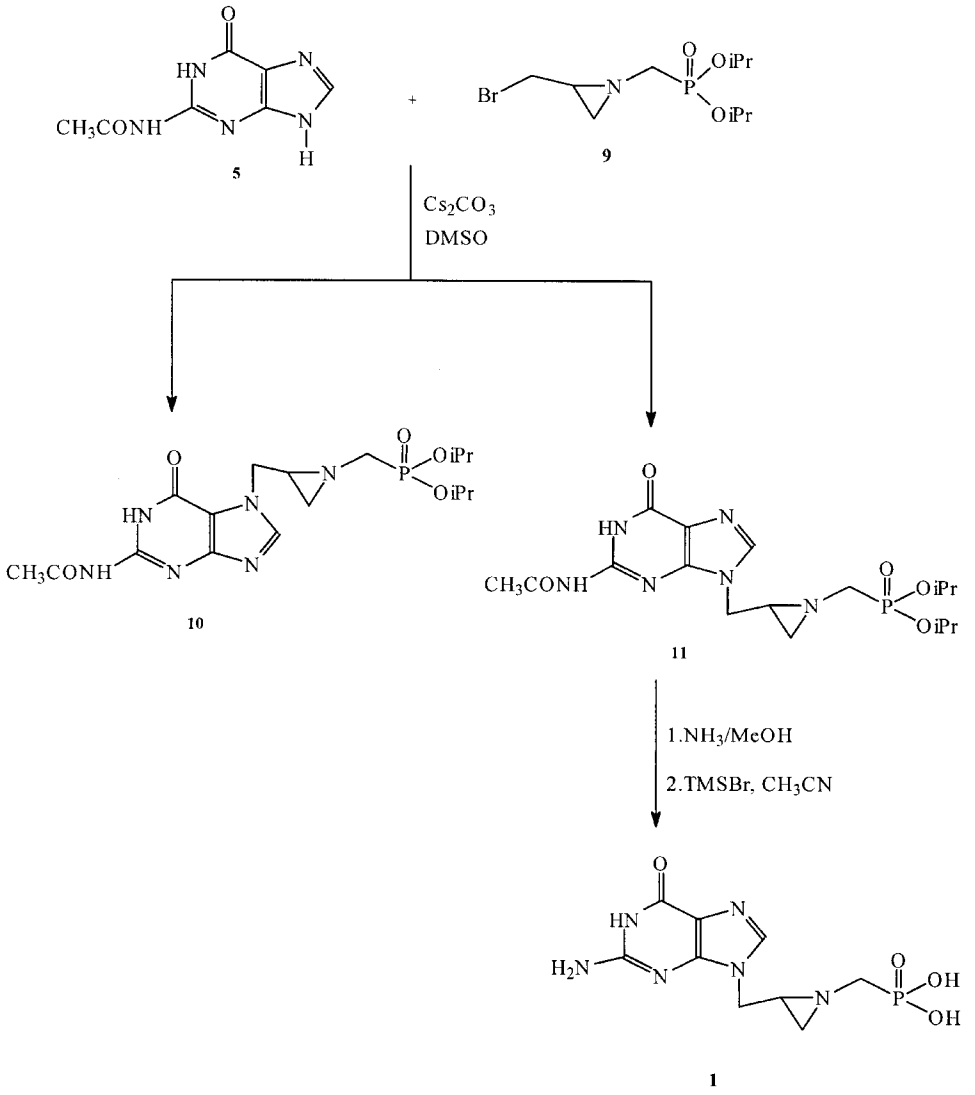
The synthesis of PMAMG (**1**) was performed by the alkylation reaction between acetylguanine (**5**) and diisopropyl {[2-(bromomethyl)aziridin-1-yl]methyl}phosphonate (**9**) as shown in Sch. 2. The synthesis of the phosphonate side-chain **9** was performed as reported by Stevens[10] (Sch. 1). Condensation reaction of allylamine with formaldehyde led to the formation of 1,3,5-triallylhexahydro[1,3,5]triazine **6**[10] and was then treated with



Scheme 1.

