Antiviral Activity of Triazine Analogues of 1-(*S*)-[3-Hydroxy-2-(phosphonomethoxy)propyl]cytosine (Cidofovir) and Related Compounds

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Treatment of 5-azacytosine sodium salt with diisopropyl [(2-chloroethoxy)methyl]phosphonate followed by removal of ester groups with $BrSi(CH_3)_3$ afforded 1-[2-(phosphonomethoxy)ethyl]-5-azacytosine (**3**). Reaction of 5-azacytosine with [(trityloxy)methyl]-(2*S*)-oxirane followed by etherification with diisopropyl (bromomethyl)phosphonate and removal of ester groups gave 1-(*S*)-[3-hydroxy-2-(phosphonomethoxy)propyl]-5-azacytosine (**1**). The synthesis of 6-azacytosine congener **2** was analogous using N^4-benzoylated intermediates. Compound **1** was shown to exert strong activity against a broad spectrum of DNA viruses including adenoviruses, poxviruses, and herpesviruses (i.e., herpes simplex viruses, varicella zoster virus, and human cytomegalovirus). Decomposition of **1** in alkaline solutions resulted in products **17** and **18**. While the *N*-formylguanidine derivative **17** proved active, the carbamyolguanidine derivative **18** was devoid of antiviral activity.

Introduction

Cidofovir {HPMPC, CDV,[a] 1-(*S*)-[3-hydroxy-2-(phosphonomethoxy)propyl]cytosine} is a potent and selective anti-DNA virus agent that was developed and discovered in our laboratories.[1] Cidofovir suppresses the in vitro growth of all human and animal DNA viruses thus far examined.[2] It is officially approved for the treatment of cytomegalovirus retinitis,[3] but it is also used off label, either systemically or topically, for the treatment of papillomatosous (anogenital or laryngeal)[4] infections, progressive multifocal leukoencephalopathy (caused by JC polyomavirus),[5] adenovirus[6] infections, and some severe infections caused by poxviruses[7] (e.g., vaccinia, orf, and molluscum contagiosum). The potential use of cidofovir as an antipoxvirus agent is supported by its potent activity against smallpox virus[8] and monkeypox virus.[9] Both are highly infectious viruses for human beings and could be purposely used in a bioterrorist attack.

In the ongoing search for new cidofovir analogues and derivatives, accruing attention is given to the development of neutral ester prodrugs to enhance oral absorption and improve pharmacological parameters.[10] So far, our effort in this area has mainly been focused at the modification of the phosphonate-bearing side chain (ref 11 and references therein) and on substitutions in the cytosine base; other data published on the modification of the cytosine moiety in the literature are rather scarce.[12]

Aza analogues of cytosine and its nucleosides have been investigated in our institute for several decades. The clinically most important compounds of this group are the antileukemic agents[13−15] 5-azacytidine[16,17] (azacitidine) and 5-aza-2′-deoxycytidine[18−20] (decitabine; Figure 1). Also the related 1-β-D-arabinofuranosyl-5-azacytosine[21,22] (fazarabine) exhibits remarkable antileukemic activity and was investigated in clinical trials.[23,24] The most promising drug candidate from this group is decitabine, which has progressed to phase III clinical studies for myelodisplastic syndrome (MDS). 5-Azacytidine and to a higher extent its 2′-deoxy congener have shown considerable anti-HIV activity even at 1 μM concentrations.[25] 2′,3′-Dideoxy-5-azacytidine[26] also exhibited a potent anti-HIV activity, yet with higher cytotoxicity than 2′,3′-dideoxycytidine. A selective antiviral effect against hepatitis B virus (HBV) was observed with 2′,3′-dideoxy-L-5-azacytidine[27] (EC_{50} = 1.5 μM), which was active as 2′,3′-dideoxy-D-cytidine (EC_{50} = 1 μM) and was not cytotoxic. The acyclic 5-azapyrimidine nucleoside 1-[(1,3-dihydroxy-2-propoxy)methyl]-5-azacytosine[28] was shown to be strongly inhibitory against human cytomegalovirus (HCMV) and Epstein−Barr virus (EBV).[29]

In this paper we describe the synthesis of 5-aza- and 6-azacytosine congeners of acyclic nucleotide analogues. Focus is given to their (*S*)-1-[3-hydroxy-2-(phosphonomethoxy)propyl] derivatives (*S*)-HPMP-5-azaC (**1**) and (*S*)-HPMP-6-azaC (**2**) (Figure 1), which are analogous to cidofovir.[30] In the series of 2-(phosphonomethoxy)ethyl derivatives, we have previously reported on the synthesis of the 6-azacytosine derivative,[31] and we describe here the synthesis of *N*-1-[2-(phosphonomethoxy)-ethyl]-5-azacytosine (**3**) that we used as a model compound to examine its chemical stability and biological properties.

Chemistry

The regioselectivity of alkylation follows the pattern of regiospecificity in similar reactions of 5-azacytosine. In neutral

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[a] Abbreviations: Ac, acetyl; ANP, acyclic nucleoside phosphonate; tBuONa, sodium *tert*-butoxide; CDV, cidofovir; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; HCMV, human cytomegalovirus; HHV-6, human herpesvirus 6; HPMP, 1-[3-hydroxy-2-(phosphonomethoxy)-propyl]; HPMP-5-azaC, 1-(*S*)-[3-hydroxy-2-(phosphonomethoxy)propyl]-5-azacytosine; HPMP-6-azaC, 1-(*S*)-[3-hydroxy-2-(phosphonomethoxy)-propyl]-6-azacytosine; HSV-1, herpes simplex virus type 1; HSV-2, herpes simplex virus type 2; iPr, 2-propyl; PME, 2-(phosphonomethoxy)ethyl; PME-azaC, 1-[2-(phosphonomethoxy)ethyl]-5-azacytosine; PMEC, 1-[2-(phosphonomethoxy)ethyl]cytosine; PMP, (*R*)-2-(phosphonomethoxy)propyl; Tr, triphenylmethyl; VZV, varicella zoster virus.

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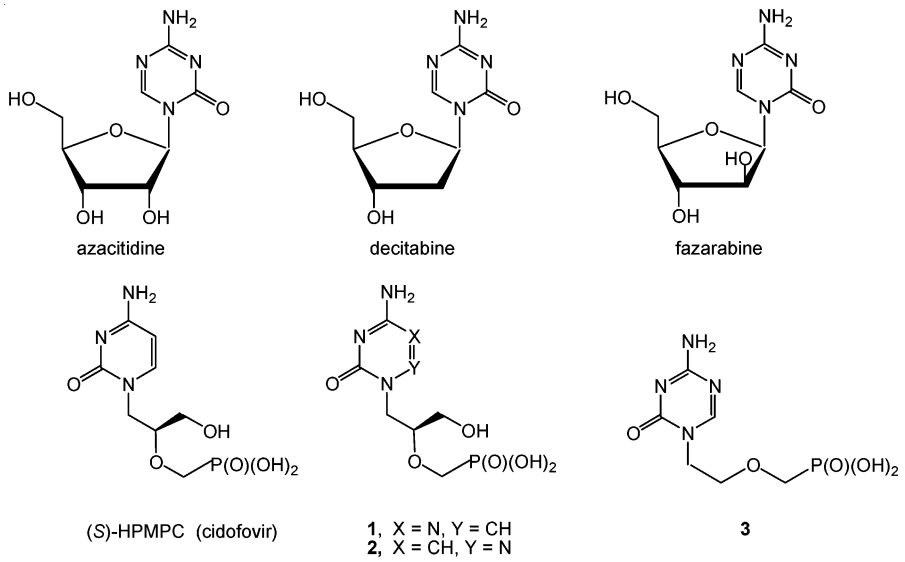
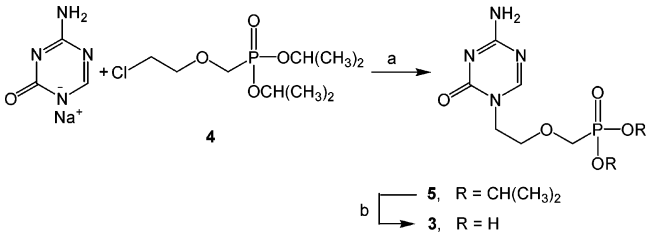


Figure 1.

