Thymidine Kinase Assay. The assay was performed by the methods of Doberson and Greer,[19] using HSV-1 (strain F)-affinity-purified kinase.[20] Reaction mixtures contained 100 mM Tris·HCl (pH 7.5), 50 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, 25 mM NaF, 1 mg BSA/mL, 0.5 mM [γ-^{32}P]ATP (20 μCi/μM), 0.2 mM nucleoside analogue, and enzyme in a 0.1 mL volume. The [γ-^{32}P]ATP required for the assay was from ICN Chemical and Radioisotope Div., Irvine, CA; thymidine came from Sigma Chemical Co., St. Louis, MO. The thymidine kinase affinity gel required for enzyme purification was prepared by the Cheng procedure.[21] and the results from the deoxynucleoside kinase assays were expressed as relative phosphorylation rates, a method that has been used by others.[20,22]

Animal Studies. Swiss-Webster female mice (Simonsen Laboratories, Gilroy, CA), weighing approximately 20 g each, were infected intraperitoneally with 5×10^4 PFU of HSV-2 (strain G). This challenge was approximately equivalent to 10 50% lethal doses. DHPG and the nucleoside analogues were administered subcutaneously once a day for 4 days starting 24 h postinfection. Deaths were recorded for 21 days after infectin.

Acknowledgment. The assistance of Dr. M. Maddox, J. Nelson, and L. Kurz in obtaining and interpreting NMR spectra is gratefully acknowledged. Technical support for the animal studies was provided by V. R. Freitas and B. P. Binko.

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Synthesis and Antiviral Activity of Certain Nucleoside 5′-Phosphonoformate Derivatives

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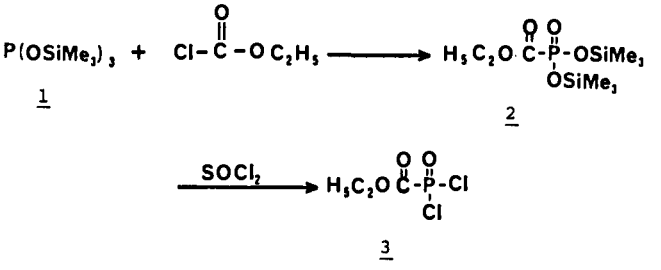
(Ethoxycarbonyl)phosphonic dichloride (**3**) was synthesized by chlorination of bis(trimethylsilyl) (ethoxycarbonyl)phosphonate with thionyl chloride. Adenosine 5′-(ethoxycarbonyl)phosphonate (**4**), guanosine 5′-(ethoxycarbonyl)phosphonate (**5**), 2′-deoxyadenosine 5′-(ethoxycarbonyl)phosphonate (**18**) and 2′-deoxyguanosine 5′-(ethoxycarbonyl)phosphonate (**19**) were synthesized by coupling of compound **3** with adenosine, guanosine, 2′-deoxyadenosine, and 2′-deoxyguanosine, respectively. Alkaline treatment of **4**, **5**, **18**, and **19** gave the corresponding adenosine 5′-(hydroxycarbonyl)phosphonate (**14**), guanosine 5′-(hydroxycarbonyl)phosphonate (**15**), 2′-deoxyadenosine 5′-(hydroxycarbonyl)phosphonate (**20**), and 2′-deoxyguanosine 5′-(hydroxycarbonyl)phosphonate (**21**). Treatment of **4** and **5** with methanolic ammonia resulted in the production of adenosine 5′-(aminocarbonyl)phosphonate (**12**) and guanosine 5′-(aminocarbonyl)phosphonate (**13**), respectively. The nucleotide analogue **20** exhibited the most potent antiviral activity of this group of nucleotide tested in vitro and was most active against herpes viruses especially HSV-2. The nucleotide analogue **21** had lower, but significant, activity against HSV-2. All of the compounds tested were nontoxic to confluent Vero cells at concentrations as high as 5000 μM.

Compounds which selectively inhibit virus specific functions but have little effect on host cells have been the target of many recent investigations.[1,2] Phosphonoformate (Foscarnet), an analogue of pyrophosphate, has been found to be a potent antiviral agent.[3] Phosphonoformate inhibits viral reverse transcriptase[4] and DNA polymerase[5] and is active against herpes simplex virus (HSV-1)[6,7] and certain other viruses.[8-10] Recently, elimination of replication of human T-cell lymphotropic virus type III (HTLV-III) with phosphonoformate at a nontoxic dose has been reported.[11,12] This study showed that the activity of reverse transcriptase of HTLV-III has been reduced drastically by phosphonoformate. Beldekas and co-workers studied the effect of foscarnet on the nature and frequency of T-cell differentiation defect seen in AIDS and in certain people with LAS.[13] These investigators reported that foscarnet permits T-cell colony growth and expansion in the cell culture.