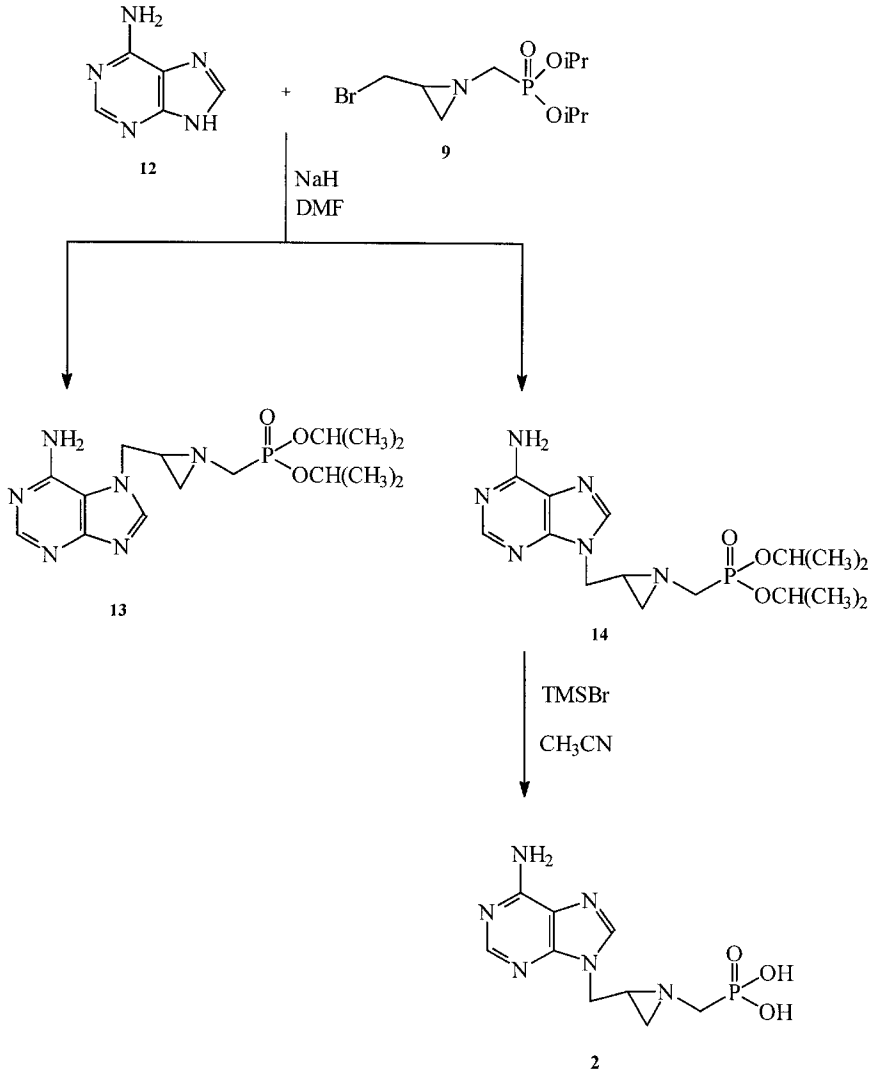
diisopropyl phosphite to give the diisopropyl allylaminomethyl phosphonate **7**. Bromination of the allyl moiety was achieved, after protecting the secondary aliphatic amine by addition of hydrobromic acid, with bromine in dichloromethane leading to intermediate **8**, which was cyclized to **9** with sodium borohydride. We have also developed a convenient method for the preparation of the intermediate **7** (Sch. 1). The reaction of allylamine with diisopropyl[(*p*-tosyloxy)-methyl]phosphonate[11a,b] in the presence of triethylamine gave the diisopropyl allylamino phosphonate **7** (98% yield) which was converted to **9** as previously described.

Compound **9** was coupled to acetylguanine (**5**) in DMSO in the presence of cesium carbonate at 90°C. The reaction mixture was stirred at the same temperature for 3 h under nitrogen atmosphere. The resulting mixture of N^7-, and N^9-isomers was separated by column chromatography on silica gel. The position of alkylation was deduced on the bases of the comparison of



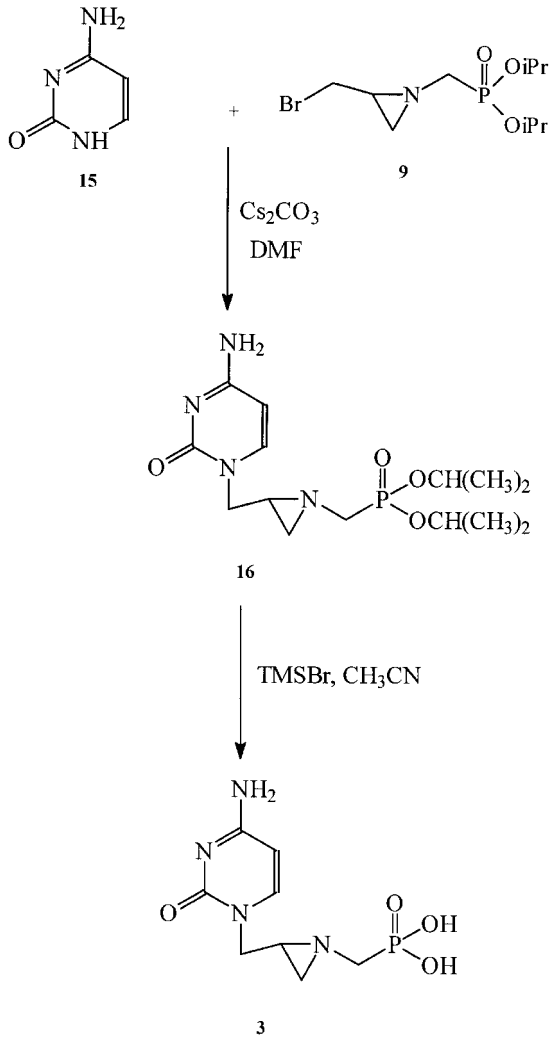
Scheme 2.

^{1}H and ^{13}C-NMR spectrum data of **10** and **11** with N^7- and N^9-alkylated derivatives reported in literature, respectively.[9] PMAMG (**1**) was obtained by treatment of **11** with ammonia in methanol and then with bromotrimethylsilane (TMSBr) in acetonitrile and was purified by precipitation from acetone and water. The structure of **1** was confirmed by UV, ^{13}C NMR and ^{1}H-NMR spectrum data.[7,9] The PMAMA (compound **2**) was obtained in a similar way to PMPAG by the reaction of **9** with adenine (**12**) (Sch. 3). The alkylation reaction resulted in the formation of N^7-, and N^9 isomers



Scheme 3.