Scheme 1[a]



[a] Conditions: (a) DMF, 110 °C, 3 days; (b) $(CH_3)_3SiBr$, CH_3CN, rt, 24 h.

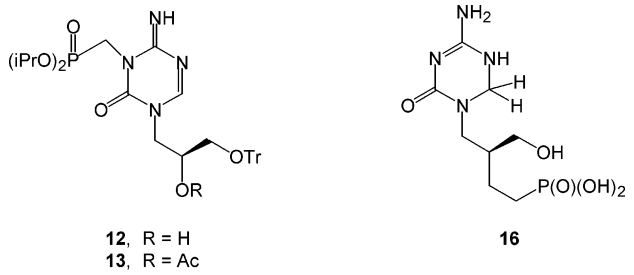


Figure 2.

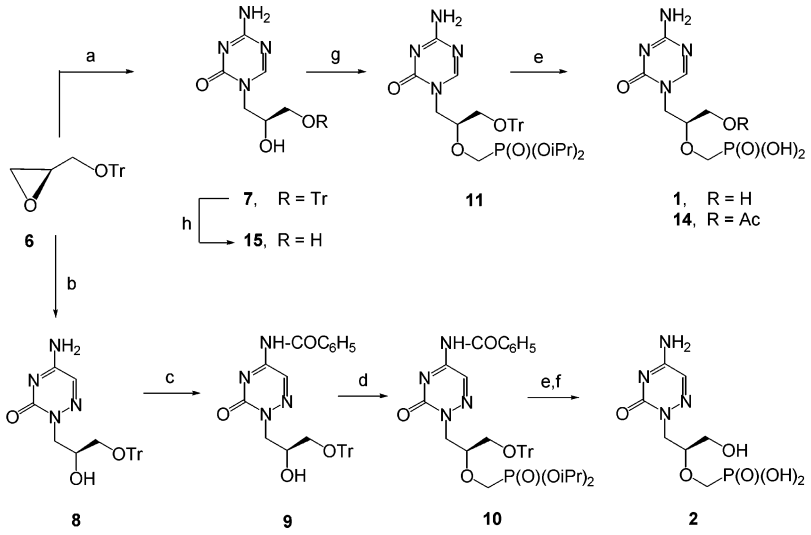
conditions alkylation of the N-3 position is dominant; in most cases mixtures of N-1- and N-3-monosubstituted and N-1,N-3-disubstituted compounds are formed. Selective alkylation of the N-1 position was described only with the sodium salt of 5-azacytosine.[32] Reaction of a sodium salt of 5-azacytosine with diisopropyl [(2-chloroethoxy)methyl]phosphonate (**4**) in dimethylformamide afforded regioselectively the desired diisopropyl 1-[2-(phosphonomethoxy)ethyl]-5-azacytosine (**5**), which was subsequently deprotected to the free phosphonic acid **3** by the action of bromotrimethylsilane in acetonitrile followed by hydrolysis (Scheme 1).

Synthesis of both (*S*)-HPMP derivatives, i.e., (*S*)-1-[3-hydroxy-2-(phosphonomethoxy)propyl] derivatives **1** and **2**, started with nucleophilic opening of an oxirane ring in (2*S*)-2-[(trityloxy)methyl]oxirane (**6**) (Scheme 2). Its reaction with 5-aza- or 6-azacytosine performed in dry dimethyl sulfoxide in the presence of a catalytic amount of sodium hydroxide gave regiospecifically in both cases N-1-substituted compounds 1-[(2*S*)-2-hydroxy-3-(triphenylmethoxy)propyl]-5-azacytosine (**7**) and 1-[(2*S*)-2-hydroxy-3-(triphenylmethoxy)propyl]-6-azacytosine (**8**) in excellent yields. Thus, it was proven that alkylation of 5-azacytosine at the N-1 position can be performed successfully with the free base instead of its sodium salt. The structure of **7** was confirmed on the basis of the proton-coupled ^{13}C NMR spectra. Whereas carbon atom C-4 (δ 166.70) has a coupling constant only with H-6, J(C-4,H-6) = 12.3 Hz, in the case of carbon atoms C-2 (δ 154.26) and C-6 (δ 159.89) we found, in addition to interaction with proton H-6, J(C-2,H-6) = 5.8 Hz and J(C-6,H-6) = 203.5 Hz, also long-range coupling constants with proton H-1′, J(C-2,H-1′) = 2.9 Hz and J(C-6,H-1′) = 3.9 Hz. Another structural proof is the IR spectrum, where the characteristic vibrations of **7** correspond to those of other 1-substituted 5-azacytosines, e.g., 1-methyl-5-azacytosine,[33] but differ from those of N-3-substituted derivatives. Still another difference distinguishing N-1- and N-3-alkylated 5-azacytosines consists in their UV spectra: while λ_{max} of *N*-1-alkyl derivatives in neutral solutions is about 247 nm and shifts at pH 2 to a value of about 254 nm, λ_{max} values of N-3-substituted derivatives in neutral solution are much higher (about 265 nm).[34] Compound **7** gave after deprotection the final phosphonate **1** (which showed 247.0 nm in H_2O at pH 7 and 254.4 nm at pH 2). The structure of the N-1 regioisomer thereby was definitely confirmed.

For the synthesis of phosphonomethyl ethers, **8** was benzoylated at the amino group to N^4-benzoyl-1-[(2*S*)-2-hydroxy-3-(triphenylmethoxy)propyl]-6-azacytosine (**9**) by the action of benzoic anhydride in *N*,*N*-dimethylformamide. Compound **9** gave on treatment with diisopropyl [(tosyloxy)methyl]phosphonate in the presence of excess sodium hydride in *N*,*N*-dimethylformamide fully protected phosphonate **10**. Reaction of **10** with bromotrimethylsilane followed by hydrolysis led simultaneously to removal of the trityl group and phosphonate-protecting diisopropyl ester groups. The final removal of the benzoyl group from the N^4 position was achieved by sodium methoxide-catalyzed methanolysis.