Phosphonoformate has been used topically for treatment of HSV-1 infection in laboratory animals with satisfactory results.[14] Phosphonoformate has also been used in a 3% cream formulation, which is not irritating to the skin, and in clinical testing shortens the time mucocutaneous HSV-1 and HSV-2 lesions require to reach the stage of crusting and reduces the appearance of new vesicles.[15] Topical treatment of recurrent genital herpes with foscarnet cream has also been claimed to accelerate healing in man.[16]

Scheme I



It appears that phosphonoformate binds to the pyrophosphate "well" of the viral DNA and RNA polymerase.[4,5]

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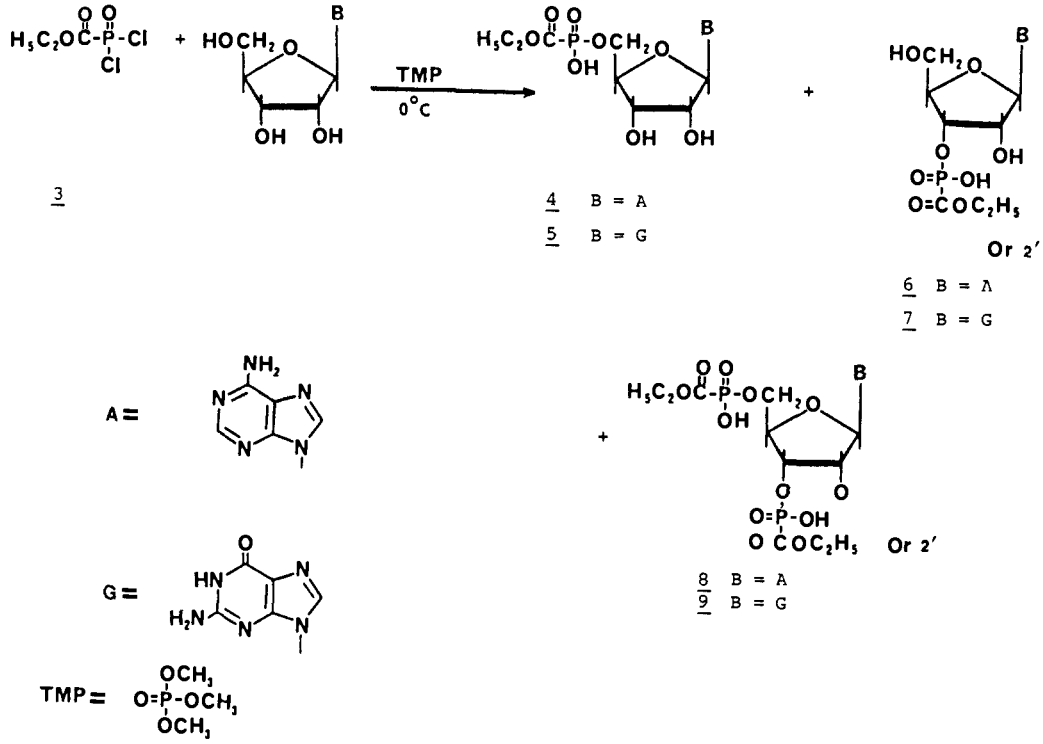
Scheme II



We have investigated the attachment of the phosphonoformate moiety to the 5'-position of a natural 2'-deoxynucleoside which we propose could provide the required specificity of an inhibitor of reverse transcriptase which would not inhibit normal cellular RNA or DNA polymerase.[17] We now report the synthesis and biological activity of certain nucleoside phosphonoformate derivatives attached through the phosphonate group to the 5'-hydroxyl of the natural nucleoside.

Results and Discussion

Tris(trimethylsilyl) phosphite[18] (1) was treated with ethyl chloroformate to yield bis(trimethylsilyl) (ethoxycarbonyl)phosphonate (2) in almost quantitative yield.[19] (Ethoxycarbonyl)phosphonic dichloride (3) was synthesized in our laboratory by treatment of 2 with thionyl chloride in 75% yield (Scheme I).

(Ethoxycarbonyl)phosphonic dichloride (3) was used as a phosphorylating agent in a reaction similar to the procedure of Yoshikawa and co-workers.[20] Compound 3 was dissolved in trimethyl phosphate and coupled with adenosine (Scheme II). The major product was adenosine 5'-(ethoxycarbonyl)phosphonate (4) in 70–80% yields. The 3'-phosphorylated 6 and 3',5'-diphosphonate of 8 were the minor products which were not studied further.

