The Presence of Substituents on the Aryl Moiety of the Aryl Phosphoramidate Derivative of d4T Enhances Anti-HIV Efficacy in Cell Culture: A Structure−Activity Relationship

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Received July 2, 1998

New substituted-aryl phosphoramidate derivatives of the anti-HIV drug d4T were synthesized as membrane-soluble intracellular prodrugs for the free bioactive phosphate to establish relationship(s) between compound structure and in vitro antiviral activity. The majority of compounds demonstrated an elevation of in vitro potency relative to that of the parent nucleoside, and unlike d4T, all retained full activity in thymidine kinase-deficient cells. The compound bearing a *p*-chloro aryl group (**8e**) expressed nanomolar activity in vitro, a 14-fold increase in activity relative to that of the unsubstituted phosphoramidate (100-fold compared to d4T). An assay using pig liver esterase was used to establish the stability of the compounds to enzymatic degradation. While there was no apparent correlation between in vitro activity and half-life of enzymatic degradation, there was a close correlation between compound lipophilicity, determined by octanol/water partition coefficient, and in vitro potency. We suggest that substitutions made to the aryl moiety of the aryl phosphoramidate of d4T that result in enhancing lipophilicity may serve to increase the cellular uptake of the prodrug by passive diffusion, leading to the expression of antiviral potency at reduced prodrug concentrations.

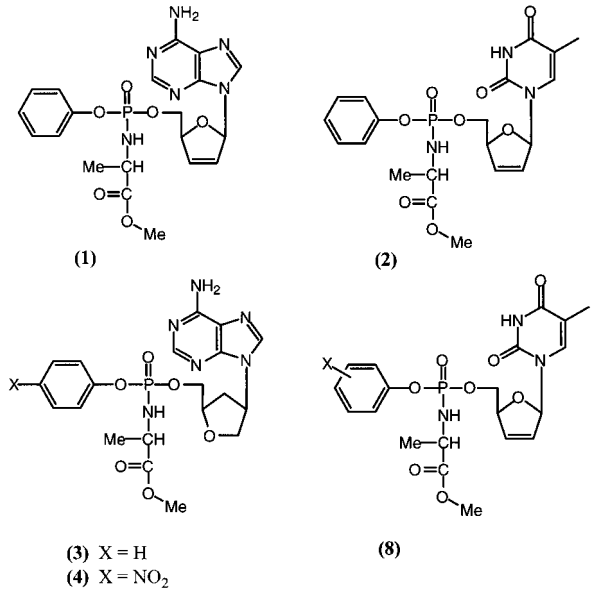
Introduction

Among the current diversity of nucleoside analogues active against human immunodeficiency virus (HIV) in cell culture, the 2′,3′-dideoxynucleosides (ddN's) remain among the most potent.[1−6] In all cases the expression of activity requires conversion to the corresponding 5′-triphosphates. These may inhibit the progression of the virus by competitive inhibition of the viral reverse transcriptase and/or incorporation and subsequent chain termination of the growing viral DNA strand. Anti-HIV ddN derivatives rely on host cell nucleoside and nucleotide kinases to deliver the corresponding 5′-triphosphates. However, many such compounds have been shown to be poor substrates for nucleoside kinases.[7−11] Furthermore their activation is restricted in cells where nucleoside kinases are less prominent or deficient (e.g., monocyte/macrophages).[12] Consequently, prodrug strategies that deliver the dideoxynucleoside have been sought to effectively bypass the initial nucleoside kinase-mediated phosphorylation step (reviewed by Jones and Bischofberger[13] and recently by Meier[14]).

Previously we have reported the considerable success of an approach using (aryloxy)phosphoramidate nucleoside prodrugs. In particular the (aryloxy)phosphoramidates **1** and **2** (Chart 1) (derivatives of d4A and d4T, respectively) were found to exhibit greatly enhanced activity against HIV-1 and HIV-2 in cell culture compared to their parent ddN's, with full retention of activity in thymidine kinase-deficient cell lines (Chart 1).[15−17] The masked-phosphate approach to d4T was particularly appealing for several reasons. This nucleoside has been shown to be a potent inhibitor of HIV and also to display reduced toxicity compared to, for example, AZT in certain cell types (e.g., bone marrow progenitor cells).[4,18,19] Furthermore the kinetics of the three phosphorylation steps leading to the nucleotide triphosphate from the original nucleoside suggests the initial monophosphorylation step to be rate-limiting in this case. This is in contrast to AZT where the rate-limiting step appears to be the phosphorylation of the monophosphate to the diphosphate.[20a,b]

Significant evidence points toward an enzyme-activated degradation pathway leading from these and