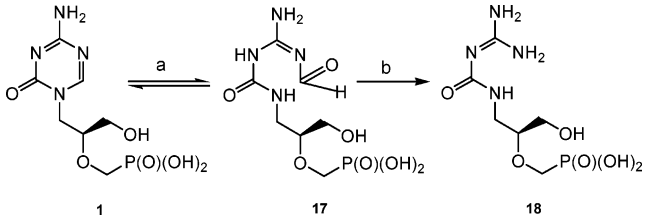
The preparation of the 5-azacytosine phosphonate **11** was more complicated due to the alkali-labile character of the 1,3,5-triazine ring in protic solvents,[34] which excludes the use of benzoyl or another alkali-labile group for amino group protection. Reaction of **7** with diisopropyl [((*p*-tolylsulfonyl)oxy)-methyl]phosphonate under standard conditions (excess of sodium hydride, *N*,*N*-dimethylformamide, room temperature) was not successful, leading to a complicated mixture of products mostly substituted at the N-3 position as witnessed by UV spectroscopy after deprotection (λ_{max} = 265 nm in H_2O). The transformation of **7** to **11** was performed by replacement of

Scheme 2[a]



[a] Conditions: (a) 5-azacytosine, NaOH, DMSO, 120 °C, 10 h; (b) 6-azacytosine, NaOH, DMSO, 120 °C, 3 h; (c) benzoic anhydride, DMF, rt, 4 days; (d) $TsOCH_2P(O)(OiPr)_2$, NaH, DMF, rt; (e) $(CH_3)_3SiBr$, CH_3CN, rt; (f) CH_3ONa/CH_3OH, rt; (g) $BrCH_2P(O)(OiPr)_2$, dioxane, NaH or tBuONa, 80−100 °C, 5 h; (h) 80% acetic acid, 80 °C, 2 h.

Scheme 3[a]



[a] Conditions: (a) 0.25 M triethylammonium hydrogen carbonate, 37 °C, 72 h, followed by 80 °C, 12 h; (b) 1 M NH_4OH, 48 h.

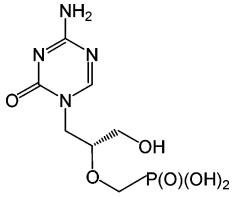
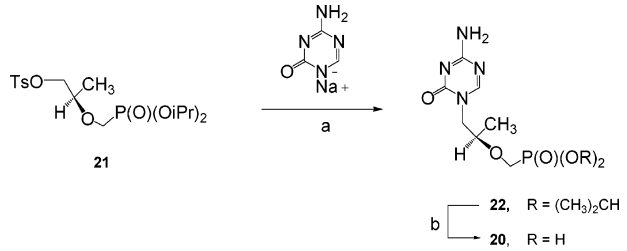


Figure 3.

Scheme 4[a]



[a] Conditions: (a) DMF, 120 °C, 3 h; (b) $(CH_3)_3SiBr$, CH_3CN, rt.

diisopropyl [((*p*-tolylsulfonyl)oxy)methyl]phosphonate[30] with diisopropyl (bromomethyl)phosphonate.[35] The conversion degree depends on the reaction conditions. Thus, reaction of **7** with diisopropyl (bromomethyl)phosphonate and sodium hydride in dimethylformamide gave a low yield (approximately 15%) of **11** contaminated by several byproducts; among them we have characterized the N-3 isomer **12** (further characterized as acetate **13**, Figure 2). However, the same reaction performed in dioxane in the presence of NaH or sodium *tert*-butoxide was much more advantageous: the yield of **11** increased (up to 30−40%), while the formation of undesired N-3-substituted byproducts was suppressed, and the residual **7** could be regenerated. Deprotection of **11** to free phosphonic acid **1** was carried out by treatment with bromotrimethylsilane in acetonitrile; to strictly avoid alkaline conditions, the final purification of the product was achieved by anion-exchange chromatography on Dowex 1 in acetate form using 1.5 M acetic acid as an eluent. The thus obtained **1** was partially contaminated with its 3-*O*-acetyl derivative **14** (up to 10%). On recrystallization of the product from water, this impurity was removed.

Besides synthesis of **1**, we also utilized the tritylated intermediate **7** for preparation of acyclic nucleoside analogue **15**, (2*S*)-(2,3-dihydroxypropyl)-5-azacytosine. Detritylation of **7** was performed by a standard procedure with acetic acid (Scheme 2). Another compound of our interest was 5,6-dihydro derivative **16** (Figure 2), i.e., (*S*)-1-[3-hydroxy-2-(phosphonomethoxy)propyl]-5,6-dihydro-5-azacytosine, a compound prepared from **1** by catalytic hydrogenation.

Similarly to other N-1-substituted 5-azacytosine derivatives (riboside, 2′-deoxyriboside, arabinoside), also the 5-aza analogue of HPMPC decomposes according to Scheme 3. The first step is a reversible ring opening of the *sym*-triazine to the *N*-formylguanidine derivative **17**, which can close back to the cyclic structure. This hydrolytic reaction is slow and reaches equilibrium within several days. However, the reversible ring-opening hydrolysis is accompanied by irreversible deformylation reaction of the intermediary formyl derivative that gives rise to antivirally inactive 2-{[(2*S*)-3-hydroxy-2-(phosphonomethoxy)-propyl]carbamoyl}guanidine (**18**).

In the HPMP series, the antiviral activity is usually connected with (*S*)-enantiomers; therefore, we prepared the triazine HPMP analogues as the (*S*)-enantiomers. However, in certain cases, an activity against DNA viruses was also observed with the (*R*)-isomers.[36] Considering the remarkable antiviral activity of compound **1**, we considered it necessary to prepare also the (*R*)-enantiomer of this compound, i.e., 1-(*R*)-[3-hydroxy-2-(phosphonomethoxy)propyl]-5-azacytosine (**19**; Figure 3). The compound was made not only to study its biological activity, but also as a standard for determination of the enantiomeric purity of **1**. The synthesis was performed following the

Table 1. Antiviral Activity and Cytostatic Activity of the Test Compounds against DNA Viruses in Cell Culture[a]

	EC_{50}[b] (μg/mL)											cytotoxicity (μg/mL)		
	HSV-1		HSV-2		VZV		HCMV		HHV-6	adenovirus	vaccinia	cell morphology (MCC)[c]		cell growth (CC_{50})[d]
compd	KOS (HEL)	KOS ACV[r] (HEL)	Lyons strain (HEL)	G strain (HEL)	OKA (HEL)	07-1 (HEL)	AD-169 (HEL)	Davis (HEL)	HHV-6A (HSB-2)	Ad2 (HEL)	Lederle (HEL)	HEL	HSB-2	HEL
3	9.90 ± 1.87	17.0 ± 6.8	39.34 ± 7.52	25.8 ± 8.2	25	10	49	37			>100	>100		>50
1	0.077 ± 0.023	0.24 ± 0.12	0.25 ± 0.14	0.35 ± 0.21	0.027 ± 0.030	0.019 ± 0.013	0.078 ± 0.039	0.054 ± 0.031	0.57 ± 0.16	0.71 ± 0.30	2.56 ± 1.84	>50	16	≥140 ± 60
2	8.84 ± 3.54	24.9 ± 9.8	≥43.4 ± 4.93	31.5 ± 16.2	38.2 ± 2.0	18.4 ± 10.7	30.0 ± 14.1	37.3 ± 36.3		>100	63.1	>100		>50
15	>100	>100	>100	>100	>100	>100	>100	>100			>100	>100		>100
16	9.35 ± 0.64	11.8 ± 1.2	10.3 ± 2.0	11.7 ± 4.0	3.2	3.8	5	3.7			38.1	>50		>50
17	1.35 ± 0.47	2.85 ± 1.20	3.16 ± 0	6.58 ± 4.84	0.042	0.046	0.10 ± 0.03	0.12 ± 0.06	10.9 ± 7.2	17.8 ± 5.8	8.35 ± 1.28	>50	≥200	>50
18	>50	>50	>50	>50	>50	>50	35.5	36.8	>200	>100	>100	>50	>200	>50
19	11.8 ± 1.2	17.8 ± 3.3	14.8 ± 0.4	12.6 ± 0	13.8		7.3	8.9			≥81.6	>100		>100
20	>100	ND	ND	>100	>100	>100	>100	>100			>100	>50		>50
HPMPC	0.09 ± 0.040	0.38 ± 0.21	0.43 ± 0.39	0.35 ± 0.21	0.077	0.073	0.20 ± 0.14	0.30 ± 0.08	4.03 ± 0.76	1.53 ± 0.63	2.60 ± 1.90	>100	37 ± 22	60.9 ± 30.5
brivudin	0.0025 ± 0.0011	>20	8.8 ± 5.4	8.97 ± 1.46	0.0069 ± 0.0042	≥122.1 ± 92.1						>100		≥100
acyclovir	0.015 ± 0.002	>20	0.018 ± 0.009	0.048 ± 0.018	0.75 ± 0.48	≥25.1 ± 13.2						>100		173.5 ± 29.1
ganciclovir	0.00038 ± 0.00015	0.91 ± 0.20	0.0014 ± 0.0009	0.0096 ± 0.0120			1.50 ± 1.26	1.50 ± 1.08				>100		83.6 ± 58.3