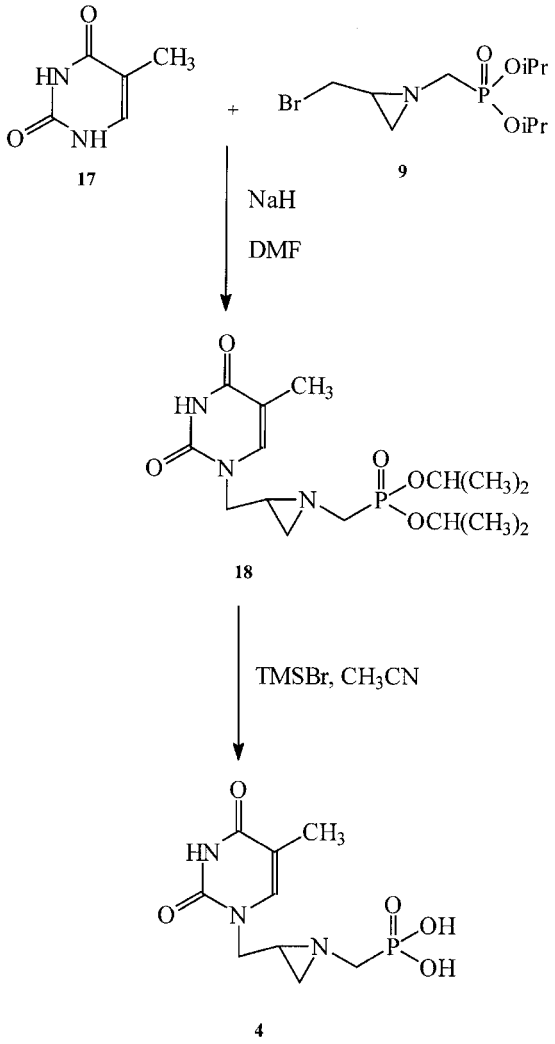
(**13** and **14**) which were separated by flash chromatography in 42% yield. PMAMA was obtained pure by treatment of **14** with TMSBr followed by water and acetone. PMAMC (**3**) was synthesized by the reaction of **9** with cytosine (**15**) in DMF in the presence of cesium carbonate affording **16** (Sch. 4). Treatment of **16** with bromotrimethylsilane followed by water gave the free phosphonate **3** (PMAMC). The synthesis of PMAMT was performed by direct alkylation of thymine (**17**) with **9** in the presence of sodium hydride in DMF to give the diester phosphonate **18** which was converted to **4** as described to compound **3** (Sch. 5).



Scheme 4.

CHEMICAL STABILITY STUDIES

The chemical stability studies were done at 37°C (1) in water, (2) in pH 7.2 (ammonium acetate buffer, 0.02 M), and (3) in pH $\sim$1 for 7 days. The use of reverse-phase RP-18 F254 S plates (Merck) allowed to exclude the chemical instability, with no chemical decompositions of the aziridinyl acyclic nucleoside phosphonates, compounds PMAMG, PMAMA, PMAMT, and PMAMC.



Scheme 5.

RESULTS AND DISCUSSION

The aziridine acyclic nucleoside phosphonates compounds, in the free acid form, PMAMG, PMAMA, PMAMT and PMAMC were evaluated in vitro for cytotoxicity and for their antiviral activity against a wide variety of RNA viruses (YFV, BVDV, Sb-1, VSV), retroviruses including HIV-1, and DNA viruses (HSV-1 and HBV). Title compounds were also tested for their capability to inhibit the multiplication of various human fungi and Gram positive, negative and acid fast microorganisms. Ribavirine (YFV,

BVDV), Guadine (Sb-1), Acyclovir (HSV-1), AZT and PMPA (HIV-1), 3TC (HBV), Bifonazole (fungi) and Streptomycin (bacteria) were used as reference drugs.

Compounds showed no cytotoxicity in a variety of cell lines. Moreover, they showed neither antiviral nor antimicrobial activity at the highest concentration tested (generally 100 μM, data not shown). All reference drugs confirmed their activity in specific assays.

The lack of biological activity might be due to the substitution of oxygen with the nitrogen atom in the phosphonate side-chain or to the rigid system, leading to one or more of the following effects: i) a decreased transport through the cell membrane; ii) inefficient phosphorylation; iii) poor affinity of the diphosphate derivatives for target polymerases.

Studies are in progress to explain which of the above mentioned modifications is responsible for the inactivity of the compounds.

EXPERIMENTAL SECTION

Melting points were determined on Stuart Scientific SMP1-7630 and are not corrected. Ultraviolet spectra were recorded on JASCO V-530 double beam or GBC 918 double beam UV/VIS spectrophotometer. Infrared pectra were recorded on FT/IR JASCO 300 E. Proton and carbon-13 magnetic resonance (^{1}H and ^{13}C-NMR) spectra were recorded on a Bruker Avance DPX 300 spectrometer and determined at 300 and 75 MHz respectively. All spectra were determined in $CDCl_3$, DMSO-d_6, and chemical shifts are reported in δ units (parts per million) relative to tetramethylsilane as an internal standard. All exchangeable protons were confirmed by the addition of D_2O. Thin layer chromatography (TLC) was run on silica gel 60 F254 and RP-18 F254 S plates (Merck); silica gel 60 (Merck) (70–230 and 230–400 mesh) for column chromatography was used.

1,3,5-Triallylhexahydro[1,3,5]triazine (6).[10] Allylamine (9.9 g, 173.6 mmol) was added to formaldehyde solution 37% in water and stirred at room temperature for 1 h led to the formation of **6** as yellow oil (11.5 g, 95.8%).

Diisopropyl allylaminomethyl phosphonate (7). Method A. To 1,3,5-Triallylhexahydro[1,3,5]-triazine (**6**) (10 g, 48.2 mmol) was added diisopropylphosphite (40.1 g, 144.6 mmol). The reaction mixture was heated at 100°C for 15 h.The resulted crude product was purified by flash chromatography on silica gel column eluting with ($CHCl_3$-MeOAc, 80:20) to give **7** as yellow oil (9.6 g, 85%). TLC ($CHCl_3$:MeOAc, 50:50): *R*f 0.45. IR (neat):ν max 3460 (br.), 3000, 1245, 1032, 980 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 1.35 (d, $J = 6.2$ Hz, 12 H, 2xOCH(CH_3)$_2$), 2.05 (br s, 1H, NH), 2.9 (d, $J = 12.7$ Hz, 2H, CH_2P), 3.35

(d, $J = 6$ Hz, 2H, CH_2N), 4.75 (m, 2H, 2xOCH$(CH_3)_2$), 5.15 (m, 2H, CH_2=), 5.85 (m, 1H, =CH).