The nucleotide analogue 4 was also prepared from 2',3'-isopropylideneadenosine and (ethoxycarbonyl)-phosphonic dichloride (3) in over 90% yield (Scheme III). The product of the above reaction was hydrolyzed with 80% acetic acid at room temperature to give nucleotide analogue 4 in an overall 85% yield. The nucleotide 4 reacted with 1 N sodium hydroxide to give adenosine-5'-(hydroxycarbonyl)phosphonate (14) which was isolated as sodium salt. Treatment of 4 with methanolic ammonia gave adenosine 5'-(aminocarbonyl)phosphonate (12).

The reaction of (ethoxycarbonyl)phosphonic dichloride (3) with 2',3'-isopropylideneguanosine in a similar manner as adenosine provided guanosine 5'-(ethoxycarbonyl)-phosphonate (5) which was obtained in 85% yield. Treatment of 5 with methanolic ammonia gave guanosine 5'-(aminocarbonyl)phosphonate (13). Hydrolysis of 5 with sodium hydroxide gave guanosine 5'-(hydroxycarbonyl)-phosphonate (15). 3'-*O*-Acetyl-2'-deoxyadenosine (16)[21] was similarly treated with 3 to provide 2'-deoxyadenosine 5'-(ethoxycarbonyl)phosphonate (18), which was hydrolyzed with 0.5 N sodium hydroxide to yield 2'-deoxyadenosine 5'-(hydroxycarbonyl)phosphonate (20) (Scheme IV) in over 90% yield after purification on DEAE Sephadex column. In a similar manner, 2'-deoxyguanosine 5'-(hydroxycarbonyl)phosphonate (21) was also prepared in an overall 72% yield.

Antiviral Evaluations. The results of the in vitro evaluation of the antiviral activity of the nucleoside 5'-phosphonoformates, as compared to trisodium phosphonoformate (Foscarnet), are shown in Table I. Foscarnet

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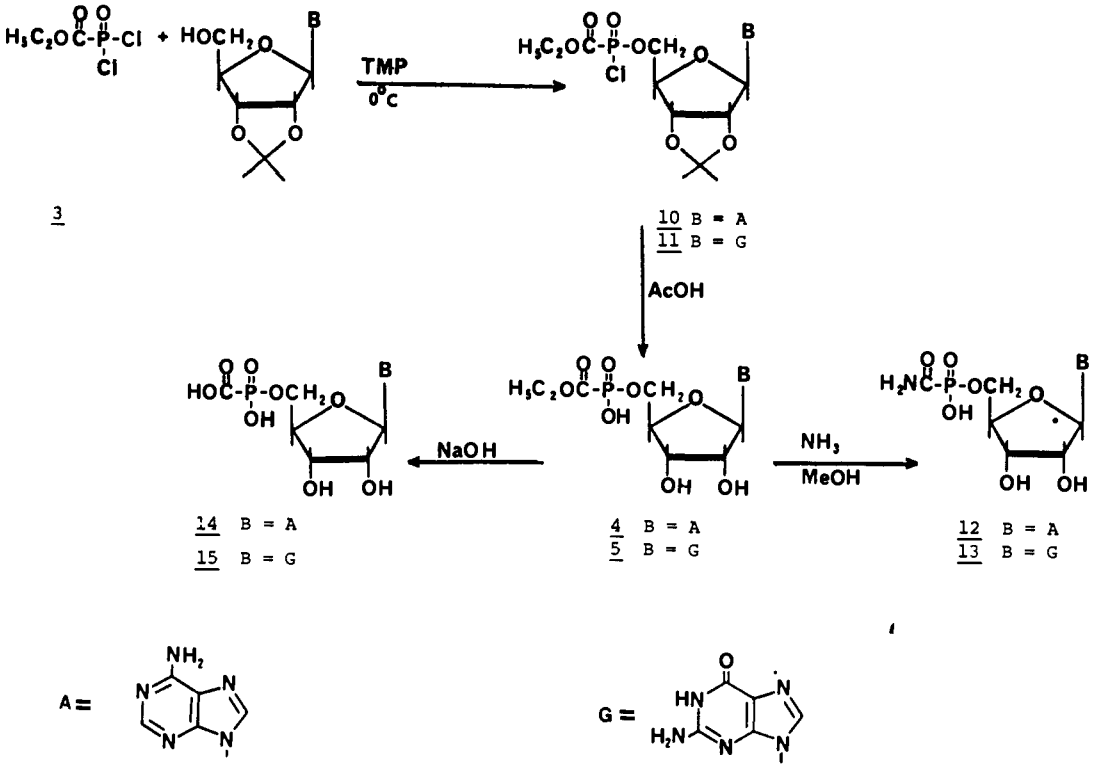
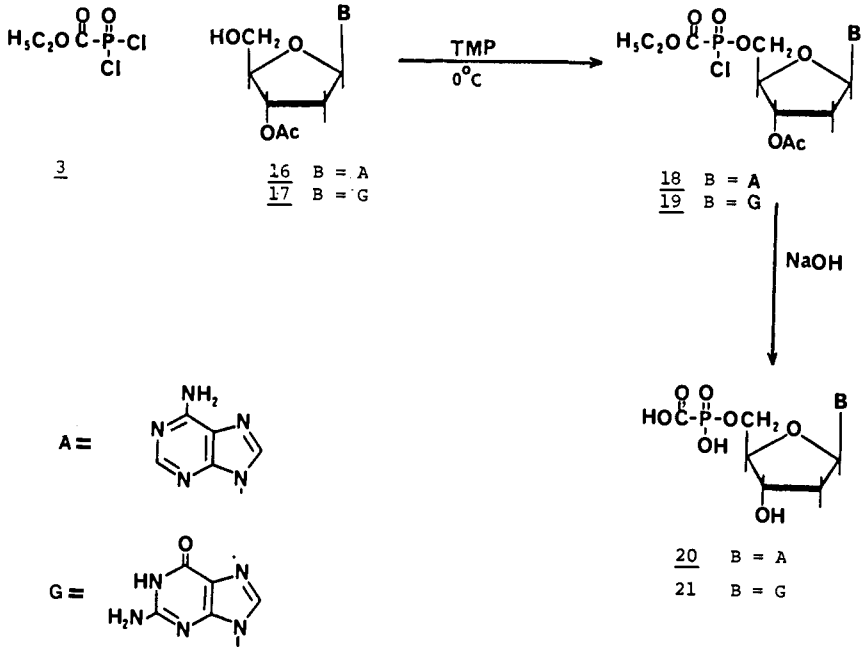
Scheme III