[a] Data shown are the mean ± standard deviation of at least two independent experiments. [b] Effective concentration required to reduce virus-induced cytopathicity (HSV-1, HSV-2, HCMV, HHV-6, Ad2, and vaccinia virus) or plaque formation (VZV) by 50%. [c] Minimum cytotoxic concentration that causes a microscopically detectable alteration of cell morphology. [d] Cytostatic concentration required to reduce cell growth by 50%.

procedure described for **1** with the use of commercially available optically pure (2*R*)-2-[(trityloxy)methyl]oxirane as a starting material. The enantiomeric purity of both enantiomers **1** and **19** was determined by the method of capillary electrophoresis.

In the series of so-called "PMP" derivatives we have prepared 1-[(*R*)-2-(phosphonomethoxy)propyl]-5-azacytosine (**20**). The synthesis followed standard conditions leading to PMP derivatives, i.e., condensation of the appropriate base component (5-azacytosine) with (*R*)-{2-[(diisopropoxyphosphoryl)methoxy]-propyl} *p*-toluenesulfonate (**21**) under basic conditions[37] followed by treatment of the intermediary ester **22** with bromotrimethylsilane and hydrolysis (Scheme 4).

Biological Activity

The antiviral activity spectrum of the different derivatives was evaluated against a variety of DNA viruses (Table 1), RNA viruses, and retroviruses. Among the 5-azacytosine congeners of acyclic nucleoside phosphonate analogues, compound **1** showed potent and selective activity against several DNA viruses, including different herpesviruses (HSV-1, HSV-2, VZV, HCMV, and HHV-6), adenovirus (Ad2), and poxvirus (vaccinia virus) with 50% effective concentration (EC_{50}) values of 0.71 μg/mL for Ad2, 2.56 μg/mL for vaccinia virus, and 0.02−0.6 μg/mL for herpesviruses. The antiviral activity of **1** was comparable to that of the reference drug (*S*)-HPMPC against HSV-1, HSV-2, and vaccinia virus, or 2−7-fold more active against VZV, HCMV, HHV-6, and Ad2. Compound **1** proved to be 2-fold less cytotoxic for HEL cells than (*S*)-HPMPC [50% cytostatic concentration (CC_{50}) 140 μg/mL for **1** compared to 61 μg/mL for (*S*)-HPMPC], but 2-fold more toxic for human T-lymphoblast HSB-2 cells [CC_{50} = 16 μg/mL for compound **1** compared to 37 μg/mL for (*S*)-HPMPC]. For all these DNA viruses, compound **1** showed a 2−16-fold higher antiviral selectivity index (ratio of CC_{50} to EC_{50}) than (*S*)-HPMPC. Among the potential decomposition products **17** and **18**, the *N*-formylguanidine derivative **17**, which can close back to the cyclic structure, showed activity with EC_{50} values equivalent to those obtained for the original compound **1** (VZV and HCMV) or at 3−25-fold higher EC_{50} values for HSV-1, HSV-2, HHV-6, Ad2, and vaccinia virus. In contrast, the carbamoylguanidine derivative **18**, obtained by irreversible deformylation of the intermediate derivative **17**, was devoid of antiviral activity. The fact that product **17** can irreversibly decompose into product **18** may explain the lost of activity observed with the first decomposition product as a function of time (data not shown). Whether the antiviral activity observed with the derivative **17** is due to its conversion to the cyclic structure, giving the original compound **1**, needs further investigations.

The 5-azacytosine congener in the so-called "PME" series (**3**) showed weak activity against the different herpesviruses tested (EC_{50} values ranging from 10 to 50 μg/mL) and lacked activity against vaccinia virus, while in the "PMP" series the 5-azacytosine derivative **20** was inactive against the DNA viruses (herpes-, adeno-, and poxviruses) evaluated here, similarly to other PMP derivatives already described.

The (*R*)-enantiomer **19** proved to be significantly less active than the (*S*)-enantiomer **1**, with 30−165-fold higher EC_{50} values. Also, the isomeric 6-azacytosine analogue **2** was considerably less active than **1**.

The acyclic nucleoside analogue (2*S*)-(2,3-dihydroxypropyl)-5-azacytosine (**15**) proved to be inactive against the different DNA viruses tested, while the 5,6-dihydro derivative **16** inhibited the replication of herpesviruses with EC_{50} values ranging from 3 to 12 μg/mL and proved also to be inhibitory to vaccinia virus (EC_{50} = 38 μg/mL).

None of the 5-azacytosine congeners of acyclic nucleoside phosphonate analogues showed activity against RNA viruses or HIV, except for **16**, which was able to inhibit HCV subgenomic replicon replication in Huh-5-2 cells with an EC_{50} value of 24 μg/mL. Interestingly, both the HPMP and PME 5-azacytosine derivatives (i.e., compounds **1** and **3**) were inhibitory to Moloney murine sarcoma virus (MSV)-induced transformation of C3H/3T3 cell cultures with EC_{50} values of 2.2 and 7.5 μg/mL, respectively. The isomeric 6-azacytosine analogue **2** was also able to inhibit MSV with an EC_{50} value of 17 μg/mL, as well as the potential decomposition product **17** (EC_{50} = 13 μg/mL).

Conclusion

In conclusion, we have prepared the isomeric aza analogues of HPMPC and PMEC, i.e., the *sym*-triazine (5-azacytosine) and 1,2,4-triazine (6-azacytosine) derivatives. While the activity of the latter compound was marginal, the 5-aza analogue of HPMPC demonstrated antiviral activity specifically against DNA viruses, comparable or even better than that of the parent compound. These findings warrant further investigations on this new class of antiviral compounds.