Method B. Allylamine (10 g, 175.4 mmol) was added to diisopropyl[(*p*-tosyloxy)-methyl]phosphonate[11a,b] (51.2 g, 146.2 mmol) in THF (100 mL) and triethylamine (24.5 mL, 175.4 mmol). The reaction mixture was heated at 40°C for 2 h. The resulted mixture was filtered and evaporated to dryness, then the residue was purified by flash chromatography on silica gel column eluting with ($CHCl_3$-MeOH, 95:5) to give **7** (33.85 g, 98%).

Diisopropyl [(2,3-dibromopropylamino)methyl] phosphonate (8). Compound **7** (10 g, 42.3 mmol) was treated, at 0°C, with 48% aqueous hydrobromic acid (7.14 g, 42.3 mmol). Bromine (6.76 g, 42.3 mmol) in dichloromethane (10 mL) was added drop-wise at the hydrobromide salt. The resulted mixture in a two-phase system was stirred at room temperature for 5 h. The dibromo derivative **8** was obtained, after extraction with dichloromethane and purification by chromatography on silica gel column eluting with ($CHCl_3$-MeOAc, 80:20), as yellow oil (15 g, 89.77%).TLC ($CHCl_3$:MeOAc, 50:50): *R*f 0.65. IR (neat): ν max 3460 (br.), 3000, 1245, 1032, 980 cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.4 (d, $J = 6.2$ Hz, 12 H, 2xOCH$(CH_3)_2$), 2.0 (br s, 1H, NH), 2.95–3.05 (ddd, $J = 11.5$, 12.6, 14.7 Hz, 2H, CH_2P), 3.15–3.35 (ddd, $J = 3.7$, 6.6, 13.6 Hz, 2H, CH_2N), 3.8 (d, $J = 7.3$ Hz, 2H, $BrCH_2$), 4.3 (m, 1H, CHBr), 4.75 (m, 2H, 2xOCH$(CH_3)_2$).

Diisopropyl [[2-(bromomethyl)aziridin-1-yl]methyl] phosphonate (9). To a stirring solution of **8** (14 g, 35.4 mmol) in anhydrous methanol (20 mL) at 0°C was added sodium borohydride (1.34 g, 35.4 mmol). The reaction mixture was stirred a reflux for 6 h, the solvent was evaporated and the residue was taken with $CHCl_3$ and aqueous work-up extraction. Further purification on chromatography silica gel column eluting with $CHCl_3$ allow to give compound **9** as pure yellow oil (9.5 g, 85%).TLC ($CHCl_3$:MeOAc, 50:50): *R*f 0.52. IR (neat): ν max 3460 (br.), 3000, 1245, 1032, 980 cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.35 (d, $J = 6.2$ Hz, 12 H, 2xOCH$(CH_3)_2$), 1.65 (d, $J = 6.1$ Hz, 1H, CH_2N), 1.85 (d, $J = 3.5$ Hz, 1H, CH_2N), 1.95 (m, 1H, CHN), 2.55–2.8 (ddd, $J = 11.2$, 13.9, 14.1 Hz, 2H, CH_2P), 3.15–3.5 (ddd, $J = 6.2$, 6.6, 10.5 Hz, 2H, $BrCH_2$), 4.8 (m, 2H, 2xOCH$(CH_3)_2$).

7-{[((Diisopropylphosphono)methyl)-aziridin-1-yl]methyl}N-acetylguanine (10) and 9-{[((Diisopropyl-phosphono)methyl)-aziridin-1-yl]methyl} N-acetylguanine (11). Diisopropyl{[2-(bromomethyl)-aziridin-1-yl]methyl}-phosphonate[10] (**9**) (1.25 g, 3.97 mmol) was added to a stirring mixture of N-acetylguanine (**5**) (0.77 g, 3.97 mmol) and cesium carbonate (1.68 g, 5.16 mmol) in anhydrous DMSO (10 mL) under nitrogen atmosphere at room temperature. The mixture of reaction was heated at 80°C for 3 h

and then was evaporated. The residue was taken with $CHCl_3$-MeOH (80:20) and filtered. The filtrate was evaporated to dryness and the resulted residue was purified by chromatography on silica gel column eluting with ($CHCl_3$-MeOH-NH_4OH, 95:4.8:0.2) to give **10** as a yellow solid (0.38 g, 25%). Mp: 170–172°C. TLC ($CHCl_3$-MeOH, 80:20): *R*f 0.62. ^{1}H NMR (DMSO-d_6): δ 1.2–1.25 (dd. $J = 4.3$, 6.3 Hz, 12 H, 2xOCH(CH_3)$_2$), 1.45 (d, $J = 6.2$ Hz, 1H, CH_2N), 1.73 (d, $J = 3.1$ Hz, 1H, CH_2N), 2.08 (m,1H, CH-N), 2.4 (s, 3H, NHCO*CH*$_3$), 2.52 (dd, $J = 8.8$, 12.9 Hz, 1H, CH_2P), 2.66 (dd, $J = 9.0$, 13.1 Hz, 1H, CH_2P), 4.1 (dd, $J = 3.8$, 5.5 Hz, 1H, NCH_2), 4.25 (dd, $J = 4.1$, 5.9 Hz, 1H, NCH_2), 4.6 (m, 2H, 2xOCH(CH_3)$_2$), 6.2 (br s, 2H, NH_2), 7.8 (s, 1H, H-8), 10.95 (br s, 1H, NH), 13.2 (br s, 1H, *NH*COCH$_3$). ^{13}C NMR (DMSO-d_6): δ 158.1, 154.7, 153.1, 143.4, 108.6, 72.0, 71.9, 62.1 (d, $J = 163.0$ Hz), 53.9, 42.5, 40.5, 24.2, 24.1, 23.95, 23.90.