Scheme IV



exhibits potent activity against both HSV-1 and HSV-2, with the most pronounced activity against HSV-2. The ED_{50} values of Foscarnet against the HSV-1 strains tested are approximately 2–10 times higher than those for HSV-2 strains. Foscarnet is inactive against vaccina, para 3, and measles. In general, the nucleoside 5'-phosphonoformates tested also were most active against herpes viruses especially HSV-2. The 2'-deoxyadenosine 5'-(hydroxycarbonyl)phosphonate exhibited the most potent antiviral activity of any nucleoside tested. ED_{50} and VR values for **20** were similar to those obtained for Foscarnet against both strains of HSV-2. The nucleotide analogue **20** was somewhat less active than Foscarnet against HSV-1, with ED_{50} values 3 times higher than Foscarnet against strain HF and 6.5 times higher against strain KOS. Compound **20** also had moderate activity against measles and was inactive against vaccinia and para 3. The 2'-deoxyguanosine 5'-(hydroxycarbonyl)phosphonate (**21**) had lower, but significant, activity against HSV-2 with ED_{50} values 4–10 times higher than **20** or Foscarnet. However, **21** was inactive against HSV-1 as well as vaccinia, para 3, and measles. Adenosine 5'-(hydroxycarbonyl)phosphonate (**14**) was considerably less active than the 2'-deoxyadenosine-5'-(hydroxycarbonyl)phosphonate analogue (**20**) against HSV-2, with lower VR values and ED_{50} values 9 times (strain MS) or 30 times (strain 333) higher than **20**. Nucleotide analogue **14** lacks the activity against HSV-1 exhibited by the 2'-deoxyadenosine analogue and is less active against measles. Compound **14** is the only nucleoside-5'-phosphonoformate to exhibit even moderate ac-

Table I. Comparative in Vitro Antiviral Activity of Nucleoside 5′-Phosphonoformates