Experimental Section

Unless stated otherwise, the solvents were evaporated at 40 °C/2 kPa and the compounds were dried at 13 Pa. Melting points were determined on a Kofler block and are uncorrected. Analytical TLC was performed on silica gel 60 F_{254} plates (Merck KGaA, Darmstadt, Germany); chromatographic systems are described in the text. Column chromatography was performed on silica gel 60 μm (Fluka) or aluminum oxide (50−150 μm, pH 7.0 ± 0.5, Fluka). Reversed-phase HPLC separations were performed on a Waters Delta 600 instrument with a Waters 2487 dual λ absorbance detector using columns XTerra RP_{18} (3.9 × 150 mm, analytical column) and XTerra RP_{18} (10 × 150 mm, preparative column). ^{1}H NMR spectra were measured on a Varian Unity 500 instrument (at 500 MHz) in DMSO-d_6 solutions (referenced to the solvent signal at δ 2.50) or in D_2O solutions with internal standard sodium 3-(trimethylsilyl)propane-1-sulfonate (DSS). ^{1}H NMR chemical shifts (δ, ppm) and coupling constants (J, Hz) were obtained by first-order analysis of the spectra. ^{13}C NMR spectra were recorded on the same instrument (at 125.7 MHz) using the APT pulse sequence in DMSO-d_6 (referenced to the solvent signal δ 39.70). Mass spectra were measured on a ZAB-EQ (VG Analytical) spectrometer using FAB (ionization with xenon, accelerating voltage 8 kV, glycerol matrix). Optical rotations were measured on an AUTOPOL IV polarimeter (Rudolph Research Analytical) at 20 °C; $[\alpha]_D$ values are given in 10^{-1} deg cm^2 g^{-1}.

Materials and Solvents. Most of the chemicals and ion-exchange resins (Dowex 50WX8-200 and Dowex 1X2-400) were purchased from Sigma-Aldrich (Czech Republic). Dimethylformamide and acetonitrile were dried by distillation from CaH_2 (DMF in vacuo) and stored over molecular sieves (4 Å). Diisopropyl [(tosyloxy)methyl]phosphonate,[30] diisopropyl (bromomethyl)phosphonate,[35] and diisopropyl [(2-chloroethoxy)methyl]phosphonate[31] were prepared by previously described procedures. (2*S*)- and (2*R*)-2-[(Trityloxy)methyl]oxiranes were purchased from DAISO Co. Ltd. (Japan).

1-{[(Diisopropoxyphosphoryl)methoxy]ethyl}-5-azacytosine (5). A mixture of the sodium salt of 5-azacytosine (4.69 g, 35 mmol) and **4** (11.5 mL, 46.5 mmol) in DMF (65 mL) was stirred at 110 °C for 3 days. The solid was filtered off and washed with DMF. The combined filtrates were evaporated, the product was extracted with chloroform, and the chloroform solution was evaporated to dryness. The crude product **5** was purified by chromatography on silica gel using a gradient of methanol in chloroform (0−10%) and subsequently crystallized from a mixture of ethyl acetate−ether. Yield: 4.17 g, 36%, of white crystals. Mp: 140 °C. Anal. ($C_{12}H_{23}N_4O_5P$) C, H, N, P. FABMS: *m*/*z* 335 (MH^+) (45), 251 (M − (2 × isopropyl) + 3H) (100).

1-[2-(Phosphonomethoxy)ethyl]-5-azacytosine (3). A solution of **5** (2.5 g, 7.5 mmol) in acetonitrile (60 mL) was stirred with bromotrimethylsilane (7 mL, 51.6 mmol) in the dark for 24 h. The mixture was evaporated, the residue was codistilled with acetonitrile (3 × 50 mL), and 0.2 M triethylammonium hydrogen carbonate (50 mL) was added. After 10 min, Dowex 50 (pyridinium form) was added to reach a neutral reaction, the solid was filtered off and washed with water, and the pH of the filtrate was adjusted to 8.0 with aqueous ammonia solution. The solution was concentrated to approximately 20 mL, alkalized to pH 9, and applied to a column of Dowex 1X2 (100 mL, acetate form). Elution was performed first with water, followed by a gradient of acetic acid (0−0.4 M, 2 L); finally the product was eluted with 1 M acetic acid. The product-containing fractions were evaporated, and the residue was coevaporated with water and three times with ethanol. The solid was warmed with ethanol to crystallization, then left to stand at 4 °C overnight, collected by suction, washed with ethanol and ether, and dried in vacuo. Yield: 1.2 g, 64%, of a white solid. Mp: 228 °C. Anal. Calcd for $C_6H_{11}N_4O_5P$: C, 28.81; H, 4.43; N, 22.40; P, 12.38. Found: C, 28.52; H, 4.53; N, 21.51; P, 11.68. FABMS: *m*/*z* 251 (MH^+) (100). HRMS (QTOF): *m*/*z*, $C_6H_{12}N_4O_5P$ (MH^+), calcd 251.0545, found 251.0544.

Reaction of (2*S*)-2-[(Trityloxy)methyl]oxirane with 5-Aza- and 6-Azacytosine (General Procedure). A suspension of 5-azacytosine or 6-azacytosine (2 g, 17.8 mmol) and **6** (5.63 g, 17.8 mmol) in dry dimethyl sulfoxide (20 mL) was heated to 120 °C. One pellet of sodium hydroxide (60 mg, 1.5 mmol) was added, and heating under stirring was continued till dissolution and then for an additional 10 h (in the case of **7**) or 3 h (for the preparation of **8**). The reaction mixture was cooled to room temperature and poured onto a column of neutral alumina (150 mL) equilibrated in toluene. Elution was performed first with a mixture of toluene−ethyl acetate (1:1) till a drop in the UV absorption, followed by ethyl acetate (200 mL) and the system ethyl acetate−acetone−ethanol−water (18:3:1:1). The purity of the products was controlled by TLC in the system ethyl acetate−acetone−ethanol−water (18:3:1:1). All product-containing fractions (still containing dimethyl sulfoxide) were taken down, and the residue was codistilled with dimethylformamide (2 × 100 mL) and then with toluene (100 mL). The semisolid residue was crystallized from a toluene−acetone (2:1) mixture, and the crystalline material was collected by suction, washed with diethyl ether, and dried in the air.

1-[(2*S*)-2-Hydroxy-3-(triphenylmethoxy)propyl]-5-azacytosine (7). Yield: 6.5 g, 83%, of white crystals. Mp: 130−132 °C. Anal. Calcd for $C_{25}H_{24}N_4O_3 \cdot 0.5H_2O$: C, 68.63; H, 5.76; N, 12.81. Found: C, 68.82; H, 5.87; N, 12.25. HRMS (FAB): *m*/*z*, $C_{25}H_{25}N_4O_3$ (MH^+), calcd 429.1927, found 429.1931. FABMS: *m*/*z* 429 (MH^+) (2), 243 (trityl) (100), 113 (5-azacytosine + H) (25). IR ($CHCl_3$, cm^{-1}): 3540 [$\nu_{as}(NH_2)$], 3485 [$\nu_{as}(NH_2)$ bonded], 3422 [$\nu_s(NH_2)$], 3346, 3193 [$\nu_s(NH_2)$ bonded + ν(OH) intramolecularly bonded], 1685 [ν(C═O)], 1633 [$\beta_s(NH_2)$], 1507 [ν(ring) triazine], 1491, 1463 [ν(ring) triazine + phenyl], 1120 [$\beta_{as}(NH_2)$], 1090, 1084, 1055 [ν_{as}(C−O−C) + ν(C−OH)], 1598, 1491, 1450 [ν(ring) triazine], 708.

1-[(2*S*)-2-Hydroxy-3-(triphenylmethoxy)propyl]-6-azacytosine (8). Yield: 6.0 g, 73%, of white crystals. Mp: 199−200 °C. Anal. ($C_{25}H_{24}N_4O_3 \cdot 2H_2O$) C, H, N. FABMS: *m*/*z* 429 (MH^+) (0.6), 243 (trityl) (100).