Further elution gave compound **11** as a yellow solid (0.49 g, 32%). Mp: 146–148°C. TLC ($CHCl_3$-MeOH, 80:20): *R*f 0.56. ^{1}H NMR (DMSO-d_6): δ 1.23 (d, $J = 6.1$ Hz, 12 H, 2xOCH(CH_3)$_2$), 1.48 (d, $J = 6.2$ Hz, 1H, CH_2N), 1.7 (d, $J = 3.1$ Hz, 1H, CH_2N), 1.97 (m, 1H, CHN), 2.3 (s, 3H, NHCO*CH*$_3$), 2.6 (dd, $J = 8.7$, 12.7 Hz, 1H, CH_2P), 2.8 (dd, $J = 9.2$, 13.3 Hz, 1H, CH_2P), 3.8 (dd, $J = 3.8$, 5.6 Hz, 1H, NCH_2), 3.95 (dd, $J = 3.9$, 5.7 Hz, 1H, NCH_2), 4.6 (m, 2H, 2xOCH(CH_3)$_2$), 6.5 (br s, 2H, NH_2), 7.6 (s, 1H, H-8), 10.8 (br s, 1H, NH), 12.9 (br s, 1H, *NH*COCH$_3$). ^{13}C NMR (DMSO-d_6): δ 157.6, 153.7, 151.6, 138.9, 116.6, 72.0, 71.9, 62.2 (d, $J = 163.2$ Hz), 52.1, 42.3, 40.4, 24.2, 24.1, 23.95, 23.90.

9-{[((Phosphono)methyl)-aziridin-1-yl]methyl}guanine (1). To **11** (0.45 g, 1.06 mmol) was added NH_3/MeOH saturated solution (20 mL) and left stirring at room temperature for 2 days. Filtration and recrystallization from hot absolute ethanol gave white solid (0.36 g, 90%). To a stirring solution of the resulted solid (0.3 g, 0.78 mmol) in anhydrous acetonitrile (20 mL) was added bromotrimethylsilane (1.19 g, 7.8 mmol) drop-wise at room temperature under a nitrogen atmosphere. The mixture of reaction was stirred at the same temperature for 18 h, and the solvent was removed under reduced pressure. The oil residue was treated with water-acetone (10:20 mL), the precipitate was collected and washed with acetone. Compound **1** was obtained, after crystallization used anhydrous ethanol, as pure white solid (0.18 g, 78%). Mp: 280–282°C dec. TLC (H_2O-CH_3CN, 85:15): *R*f 0.80. UV (pH 12): λmax 256 nm (ϵ 12,000), 270 (ϵ 15,000). ^{1}H NMR (DMSO-d_6): δ 1.3 (d, $J = 6.1$ Hz, 1H, CH_2N), 1.6 (d, $J = 3.1$ Hz, 1H, CH_2N), 2 (m, 1H, CH-N), 2.4 (dd, $J = 9.2$, 13.4 Hz, 1H, CH_2P), 2.65 (dd, J = 9.1, 13.3 Hz, 1H, CH_2P), 4.0 (dd, $J = 3.7$, 5.7 Hz, 1H, N-CH_2), 4.2 (dd, $J = 3.9$, 5.8 Hz, 1H, NCH_2), 6.65 (br s, 2H, NH_2), 7.75 (s, 1H, H-8), 10.9 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 157.4, 153.9, 151.7, 138.5, 116.3, 76, 58.6 (d, $J = 147$ Hz), 52.1, 40.2. *Anal*. Calcd. For $C_9H_{13}N_6O_4P$: C 36.01, H 4.36, N 27.99. Found: C 36.26, H 4.31, N 28.04.

7-{[((Diisopropylphosphono)methyl)-aziridin-1-yl]methyl}adenine (13) and 9-{[((Diisopropyl-phosphono)methyl)-aziridin-1-yl]methyl}adenine(14). To a solution of adenine (**12**) (1 g, 7.4 mmol) in anhydrous DMF (50 mL) under a nitrogen atmosphere, sodium hydride (60% in mineral oil, 0.18 g, 7.4 mmol) was added and the mixture was stirred at 80°C for 1 h. A solution of diisopropyl{[2-(bromomethyl)-aziridin-1-yl]methyl}phosphonate (**9**) (2.33 g, 7.4 mmol) (10) in anhydrous DMF (30 mL) was added and the resulting reaction mixture was heated at the same temperature for 3 h. The solvent was removed under reduced pressure and the residue was treated with boiled $CHCl_3$ (3×50 mL). After filtration of insoluble material, the filtrate was evaporated and the residue was chromatographed on silica gel using $CHCl_3$-MeOH (94:6) to give **13** as yellow oil (0.6 g, 22%).TLC ($CHCl_3$-MeOH 90: 10): *Rf* 0.56. ^{1}H NMR (DMSO-d_6): δ 1.2–1.3 (dd, $J = 4.3$, 6.3 Hz, 12 H, 2xOCH(CH_3)$_2$), 1.55 (d, $J = 6.3$ Hz, 1H, CH_2N), 1.83 (d, $J = 3.3$ Hz, 1H, CH_2N), 2.08 (m, 1H, CH-N), 2.62 (dd, $J = 8.9$, 13.1 Hz, 1H, CH_2P), 2.72 (dd, $J = 9.1$, 13.3 Hz, 1H, CH_2P), 4.15 (dd, $J = 3.9$, 5.7 Hz, 1H, NCH_2), 4.42 (dd, $J = 4.1$, 6.0 Hz, 1H, NCH_2), 4.75 (m, 2H, 2xOCH(CH_3)$_2$), 5.9 (br s, 2H, NH_2), 7.95 (s, 1H, H-8), 8.37 (s, 1H, H-2). ^{13}C NMR (DMSO-d_6): δ 156.9, 146.7, 141.8, 141.7, 107.7, 70.8, 70.7, 61.3 (d, $J = 165.0$ Hz), 55.6, 51.2, 42.7, 24.15, 24.0, 23.95, 23.90.