compd	MTC,[a] (μM)	HSV-2 (333) VR[b]	HSV-2 (333) ED_{50},[c] μM	HSV-2 (MS) VR	HSV-2 (MS) ED_{50}, μM	HSV-1 (HF) VR	HSV-1 (HF) ED_{50}, μM	HSV-1 (KOS) VR	HSV-1 (KOS) ED_{50}, μM	vaccinia VR	vaccinia ED_{50}, μM	para 3 VR	para 3 ED_{50}, μM	measles VR	measles ED_{50}, μM
4	>5000	0.72	1000	1.12	820	0.30	>5000	0.00	>5000	0.23	>5000	0.00	>5000	0.18	>5000
5	>5000	1.02	600	0.90	820	0.22	>5000	0.00	>5000	0.00	>5000	0.10	>5000	0.68	500
12	>5000	1.24	440	1.00	830	0.28	>5000	0.00	>5000	0.00	>5000	0.10	>5000	0.45	2800
13	>5000	1.16	300	0.91	970	0.00	>5000	0.00	>5000	0.00	>5000	0.10	>5000	0.70	900
14	>5000	0.70	1600	1.15	360	0.18	>5000	0.00	>5000	0.65	740	0.00	>5000	0.38	3000
15	>5000	0.22	>5000	0.18	>5000	0.20	>5000	0.00	>5000	0.00	>5000	0.10	>5000	0.62	330
20	>5000	1.77	54	1.85	40	1.08	730	0.65	1800	0.00	>5000	0.20	5000	0.70	900
21	5000	1.26	230	1.18	350	0.22	>5000	0.10	>5000	0.00	>5000	0.25	2800	0.10	>5000
Foscarnet	>5000	2.08	20	1.74	89	1.30	230	1.37	170	0.10	>5000	0.40	5000	0.00	>5000

[a] The maximum tolerated concentration (MTC) is the lowest concentration of compound that produced microscopically evident cytotoxicity to confluent Vero cells. Determined as described in text. [b] The virus rating (VR) was determined as described in text. Significance of VR values were assigned as follows: <0.5 inactive, 0.5–0.95 moderate activity, >1.0 marked activity. [c] The ED_{50} is the concentration of compound that produced 50% inhibition of viral CPE as compared with infected but untreated control cells. Determined as described in text.

tivity against vaccinia. Guanosine 5′-(hydroxycarbonyl)-phosphonate (**15**) was inactive against all viruses tested except for slight activity against measles. In the adenosine series, the adenosine 5′-(ethoxycarbonyl)phosphonate (**4**) and adenosine 5′-(aminocarbonyl)phosphonate (**12**) were not significantly different from the hydroxycarbonyl **14** in their activity patterns, with ED_{50} values against both HSV-2 strains varying by only two- to four-fold. However, in the guanosine series, the guanosine 5′-(ethoxycarbonyl)phosphonate (**5**) and guanosine 5′-(aminocarbonyl)phosphonate (**13**) were more active than the hydroxycarbonyl **15** and were essentially comparable to the 2′-deoxyguanosine 5′-(hydroxycarbonyl)phosphonate analogue (**21**) in activity against HSV-2 with ED_{50} values only two- to three-fold greater than **21**. Compounds **5** and **13** were the only guanosine analogues tested that exhibited antiviral activity approximately as potent as the corresponding adenosine analogues **4** and **12**.

All compounds tested were nontoxic to confluent Vero cells at concentrations as high as 5000 μM, as assessed by microscopic observation, except **21** which was slightly toxic at 5000 μM.

Inhibition of Tumor Cell Growth in Vitro. All compounds were tested and found to be inactive in inhibiting the growth of P388 and L1210 leukemia at concentrations of up to 100 μM.

Experimental Section

General Information. Ultraviolet spectra were recorded on a Cary Model 118 spectrophotometer. Infrared (IR) spectra were obtained on a Beckman Acculab 2 spectrophotometer. Melting points were taken on a Fisher-Johns plate melting point apparatus and are uncorrected. Nuclear magnetic resonance (NMR) spectra were taken on a JEOL FX 90Q spectrometer. The chemical shift values are expressed in values (parts per million) and referenced with tetramethylsilane (for ^1H NMR), phosphoric acid (85%) (for ^{31}P NMR), and dioxane (for ^{13}C NMR). High-performance liquid chromatography (HPLC) studies were performed on a Beckman Model 110A gradient liquid chromatograph with a Hitachi Model 110-40 UV monitor and an Altex C-RIA recorder. A column of ODS-2 (reversed-phase silica gel) was used, and the elutions for HPLC were performed with both 5% acetonitrile in water and 0.05 M ammonium phosphate buffer at pH 6.5.

Thin-layer chromatograms (TLC) were run on silica gel 60 F-254 (EM Reagent) plates. EM silica gel (200–400 mesh) was used for flash column chromatography. Bio-Rad AG 2-X8 and Sigma DEAE-Sephadex A-25 was used for ion exchange chromatography. The components were detected on TLC plates by UV light and with 10% H_2SO_4 in methanol spray followed by heating. The solvent systems used for TLC were as follows: (A) 2% MeOH in $CHCl_3$, (B) 5% MeOH in $CHCl_3$ (C) 15% MeOH in $CHCl_3$, (D) 0.1 N NH_4Cl, CH_3CN (3:7), and (E) acetone, hexane (3:7).