***N*^{4}-Benzoyl-1-[(2*S*)-2-hydroxy-3-(triphenylmethoxy)propyl]-6-azacytosine (9)**. Benzoic anhydride (814 mg, 3.27 mmol) was added to a solution of **8** (1.4 g, 3.27 mmol) in dimethylformamide (15 mL) and the mixture stirred for 4 days at room temperature. Ethanol (5 mL) was added, the solution taken down, and the residue codistilled with toluene (100 mL) and then chromatographed on a column of silica gel (300 mL) in the system toluene−ethyl acetate (1:1). Yield: 1.12 g, 64%, of a solid foam. Anal. Calcd for $C_{32}H_{28}N_4O_4 \cdot 0.25H_2O$: C, 71.56; H, 5.35; N, 10.43. Found: C, 71.49; H, 5.44; N, 9.95. HRMS (FAB): *m*/*z*, $C_{32}H_{29}N_4O_{43}$ (MH^+),

calcd 533.2189, found 533.2204. FABMS: *m*/*z* 533 (MH^+) (1.3), 243 (trityl) (100), 105 (benzoyl) (46), 77 (phenyl) (10).

***N*⁴-Benzoyl-1-{(2*S*)-2-[(diisopropoxyphosphoryl)methoxy]-3-(triphenylmethoxy)propyl}-6-azacytosine (10).** A solution of **9** (1.04 g, 1.96 mmol) and diisopropyl [((*p*-tolylsulfonyl)oxy)methyl]-phosphonate (1.05 g, 3.0 mmol) in DMF (10 mL) was stirred at −20 °C, and a 60% dispersion of sodium hydride in mineral oil (235 mg, 5.9 mmol) was added. The reaction mixture was then slowly warmed to room temperature, stirred for an additional 2 h, and filtered through Celite. The filtrate was taken down in vacuo and the residue chromatographed on a column of silica gel (300 mL) in ethyl acetate. Yield: 1.02 g, 73%, of white foam. Anal. ($C_{39}H_{43}N_4O_7P$) C, H, N, P. FABMS: *m*/*z* 711 (MH^+) (0.6), 469 (M − trityl + 2H) (4), 243 (trityl) (100), 105 (benzoyl) (38).

(*S*)-1-[3-Hydroxy-2-(phosphonomethoxy)propyl]-6-azacytosine (2). Bromotrimethylsilane (1.9 mL, 14 mmol) was added to a solution of **10** (1.02 g, 1.44 mmol) in acetonitrile (10 mL) and the reaction mixture kept in the dark for 2 days. The yellow solution was evaporated and the residue codistilled with acetonitrile (20 mL) and with methanol (20 mL) and then treated with 0.1 M sodium methoxide in methanol (30 mL) for 24 h at ambient temperature. Dowex 50 (H^+ form) was added till the reaction was weakly acidic (pH 5) and the resulting suspension applied to a column of Dowex 50 (H^+, 50 mL). Elution with water (500 mL) removed salts, followed by 1% aqueous ammonia (elution of the product). The content of UV-absorbing fractions was analyzed by TLC in the system 2-propanol−ammonia−water (7:1:2) (R_f = 0.1), and the product-containing fractions were evaporated. The residue was applied to a column of Dowex 50 (Na^+, 20 mL) and the column eluted with water. The product-containing fractions were evaporated to dryness, and the resulting solid was stirred with a mixture of ethanol−hexane (1:1) (10 mL), filtered off, and dried in vacuo. Yield: 250 mg, 54%, of the sodium salt of **3**, white solid. Mp: 254 °C. $[\alpha]_D$: −19.4 (*c* 0.508, H_2O). HRMS (FAB): *m*/*z*, $C_7H_{12}N_4Na_2O_6P$ (MH^+), calcd 325.0290, found 325.0302. FABMS: *m*/*z* 325 (MH^+) (22).

Reaction of Compound 7 with Diisopropyl (Bromomethyl)-phosphonate (Method A). A suspension of **7** (785 mg, 1.8 mmol) in dry dioxane (4 mL) was stirred with sodium *tert*-butoxide (220 mg, 2.3 mmol). After a complete dissolution of starting material (approximately 15 min) diisopropyl (bromomethyl)phosphonate (700 mg, 2.7 mmol) was added and the mixture heated at 80 °C for 6 h, then cooled to ambient temperature, neutralized dropwise with acetic acid to pH 7, and taken down. The residue was chromatographed on silica gel (300 mL) in the system chloroform−methanol−triethylamine (100:5:1). Three fractions were obtained in the following elution sequence: products **12** and **11** and regenerated starting compound **7** (215 mg, 27%).

***N*-3-[(Diisopropoxyphosphoryl)methyl]-1-[(2*S*)-2-hydroxy-3-(triphenylmethoxy)propyl]-5-azacytosine (12)**. Yield: 200 mg, 18%, of colorless syrup (80% purity), for characterization transformed to **13**.

1-{(2*S*)-2-[(Diisopropoxyphosphoryl)methoxy-3-(triphenylmethoxy)]propyl}-5-azacytosine (11). Yield: 400 mg, 36%, of a white foam. $[\alpha]_D$: −36.3 (*c* 0.715, $CHCl_3$). Anal. ($C_{32}H_{39}N_4O_6P$) C, H, N, P. FABMS: *m*/*z* 629 (M + Na) (0.6), 365 (M − trityl + 2H) (0.2), 243 (trityl) (100).

Reaction of Compound 7 with Diisopropyl (Bromomethyl)-phosphonate (Method B). Sodium hydride (60% suspension in mineral oil, 100 mg, 2.5 mmol) was added to a suspension of compound **7** (830 mg, 2.0 mmol) in dioxane (5 mL). The mixture was stirred for 30 min at room temperature, then diisopropyl (bromomethyl)phosphonate (674 mg, 2.6 mmol) was added, and the reaction mixture was stirred at 100 °C for 5 h. After being cooled to room temperature, the mixture was applied to a column of silica gel (150 mL) and chromatographed in the system chloroform−methanol−triethylamine (100:5:1). The following products were obtained: **12** (260 mg, 20%), **11** (500 mg, 41%), and regenerated **7** (250 mg, 30%).

***N*-3-[(Diisopropoxyphosphoryl)methyl]-1-[(2*S*)-2-acetoxy-3-(triphenylmethoxy)propyl]-5-azacytosine (13).** 4-(Dimethylamino)pyridine (40 mg, 0.33 mmol) and acetic anhydride (240 mg, 2.4 mmol) were added to a solution of **12** (800 mg, 1.3 mmol) in acetonitrile (20 mL), and the mixture was set aside for 24 h at ambient temperature. Methanol (20 mL) was added and the solution evaporated. The residue was chromatographed on a column of silica gel (200 mL) in the system ethyl acetate−acetone−ethanol−water (36:6:1:1). Yield: 450 mg, 53%, of a colorless syrup.