Further elution gave compound **14** as a yellow oil (1.69 g, 62%). TLC ($CHCl_3$-MeOH, 90:10): *R*f 0.53. ^{1}H NMR (DMSO-d_6): δ 1.2–1.3 (dd, $J = 4.3$, 6.3 Hz, 12 H, 2xOCH(CH_3)$_2$), 1.55 (d, $J = 6.3$ Hz, 1H, CH_2N), 1.83 (d, $J = 3.3$ Hz, 1H, CH_2N), 2.08 (m, 1H, CH-N), 2.62 (dd, $J = 8.9$, 13.1 Hz, 1H, CH_2P), 2.72 (dd, $J = 9.1$, 13.3 Hz, 1H, CH_2P), 4.15 (dd, $J = 3.9$, 5.7 Hz, 1H, NCH_2), 4.42 (dd, $J = 4.1$, 6.0 Hz, 1H, NCH_2), 4.75 (m, 2H, 2xOCH(CH_3)$_2$), 6.1 (br s, 2H, NH_2), 7.95 (s, 1H, H-8), 8.35 (s, 1H, H-2). ^{13}C NMR (DMSO-d_6): δ 156.6, 145.7, 140.6, 136.9, 116.7, 70.8, 70.7, 61.3 (d, $J = 165.3$ Hz), 55.9, 51.2, 42.9, 24.15, 24.0, 23.95, 23.90.

9-{[((Phosphono)methyl)-aziridin-1-yl]methyl}adenine (2). To a stirring solution of **14** (1 g, 2.7 mmol) in anhydrous acetonitrile (30 mL) was added bromotrimethylsilane (4.1 g, 27 mmol) drop-wise at room temperature under a nitrogen atmosphere. The mixture of reaction was stirred at room temperature over night, and the solvent was removed under reduced pressure. The oil residue was treated with water-acetone (20:40 mL), the precipitate was collected and washed with acetone. Compound **2** was obtained, after crystallization used anhydrous ethanol, as pure white solid (0.65 g, 85%). Mp: 249–251°C dec. TLC (H_2O-CH_3CN, 85:15): *R*f 0.75. UV (pH 12): λ max 265 nm (ϵ 10,600). ^{1}H NMR (DMSO-d_6): δ 1.45 (d, $J = 6.3$ Hz, 1H, CH_2N), 1.75 (d, $J = 3.3$ Hz, 1H, CH_2N), 2.0 (m, 1H, CH-N), 2.48 (dd, $J = 8.9$, 13.1 Hz, 1H, CH_2P), 2.66 (dd, $J = 9.1$, 13.3 Hz, 1H, CH_2P), 4.0 (m, 2H, NCH_2), 5.59 (br s, 2H, NH_2), 8.4 (s, 1H, H-8), 8.65 (s, 1H, H-2). ^{13}C NMR (DMSO-d_6): δ 156.3, 145.9, 141.1, 137.9, 116.5, 58.3 (d, $J = 147$ Hz),56.7,

51.4, 43.2. Anal. Calcd. For $C_9H_{13}N_6O_3P$: C 38.03, H 4.61, N 29.57. Found: C 37.98, H 4.66, N 29.50.

1-{[((Diisopropylphosphono)methyl)-aziridin-1-yl]methyl}cytosine (16). The mixture of cytosine (**15**) (1 g, 9.1 mmol) and cesium carbonate (3.7 g, 11.4 mmol) in anhydrous DMF (20 mL) under a nitrogen atmosphere was stirred at 100°C for 1 h, and after the addition of diisopropyl{[2-(bromomethyl)-aziridin-1-yl]methyl}phosphonate (**9**) (3.6 g, 11.4 mmol)[9] the mixture was heated at the same temperature for 5 h. The solvent was removed under reduced pressure and the residue was chromatographed on silica gel using $CHCl_3$-MeOH (92:8) to give **16** as yellow oil (2.6 g, 85%).TLC ($CHCl_3$-MeOH 90:10): *Rf* 0.58. ^{1}H NMR (DMSO-d_6): δ 1.3–1.4 (dd, $J = 4.3$, 6.3 Hz, 12 H, 2xOCH(CH_3)$_2$), 1.58 (d, $J = 6.4$ Hz, 1H, CH_2N), 1.82 (m, 1H, CH-N), 1.86 (d, $J = 3.4$ Hz, 1H, CH_2N), 1.99 (s, 3H, CH_3), 2.52 (dd, $J = 8.9$, 13.1 Hz, 1H, CH_2P), 2.75 (dd, $J = 9.1$, 13.3 Hz, 1H, CH_2P), 3.6 (dd, $J = 3.9$, 5.7 Hz, 1H, NCH_2), 4.18 (dd, $J = 4.1$, 6.0 Hz, 1H, NCH_2), 4.77 (m, 2H, 2xOCH(CH_3)$_2$), 5.62 (d, $J = 5.7$ Hz, 1H, H-5), 7.2 (s, 1H, H-6), 7.45 (d, $J = 7.05$ Hz, 1H, H-6), 8.7 (br s, 2H, NH_2).