Evaporations were carried out by using a rotary evaporator (Buchi Rotavapor R110) under reduced pressure applied by a tap water aspirator with the bath temperature of 30–35 °C. Freeze drying was carried out with a Precision vacuum pump, Model DD 195.

Chlorotrimethylsilane, ethyl chloroformate, and thionyl chloride were from Aldrich, Milwaukee, WI. 2′,3′-Isopropylideneadenosine and 2′,3′-isopropylideneguanosine were obtained from ICN Pharmaceutical Inc., Plainview, NY. Phosphorous acid and triethylamine were obtained from Fisher Scientific, Fair Lawn, NJ. 3′-*O*-Acetyl-2′-deoxyadenosine[21] and 3′-*O*-acetyl-2′-deoxyguanosine[21] were synthesized according to the published procedures.

Elemental analysis were performed by Galbraith Laboratories, Knoxville, TN, and by Schwarzkopf Microanalytical Laboratory, Woodside, NY.

Procedures. (Ethoxycarbonyl)phosphonic Dichloride (3). Tris(trimethylsilyl) phosphite (1), (60 g) (0.2 mol) was added dropwise to 22 g (0.2 mol) of ethyl chloroformate in an ice bath. After the addition, the ice bath was removed, and the reaction was continued at room temperature for 4 h. Bis(trimethylsilyl) (ethoxycarbonyl)phosphonate (**2**) was distilled at 68 °C (0.05 torr) [^1H NMR in $CDCl_3$ δ 0.2 (s, 9 H), 0.95 (t, 3 H), 3.9 (q, 2 H)]. The IR spectrum also matched the structure.[18] Compound **2** (60 g) (0.2 mol) was placed in a 250-mL flask and 100 mL of thionyl chloride was added. The mixture was then heated in an oil bath (70 °C) and refluxed for 4 h. The excess thionyl chloride and the chlorotrimethylsilane byproduct were removed by distillation. Compound **3** was distilled at 53 °C (0.05 torr).[18] The yield was 31 g (0.16 mol) (80.5%) [^1H NMR in $CDCl_3$, 1.45 (t, 3 H), 4.5 (q, 2 H)].

Adenosine 5′-(Ethoxycarbonyl)phosphonate (4). In a 25-mL round-bottomed flask equipped with a drying tube, 1 g (5.2 mmol) of (ethoxycarbonyl)phosphonic dichloride (**3**) was dissolved in 10 mL of trimethyl phosphate and cooled to 0 °C. One gram (3.3 mmol) of dry 2′,3′-isopropylideneadenosine (dried over P_2O_5 at 76 °C and 0.05 torr) was added and stirred at 0 °C until complete dissolution. The reaction then continued at 0 °C for another hour, and the reaction mixture was poured slowly into 250 mL of anhydrous ether with stirring. The precipitate was separated by decantation and washed with 2 × 50 mL of ether. The crude product was dried and dissolved in 50 mL of 80% formic acid. After 2 h at room temperature or $^1/_2$ h at 50 °C, the formic acid was removed by distillation in vacuo at 30 °C. The compound was coevaporated with water to dryness, and the final product was purified on a Dowex 2 (X8) (200–400 mesh) (Cl^- form) column (3 × 50 cm). The column was eluted with 300 mL of water and a gradient of 0.0–0.15 M LiCl. The water was evaporated to near dryness at 30 °C and 100 mL ethanol was added. The precipitate was centrifuged, and the solution was discarded. The precipitate was washed with 3 × 50 mL of ethanol and separated by centrifugation. The lithium was removed by a column of Dowex 50 (X8) H^+, and the resulting solution was freeze dried. The yield of compound **4** was 650 mg (1.62 mmol) (49.5%). HPLC showed a single peak on elution with 5% acetonitrile in water: ^{31}P NMR in Me_2SO-d_6 δ −6.999 (s); UV spectrum λ_{max} 257 nm (pH 7), λ_{min} 229 nm, ε 14.9×10^3. Anal. $C_{13}H_{18}N_5O_9P \cdot 1.8H_2O$) C, H, N, P.

Guanosine 5'-(Ethoxycarbonyl)phosphonate (5). In a 25-mL flask, 1 g (5.2 mmol) of (ethoxycarbonyl)phosphonic dichloride (3) was dissolved in 10 mL of trimethyl phosphate and cooled to 0 °C in an ice bath. To the cold solution was added and stirred at 0 °C for 2 h 1.2 g (3.7 mmol) of dry 2',3'-isopropylideneguanosine. The reaction mixture was then treated in a similar manner to the adenosine analogue 4. The lithium was then replaced with ammonium by using a Dowex-50 ion-exchange column. The yield of compound 5 was 1.1 g (68.3%). HPLC showed a single peak: ^{31}P NMR in Me_2SO-d_6 δ −7.067 (s); UV spectrum λ_{max} 252 nm (pH 7), λ_{min} 223 nm, ϵ 15.5 × 10^3. Anal. ($C_{13}H_{21}N_8O_9P \cdot 1.5H_2O$) C, H, N, P.