(*S*)-1-[3-Hydroxy-2-(phosphonomethoxy)propyl]-5-azacytosine (1). Bromotrimethylsilane (4.7 mL, 35 mmol) was added to a solution of **11** (1.34 g, 3.13 mmol) in acetonitrile (30 mL), and the mixture was set aside at ambient temperature for 20 h. The mixture was then evaporated at 30 °C, the residue was coevaporated with acetonitrile (2 × 30 mL), and 90% aqueous methanol (50 mL) was added. The solution was neutralized with 1 M triethylammonium hydrogen carbonate to pH 7 and evaporated. The residue was partitioned between water (100 mL) and ether (100 mL) and the aqueous layer evaporated to a volume of 5 mL and applied to a column of Dowex 1 (AcO^- form, 100 mL). Elution was performed with water (500 mL) and then with a linear gradient of acetic acid (0.1−1 M, 1.5 L), and last, the product was eluted with 1.5 M acetic acid. UV-absorbing fractions were taken down in vacuo, and the residue was coevaporated several times with water (20 mL) till complete removal of acetic acid. The thus obtained product **1** was contaminated with **14** (11.5% according to HPLC). Pure **1** was obtained after double recrystallization from water. Yield: 350 mg, 40%, of white crystals. Mp: 175−178 °C. $[\alpha]_D$: −43.7 (*c* 0.308, H_2O). Enantiomeric purity: 95% (determined by capillary electrophoresis). UV: λ_{max} = 247 nm (pH 7), 254 nm (pH 2). Anal. ($C_7H_{13}N_4O_6P{\cdot}H_2O$) C, H, N, P. FABMS: *m*/*z* 281.1 (MH^+) (4). HRMS (FAB): *m*/*z*, $C_7H_{14}N_4O_6P$ (MH^+), calcd 281.0651, found 281.0657.

(*R*)-1-[3-Hydroxy-2-(phosphonomethoxy)propyl]-5-azacytosine (19). 19 was prepared analogously to **1**, starting from (2*R*)-2-[(trityloxy)methyl]oxirane. The spectral data are identical with those of compound **1**. $[\alpha]_D$: +47.8 (*c* 0.189, H_2O). Enantiomeric purity: 100% (determined by capillary electrophoresis).

(*S*)-1-[3-Acetoxy-2-(phosphonomethoxy)propyl]-5-azacytosine (14). This compound formed as an impurity in **1** during its elution from Dowex 1 with 1.5 M acetic acid (method A). To confirm its structure, a sample of a mother liquor after crystallization of **1** (5 mL, mass content approximately 50 mg) was separated by preparative HPLC using 0.02 M triethylammonium acetate buffer (isocratic elution, flow rate 5 mL/min); two products were obtained: free phosphonic acid **1** (retention time 1.9 min) and acetate **14** (retention time 3.8 min). Fractions containing **14** were evaporated to dryness, and the residue was coevaporated with water (5 × 50 mL) and dried in vacuo at 60 °C for 8 h to give the triethylammonium salt of **14** as a colorless oil. FABMS: *m*/*z* 345.1 (M + Na) (6), 323.2 (MH^+) (4).

(2*S*)-(2,3-Dihydroxypropyl)-5-azacytosine (15). A mixture of **7** (1.16 g, 2.7 mmol) in 80% acetic acid (100 mL) was heated at 80 °C for 2 h. The solution was evaporated in vacuo and the residue codistilled with water (4 × 30 mL). The crude product was adsorbed onto silica gel (20 mL) from methanol, applied to a silica gel column (100 mL) equilibrated in the system ethyl acetate−acetone−ethanol−water (15:3:4:3), and chromatographed in this system. The residue of **15** was finally crystallized from aqueous ethanol. Yield: 211 mg, 40%. $[\alpha]_D$: −73.9 (*c* 0.187, H_2O). Anal. ($C_6H_{10}N_4O_3{\cdot}{}^1/_2H_2O$) C, H, N. FABMS: *m*/*z* 187 (MH^+) (100).

(*S*)-1-[3-Hydroxy-2-(phosphonomethoxy)propyl]-5,6-dihydro-5-azacytosine (16). A mixture of **1** (110 mg, 0.39 mmol) in methanol (15 mL) and acetic acid (0.5 mL) was stirred till complete dissolution (1 h, approximately) and then hydrogenated on 10% palladium on charcoal (20 mg) under atmospheric pressure at room temperature till UV absorption disappeared (48 h). The catalyst was filtered off through a Celite pad, the filtrate passed through a Nylon membrane filter (Whatman, 0.2 μm) and evaporated in vacuo, and the product lyophilized. Yield: 80 mg, 73%. $[\alpha]_D$: −2.9 (*c* 0.259, H_2O). FABMS: *m*/*z* 283 (MH^+) (16). HRMS (FAB): *m*/*z*, $C_7H_{16}N_4O_6P$ (MH^+), calcd 283.0807, found 283.0807.

3-Formyl-2-{[(2*S*)-3-hydroxy-2-(phosphonomethoxy)propyl]-carbamoyl}guanidine (17). A solution of **1** (100 mg, 0.34 mmol) in 0.25 M triethylammonium hydrogen carbonate (5 mL) was incubated at 37 °C for 72 h and then for 12 h at 80 °C. The reaction course was monitored by measurement of the absorption maximum decrease in the UV spectrum at λ_{max} = 245 nm. For each measurement, a 10 μL sample of the reaction mixture was diluted with water to an overall volume of 3 mL and the absorbance value at 245 nm determined. The reaction reached equilibrium when no further decrease of absorbance occurred. The reaction mixture was evaporated to dryness and the residue coevaporated with water (5 × 3 mL) and then with methanol (2 × 3 mL). The residue in water (3 mL) was applied to a column of Dowex 50 (Na^+ form, 20 mL) and the column eluted with water. The UV-absorbing eluate was concentrated in vacuo and lyophilized to give 105 mg (92%) of a sodium salt of **17** as a colorless amorphous material.

2-{[(2*S*)-3-Hydroxy-2-(phosphonomethoxy)propyl]carbamoyl}-guanidine (18). A solution of **1** (200 mg, 0.67 mmol) in 1 M aqueous ammonia (2.5 mL) was stirred at room temperature for 48 h and then evaporated and the residue coevaporated with water (5 mL). The crude product was purified by reversed-phase HPLC, isocratic elution with water. The desired product in fractions (not absorbing in UV) was detected by TLC on silica gel plates in the system 2-propanol−25% NH_4OH−water (7:1:2) followed by spraying of the plate with a mixture of 5% NaOH−5% $K_3[Fe(CN)_6]$−5% $Na[Fe(CN)_5NO]$ (1:1:1) (which gave orange spots of **18**). The product-containing fractions were evaporated and dried in vacuo. Yield: 150 mg, 83%, as a white foam. $[\alpha]_D$: +31.2 (*c* 0.226, H_2O). FABMS: *m*/*z* 271(MH^+) (100). HRMS (FAB): *m*/*z*, $C_6H_{16}N_4O_6P$ (MH^+), calcd 271.0807, found 271.0808.

1-{(*R*)-2-[(Diisopropoxyphosphoryl)methoxy]propyl}-5-azacytosine (22). A suspension of a sodium salt of 5-azacytosine (280 mg, 2.1 mmol), **21** (830 mg, 2.03 mmol), and a catalytic amount of cesium carbonate (approximately 10 mg) in DMF (6 mL) was heated at 120 °C for 3 h. After being cooled to room temperature, the mixture was filtered through a Celite pad, the filtrate was evaporated, and the residue was coevaporated with toluene and chromatographed on a column of silica gel (300 mL) in the system chloroform−methanol (9:1). Yield: 310 mg, 44%, of a white solid. $[\alpha]_D$: −78.4 (*c* 0.257, $CHCl_3$). Anal. ($C_{13}H_{25}N_4O_5P$) C, H, N, P. FABMS: *m*/*z* 349 (MH^+) (64), 265 (M − (2 × isopropyl) + 3H) (100), 113 (BH^+) (42).