1-{[((Phosphono)methyl)-aziridin-1-yl]methyl}cytosine (3). To a stirring solution of **16** (1 g, 2.9 mmol) in anhydrous acetonitrile (30 mL) was added bromotrimethylsilane (4.4 g, 29 mmol) drop-wise at room temperature under a nitrogen atmosphere. The mixture of reaction was stirred overnight, and the solvent was removed under reduced pressure. The oil residue was treated with water-acetone (15:30 mL), the precipitate was collected and washed with acetone. Compound **3** was obtained, after crystallization used anhydrous mixture of ethanol and acetone, as pure white solid (0.63 g, 83%). Mp: 250–252°C dec. TLC (H_2O-CH_3CN, 85:15): *R*f 0.64. UV (pH 12): λ max 291 nm (ϵ 6800). ^{1}H NMR (DMSO-d_6): δ 1.52 (d, $J = 6.4$ Hz, 1H, CH_2N), 1.76 (m, 1H, CH-N), 1.82 (d, $J = 3.4$ Hz, 1H, CH_2N), 1.99 (s, 3H, CH_3), 2.5 (dd, $J = 8.9$, 13.1 Hz, 1H, CH_2P), 2.65 (dd, $J = 9.1$, 13.3 Hz, 1H, CH_2P), 3.62 (dd, $J = 3.9$, 5.7 Hz, 1H, NCH_2), 4.18 (dd, $J = 4.1$, 6.0 Hz, 1H, NCH_2), 5.62 (d, $J = 5.7$ Hz, 1H, H-5), 7.2 (s, 1H, H-6), 7.45 (d, $J = 7.05$ Hz, 1H, H-6), 8.7 (br s, 2H, NH_2). Anal. Calcd. For $C_8H_{13}N_4O_4P$: C 36.93, H 5.04, N 21.53. Found: C 36.84, H 4.97, N 21.6.

1-{[((Diisopropylphosphono)methyl)-aziridin-1-yl]methyl}thymine (18). To a solution of thymine (**17**) (1 g, 7.9 mmol) in anhydrous DMF (50 mL) under a nitrogen atmosphere, sodium hydride (60% in mineral oil, 0.19 g, 7.9 mmol) was added and the mixture was stirred at 80°C for 1 h. A solution of diisopropyl{[2-(bromomethyl)-aziridin-1-yl]methyl}phosphonate(**9**) (2.49 g, 7.9 mmol) (10) in anhydrous DMF (30 mL) was added and the resulting reaction mixture was heated at the same temperature for 2 h. The solvent was removed under reduced pressure and the residue was

chromatographed on silica gel using $CHCl_3$-MeOH (94:6) to give **18** as yellow oil (2.5 g, 89.3%).TLC ($CHCl_3$-MeOH 92:8): *Rf* 0.58. ^{1}H NMR (DMSO-d_6): δ 1.3–1.4 (dd, $J = 4.3$, 6.3 Hz, 12 H, 2xOCH(CH_3)$_2$), 1.52 (d, $J = 6.4$ Hz, 1H, CH_2N), 1.78 (m, 1H, CH-N), 1.8 (d, $J = 3.4$ Hz, 1H, CH_2N), 1.93 (s, 3H, CH_3), 2.5 (dd, $J = 8.9$, 13.1 Hz, 1H, CH_2P), 2.75 (dd, $J = 9.1$, 13.3 Hz, 1H, CH_2P), 3.5 (dd, $J = 3.9$, 5.7 Hz, 1H, NCH_2), 4.08 (dd, $J = 4.1$, 6.0 Hz, 1H, NCH_2), 4.77 (m, 2H, 2xOCH(CH_3)$_2$), 7.2 (s, 1H, H-6), 8.7 (s, 1H, NH).

1-{[((Phosphono)methyl)-aziridin-1-yl]methyl}thymine (4). To a stirring solution of **18** (1 g, 2.78 mmol) in anhydrous acetonitrile (30 mL) was added bromotrimethylsilane (4.2 g, 27.8 mmol) drop-wise at room temperature under a nitrogen atmosphere. The mixture of reaction was stirred for 18 h, and the solvent was removed under reduced pressure. The oil residue was treated with water-acetone (15:30 mL), the precipitate was collected and washed with acetone. Compound **4** was obtained, after crystallization used anhydrous mixture of ethanol and acetone, as pure white solid (0.67 g, 87%). Mp: 245–247°C dec. TLC (H_2O-CH_3CN, 85:15): *R*f 0.70. UV (pH 12): λmax 291 nm (ϵ 6500). ^{1}H NMR (DMSO-d_6): 1.34 (d, $J = 6.4$ Hz, 1H, CH_2N), 1.62 (m, 1H, CH-N), 1.66 (d, $J = 3.4$ Hz, 1H, CH_2N), 1.91 (s, 3H, CH_3), 2.2 (dd, $J = 8.9$, 13.1 Hz, 1H, CH_2P), 2.45 (dd, $J = 9.1$, 13.3 Hz, 1H, CH_2P), 3.3 (dd, $J = 3.9$, 5.7 Hz, 1H, NCH_2), 3.98 (dd, $J = 4.1$, 6.0 Hz, 1H, NCH_2), 7.25 (s, 1H, H-6), 8.7 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 158.3, 148.9, 140.7, 103.5, 54.2, 52.3, 45.1, 35.5, 12.5. *Anal*. Calcd. For $C_9H_{14}N_3O_5P$: C 39.28, H 5.13, N 15.27. Found: C 38.90, H 4.87, N 14.93.