2'-Deoxyadenosine 5'-(Hydroxycarbonyl)phosphonate (20). The 2'-deoxy-3'-*O*-acetyladenosine (16) was synthesized according to the procedure of Holy.[21] In a 10-mL flask, 300 mg (1.6 mmol) of compound 3 was dissolved in 5 mL of trimethyl phosphate and cooled to 0 °C. With stirring, 250 mg (0.85 mmol) of compound 16 was added and stirred at 0 °C for 2 h. The reaction mixture was poured into 200 mL of anhydrous ether. The precipitate was separated by decantation and washed with 2 × 50 mL of ether and dried. The dry compound was dissolved in 5 mL of water and 20 mL of 1 N sodium hydroxide was added to this solution and stirred for 1 h. The mixture was neutralized to pH 10 with Dowex-50 (X8) H^+ and filtered. The solution was then loaded on a DEAE-Sephadex column (3 × 50 cm) (bicarbonate form) and eluted with 100 mL of water and a gradient of 0.1–0.25 M ammonium bicarbonate. The title compound was separated and dried in vacuo at 35 °C. The ammonium bicarbonate contamination was removed by freeze drying of the frozen aqueous solution for 48 h. The yield was 275 mg (0.7 mmol) (82%). HPLC showed a single peak: ^{31}P NMR in D_2O δ −6.95; UV spectrum λ_{max} 259 (pH 7), ϵ 14.9 × 10^3. Anal. ($C_{11}H_{20}N_7O_7P \cdot 1.75H_2O$) C, H, N, P.

2'-Deoxyguanosine 5'-(Hydroxycarbonyl)phosphonate (21). To synthesize the title compound the procedure for 20 was followed using 300 mg (0.97 mmol) 2'-deoxy-3'-*O*-acetylguanosine.[21] As a result, the yield for compound 21 was 310 mg (0.76 mmol) (77.5%). HPLC showed a single peak. ^{31}P NMR in D_2O δ −6.95; UV spectrum λ_{max} 252 nm (pH 7), λ_{min} 223 nm, ϵ 15.1 × 10^3. Anal. ($C_{11}H_{20}N_7O_8P$) C, H, N, P.

Adenosine 5'-(Hydroxycarbonyl)phosphonate (14). The crude product of adenosine 5'-(ethoxycarbonyl)phosphonate (4) (200 mg) (0.477 mmol) was dissolved in 5 mL of water, and 20 mL of 1 N sodium hydroxide was added and stirred at room temperature for 1 h. The reaction mixture was neutralized to pH 9–10 with Dowex-50 (X8) H^+. The mixture was filtered and loaded on a DEAE-Sephadex column and eluted as described for compound 20. The major peak of mononucleotide was separated and dried in vacuo at 35 °C. The trace of ammonium bicarbonate was removed by freeze drying. The yield of compound 14 was 176 mg (0.429 mmol) (90%). This compound was obtained as sodium salt by passing through a Dowex 50 × 8 (Na^+ form) column. HPLC showed a single peak. Anal. ($C_{11}H_{12}N_8O_8P$-$Na_2 \cdot 2.25H_2O$) C, H, N, P.

Guanosine 5'-(Hydroxycarbonyl)phosphonate (15). The crude product of 5 (200 mg) (0.47 mmol) was dissolved in 5 mL of water and treated with 1 N sodium hydroxide and chromatographed on DEAE-Sephadex as described for adenosine 5'-(hydroxycarbonyl)phosphonate (14). The nucleotide 15 was purified on a DEAE Sephadex column and dried as described above. The yield of compound 15 was 164 mg (0.385 mmol), (81%). Anal. ($C_{11}H_{20}N_7O_9P$) C, H, N, P.

Adenosine 5'-(Aminocarbonyl)phosphonate (12). Adenosine 5'-(ethoxycarbonyl)phosphonate (4) was dried over P_2O_5, 200 mg (0.496 mmol) was placed in a pressure bottle, and 10 mL of saturated methanolic ammonia was added and stirred at room temperature for 36 h. The reaction mixture was dried in vacuo at 35 °C and dissolved in 10 mL of water and chromatographed on a DEAE-Sephadex column. The pure 12 was separated and dried in vacuum at 35 °C. The trace of ammonium bicarbonate was removed by freeze drying. The conversion of ethoxycarbonyl to aminocarbonyl was quantitative: ^1H NMR in Me_2SO-d_6 δ 3.5 (H_2O), 5.82 (d, 1 H, anomeric), 7.2–7.5 (4 H, the NH_2 of adenine and the NH_2 of aminocarbonyl), 8.06 (s, 1 H, Ar), 8.36 (s, 1 H, Ar). Anal. ($C_{11}H_{18}N_7O_7P \cdot 2.5H_2O$) C, H, N, P.