1-[(*R*)-2-(Phosphonomethoxy)propyl]-5-azacytosine (20). A solution of **22** (270 mg, 0.8 mmol) in acetonitrile (6 mL) was treated with bromotrimethylsilane (0.6 mL, 4.4 mmol) by the same procedure as described for **1**. Yield: 205 mg, 97%, of a white foam. $[\alpha]_D$: −67.4 (*c* 0.270, H_2O). FABMS: *m*/*z* 365 (MH^+) (100). HRMS (FAB): *m*/*z*, $C_7H_{14}N_4O_5P$ (MH^+), calcd 265.0702, found 265.0698.

Antiviral Activity Assays. The compounds were evaluated against the following viruses: herpes simplex virus type 1 (HSV-1) strain Kos, thymidine kinase-deficient (TK^-) HSV-1 Kos strain resistant to ACV (ACV^r), herpes simplex virus type 2 (HSV-2) strains Lyons and G, varicella zoster virus (VZV) strain Oka, TK^- VZV strain 07-1, human cytomegalovirus (HCMV) strains AD-169 and Davis, a clinical isolate of adenovirus type 2 (Ad2), human herpes virus 6 subtype A (HHV-6A) strain GS, vaccinia virus Lederle strain, respiratory syncitial virus (RSV) strain Long, vesicular stomatitis virus (VSV), Coxsackie B4, parainfluenza 3, reovirus-1, Sindbis, Punta Toro, yellow fever virus (YFV), human immunodeficiency virus type 1 strain IIIB, human immunodeficiency virus type 2 strain ROD, and hepatitis C virus (HCV). The antiviral, other than anti-HIV and anti-HCV, assays were based on inhibition of virus-induced cytopathicity or plaque formation in human embryonic lung (HEL) fibroblasts, African green monkey cells (Vero), human epithelial cells (HeLa), or human T-lymphoblasts (HSB-2), according to previously established procedures.[38] Confluent cell cultures in microtiter 96-well plates were inoculated with 100 $CCID_{50}$ of virus (1 $CCID_{50}$ being the virus dose to infect 50% of the cell cultures) or with 20 plaque-forming units (PFUs). After a 1−2 h adsorption period, residual virus was removed, and the cell cultures were incubated in the presence of varying concentrations of the test compounds. Viral cytopathicity or plaque formation (VZV) was recorded as soon as it reached completion in the control virus-infected cell cultures that were not treated with the test compounds. Antiviral activity was expressed as the EC_{50}, or the concentration required to reduce virus-induced cytopathogenicity or viral plaque formation by 50%.

Inhibition of HIV-Induced Cytopathicity in CEM Cells. The methodology for the anti-HIV assays has been described previously.[39] Briefly, human CEM cells (~3 × 10^5 cells/mL) were infected with 100 $CCID_{50}$ HIV-1 or HIV-2/mL and seeded in 200 μL well microtiter plates, containing appropriate dilutions of the test compounds. After 4 days of incubation at 37 °C, CEM giant cell formation was examined microscopically.

Inhibition of HCV Subgenomic Replicon Replication in Huh-5-2 Cells. Huh-5-2 cells (a cell line with a persistent HCV replicon, I389luc-ubi-neo/NS3-3′/5.1, a replicon with firefly luciferase−ubiquitin−neomycin phosphotransferase fusion protein and an EMCV-IRES-driven NS3-5B HCV polyprotein) were cultured in RPMI medium (Gibco) supplemented with 10% fetal calf serum, 2 mM L-glutamine (Life Technologies), 1× nonessential amino acids (Life Technologies), 100 IU/mL penicillin, 100 μg/mL streptomycin, and 250 μg/mL G418 (Geneticin, Life Technologies). The cells were seeded at a density of 7000 cells/well in a 96-well View Plate (Packard) in medium containing the same components as described above, except for G418. The cells were allowed to adhere and proliferate for 24 h. At that time, the culture medium was removed and five serial dilutions (5-fold dilutions starting at 100 μg/mL or 100 μM) of the test compounds were added in culture medium lacking G418. Interferon α 2a (500 IU) was included as a positive control in each experiment for internal validation, and an HCV polymerase (2′*C*-methylcytidine) and/or an HCV protease inhibitor (VX-950), can be included as well as a reference. The plates were further incubated at 37 °C and 5% CO_2 for 72 h. Replication of the HCV replicon in Huh-5-2 cells results in luciferase activity in the cells. Luciferase activity was measured by adding 50 μL of 1× Glo-lysis buffer (Promega) for 15 min followed by 50 μL of the Steady-Glo luciferase assay reagent (Promega). Luciferase activity was measured with a luminometer, and the signal in each individual well was expressed as a percentage of the untreated cultures. The 50% effective concentrations (EC_{50}) were calculated from these data sets. Parallel cultures of Huh-5-2 cells, seeded at a density of 7000 cells/well on classical 96-well cel culture plates (Becton-Dickinson), were treated in a similar fashion except that no Glo-lysis buffer or Steady-Glo luciferase reagent was added. The effect of the compounds on the proliferation of the cells was measured 3 days after addition of the various compounds by means of the CellTiter 96 AQ_{ueous} nonradioactive cell proliferation assay (MTS, Promega). In this assay 3-(4,5-dimethylthiazol-2-yl)-5-[3-(carboxymethoxy)phenyl]-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) was bioreduced by cells into a formazan that is soluble in tissue. The number of cells correlates directly with the production of the formazan. The MTS-stained cultures were quantified in a plate reader at 498 nm. The 50% cytostatic concentrations (EC_{50}) were calculated from these data sets.

Inhibition of MSV-Induced Transformation of Murine C3H/3T3 Embryo Fibroblasts. The anti-MSV assay was performed as described previously.[39] Murine C3H/3T3 embryo fibroblast cells were seeded at 5 × 10^5 cells/mL into 1-cm^2 wells of 48-well microplates. After 24 h, the cell cultures were infected with 80 focus-forming units of MSV (prepared from tumors induced following intramuscular inoculation of 3 day old NMRI mice with MSV, as described previously[40]) for 90−120 min at 37 °C. The medium was then replaced by 1 mL of fresh medium containing various concentrations of the test compounds. After 6 days, transformation of the cell culture was examined microscopically.

Cytotoxicity Assays. Cytotoxicity measurements were based on the inhibition of cell growth. HEL cells were seeded at a rate of 5 × 10^3 cells/well into 96-well microtiter plates and allowed to proliferate for 24 h. Then, medium containing different concentrations of the test compounds was added. After 3 days of incubation

at 37 °C, the cell number was determined with a Coulter counter. The cytostatic concentration was calculated as the CC_{50}, or the compound concentration required to reduce cell proliferation by 50% relative to the number of cells in the untreated controls. CC_{50} values were estimated from graphic plots of the number of cells (percentage of control) as a function of the concentration of the test compounds. Alternatively, cytotoxicity for cell morphology was expressed as the minimum cytotoxic concentration (MCC), or the compound concentration that caused a microscopically detectable alteration of cell morphology.

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Supporting Information Available: Elemental analysis data, ^1H and ^{13}C NMR data of all new compounds, NMR spectra of target compounds, HRMS (compounds **3**, **7**, and **9**), and activities of compounds against RNA viruses and retroviruses. This material is available free of charge via the Internet at http://pubs.acs.org.

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