BIOLOGICAL DETERMINATION

Compounds. Test compounds were dissolved in DMSO at an initial concentration of 200 mM and then were serially diluted in culture medium.

Cells and Viruses. MT-4 cells [grown in RPMI 1640 containing 10% fetal calf serum (FCS), 100 UI/mL penicillin G and 100 μg/mL streptomycin] were used for anti-HIV-1 assays. The 2.2.15 cells line [(clonal cells derived from HepG2 cells that were transfected with a plasmid containing HBV DNA) grown in DMEM supplemented with 4% fetal calf serum, 100 IU/mL penicillin G and 100 μg/mL streptomycin and 0.5 mM glutamine] were used for anti-HBV assays. Baby hamster kidney cells (BHK-21, grown in MEM Earle's with 1.5 g/L sodium bicarbonate additioned of 2 mM L-glutamine, 10% FBS and 1mM sodium pyruvate) were used for anti-YFV and Reovirus-1 assays. Bovine Turbinate cells (BT, grown in DMEM with 1.5 g/L sodium bicarbonate, 10% horse serum and 1.0 mM sodium pyruvate) were used for anti-BVDV assays. VERO cells (grown in DMEM with 3.7 g/L sodium bicarbonate and 10% FBS) were used for anti-HSV-1, Sb-1, VSV assays.

Cell cultures were checked periodically for the absence of mycoplasma contamination with a MycoTect Kit (Gibco).

Anti-HIV Assay. Activity against HIV-1 (HIV-1, III_B strain, obtained from supernatants of persistently infected $H9/III_B$ cells) multiplication in acutely infected cells was based on inhibition of virus-induced cytopathogenicity in MT-4 cells. Briefly, 50 μL of RPMI 10% FCS containing 1×10^4 cells were added to each well of flat-bottomed microtiter trays containing 50 μL of medium and serial dilutions of test compounds. 20 μL of an HIV-1 suspension containing 100 $CCID_{50}$ were then added. After a 4 day incubation at 37°C, the number of viable cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) method. Cytotoxicity of compounds, based on the viability of mock-infected cells as monitored by the MTT method, was evaluated in parallel with their antiviral activity.

Anti-Reovirus-1 and BVDV Assays. Activity against the Reovirus-1 and BVDV multiplication in acutely infected cells was based on inhibition of virus-induced cytopathogenicity in BHK-21 and BT cells, respectively. Cells were seeded overnight at a rate of 5×10^4/well into 96-well plates in growth medium. Cell monolayers were infected with 50 μL of a proper virus dilution to give an m.o.i = 0.01. Serial dilutions of test compounds in DMEM with 2% inactivated fetal bovine serum, were then added. After a 4 day incubation at 37°C, the number of viable cells was determined by MTT method. Cytotoxicity of compounds was evaluated in parallel with their antiviral activity.

Anti-HBV Assay. As previously described,[12] cells were cultured for 11 days in the presence of drug with medium changes every 3 days. At the end of the subsequent 3-day period, an aliquot of the culture medium was harvested and processed to obtain extracellular HBV-DNA by slot blot analysis.

For intracellular HBV DNA analysis cells were lysed (10 mM Tris-HCl pH 7.5, 5mM EDTA, 150 mM NaCl, 1%SDS). Total intracellular DNA was extracted, HBV DNA was digested with HindIII restriction endonuclease, separated by electrophorese and transferred to a nylon membrane. Filters from Slot and Southern blot were hybridized with a HBV-specific probe, prepared from a full length HBV DNA genome template excised from plasmid. Quantification was performed on a Personal Molecular Imager FX (Bio-Rad).

Plaque Reduction Assays. Antiviral assays against HSV-1, Sb-1, VSV and YFV viruses were based on the plaque reduction test, as previously described.[13] For each compound the 50% effective concentration (EC_{50}) was determined in duplicate 24-well plates by plaque reduction assays. Cell monolayers were infected with 100 PFU/well of virus. Then, serial dilutions

of test compounds in medium supplemented with 2% inactivated serum and 0.75% of methyl cellulose were added to the monolayers. Cultures were further incubated at 37°C for 3 days, then fixed with 50% ethanol and 0.8% Crystal Violet, washed and air-dried. Then plaques were counted.

Antibacterial Assay. Assays against *Staphylococcus aureus*, group D *Streptococcus*, *Salmonella* and *Shigella spp*. were carried out in nutrient broth, pH 7.2, with an inoculum of 10^3 bacterial cells/tube. Minimum inhibitory concentrations (MIC) were determined after incubation at 37°C for 18 h in the presence of serial dilutions of test compounds. The minimal bactericidal concentration (MBC) were determined by subcultivating in Triptosio agar samples from cultures with no apparent growth.

Antimycotic Assay. Yeast inocula were obtained by properly diluting cultures incubated at 37°C for 30 h in Sabouraud dextrose broth to obtain 5×10^3 cells/mL. On the contrary, dermatophyte inocula were obtained from cultures grown at 37°C for 5 days in Sabouraud dextrose broth by finely dispersing clumps with a glass homogenizer before diluting to 0.05 OD 590/mL. Then, 20 µl of the above suspensions were added to each well of flat-bottomed microtiter trays containing 80 µL of medium with serial dilutions of test compounds, and were incubated at 37°C. Growth controls were visually determined after 2 days (yeasts) or 3 days (dermatophytes). MIC was defined as the compound concentration at which no macroscopic sign of fungal growth was detected. The minimal fungicidal concentrations (MFC) were determined by subcultivating in Sabouraud dextrose agar samples from cultures with no apparent growth.

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