Guanosine 5'-(Aminocarbonyl)phosphonate (13). Guanosine 5'-(ethoxycarbonyl)phosphonate (5) was dried, 200 mg (0.47 mmol) was treated with methanolic ammonia as described for 12, and the resulting title compound was purified by the same procedure. The yield of compound 13 was 167 mg (0.447 mmol) (95%): ^1H NMR in Me_2SO-d_6 δ 3.5 (H_2O), 5.7 (d, 1 H, anomeric), 6.6 (2 H, NH_2), 7.1 (2 H, other NH_2), 7.3 (1 H, N_1H), 7.92 (1 H, Ar). Anal. ($C_{11}H_{18}N_7O_8P \cdot 0.5H_2O$) C, H, N, P.

In Vitro Antiviral Evaluations. The compounds were evaluated for their ability to inhibit virus-induced cytopathic effect (CPE) produced by seven viruses in African green monkey kidney (Vero) cells (American Type Culture Collection, Rockville, MD). Viruses used in this evaluation included parainfluenza virus type 3 (para 3) strain C243, measles virus strain Edmonston, vaccinia virus strain Elstree, herpes simplex virus type 1 (HSV-1) strain KOS and strain HF, and herpes simplex virus type 2 (HSV-2) strain MS and strain 333. Vero cells were maintained in antibiotic free Dulbecco's modified Eagle medium (DMEM) supplemented with 10% newborn bovine serum (Grand Island Biological Co., Grand Island, NY).

For antiviral experiments, cells were inoculated into 96 well tissue culture plates (Corning Glassworks, Corning, NY) at a concentration of 4 × 10^4 cells/0.2 mL per well and cultured to confluency for 24 h at 37 °C in 5% CO_2 atmosphere. Following incubation, growth medium was removed, and monolayers were inoculated (0.1 mL per well) with a predetermined amount of virus that will produce complete destruction of the cell monolayers in 72 h. After 30-min adsorption of virus at 37 °C, test compounds were added (0.1 mL/well) in seven 0.5 log concentrations ranging from 10.0 to 10000 μM, resulting in final well concentrations of 5.0 to 5000 μM. At each concentration, duplicate wells were used for evaluation of antiviral activity and single uninfected wells for cytotoxicity estimation.

The degree of virus-induced CPE and compound cytotoxicity was observed microscopically after 72 h incubation at 37 °C in humidified 5% CO_2 atmosphere. CPE was scored numerically from 0 (normal, uninfected control cells) to 4 (100% cell destruction as in virus-infected controls), and a virus rating (VR) was calculated as previously reported.[22] The higher the VR the better the antiviral activity of the compound, with values greater than 1.0 being considered indicative of significant activity. The CPE scores were also used to estimate the concentration of compound that produced 50% inhibition of viral CPE (ED 50).

A given concentration of the compound was considered to be cytotoxic if it produced any microscopically visible change in cellular morphology or in the density of the cell monolayer due to lysis, rounding up or detachment of cells. The lowest concentration where cytotoxicity to uninfected, confluent cell monolayers was observed was considered to be the maximum tolerated concentration (MTC).

Inhibition of Tumor Cell Growth in Vitro. Compounds were evaluated for their ability to inhibit growth of murine P388 and L1210 leukemia (American Type Culture Collection, Rockville, MD) maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (Grand Island Biological Co.) and 20 mM HEPES buffer. For growth experiments, cells were adjusted to 1 × 10^5 cells/mL and distributed into 24 well tissue culture plates (0.5 mL/well). Test compounds were dissolved in growth medium, sterilized by passage through a 0.22-μm membrane filter, serially diluted, and added to wells (0.5 mL/well). Compounds were tested in duplicate at log concentrations ranging from 0.1 to 100 μM. Following 48 h incubation at 37 °C in 5% CO_2 atmosphere, cell counts were determined by using a Coulter Model ZF cell counter. Cell growth in the presence of test compounds was expressed as a percentage of growth in untreated control cells, and the concentration of compound producing 50% inhibition of cell growth was determined (ID_{50}).

(22) Sidwell, R. W.; Huffman, J. H. *Appl. Microbiol.* 1971, *22*, 797.