Chart 1



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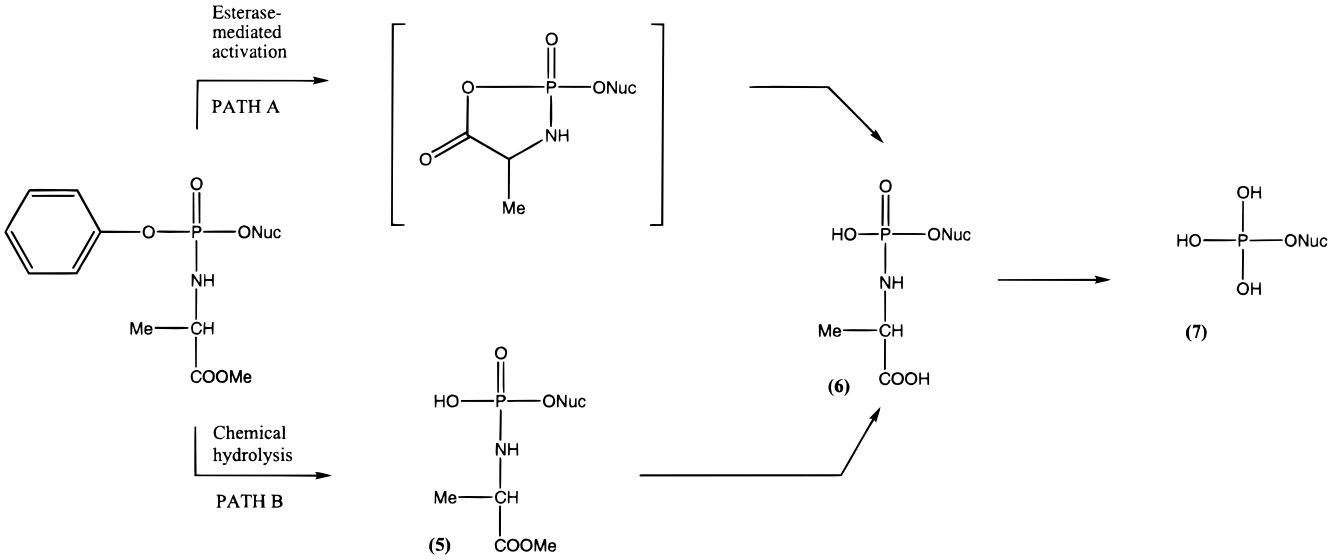
10.1021/jm9803931 CCC: $18.00
Published on Web 01/26/1999

Scheme 1



similar phosphate prodrugs to their corresponding free nucleotides. Two possible degradation pathways have been proposed (Scheme 1). Path A involves initial esterase-catalyzed cleavage of the carboxy ester, followed by the internal nucleophilic attack of the acid residue on the phosphorus center, displacing the aryloxy group.[21] The putative transient, cyclic mixed-anhydride is then rapidly hydrolyzed to the corresponding alaninyl phosphomonoester **6**. Path B involves hydrolysis of the phosphodiester amidate to the phosphomonoester amidate followed by enzymatic cleavage of the carboxy ester to give the alaninyl phosphomonoester. The alaninyl residue of the phosphomonoester (formed by either pathway) is then presumed to be cleaved by either chemical or enzymatic means to give the nucleoside monophosphate **7**.

Previous metabolic studies on the (aryloxy)phosphoramidate of d4T (**2**)[22] have revealed the presence of significant amounts of alaninyl phosphomonoester **6** (in addition to mono-, di-, and triphosphorylated nucleosides) following incubation of **2** with cells. Similarly studies by Imbach and co-workers on the (aryloxy)-phosphoramidate (**3**) of isoddA[21] revealed significant amounts of alaninyl phosphomonoester on incubation of **3** with cell extracts. Conversely when the corresponding phosphomonoester amidate was incubated with cell extracts, neither the alaninyl phosphomonoester nor the nucleoside monophosphate were observed. These results suggest that path A is the major route to the free nucleoside monophosphate.

Exhaustive modifications to the amino acid moiety in **2** have established L-alanine as the moiety for optimal antiviral activity.[23] A small series of substituted (aryloxy)phosphoramidate analogues of **2** was previously synthesized to determine the effects on potency of modifying the aryloxy moiety.[17] The compounds were found to be less active than the parent structure against HIV-1 and HIV-2 in cell culture. However, comparisons between the phenyl and *p*-nitrophenyl phosphoramidates of isoddA **3** and **4** made by Imbach and co-workers reveal interesting differences in the stability of the two compounds.[21] In culture medium and in human serum (where esterase activity is less than in cell extracts), the *p*-nitrophenyl phosphoramidate **4** is less stable than the phenyl phosphoramidate **3** (likely due to the electron-withdrawing characteristics of the nitro group). By contrast, in cell extracts the *p*-nitrophenyl phosphoramidate **4** is twice as stable as the phenyl phosphoramidate **3**, indicating that the polar *p*-nitro substituent may adversely affect the compound's interaction with cellular esterase(s). Furthermore, substitutions at the aryloxy part of **2** may have the effect of altering not only the rate and/or nature of prodrug degradation but also the rate of membrane transport.

We present here a comprehensive structure−activity relationship study on the effect of various substitutions of the aryloxy moiety of **2** on anti-HIV activity in cell culture. We will define some of the key chemical characteristics required for lead optimization including correlations between antiviral activity, compound partition coefficient, and compound stability to both enzymatic and chemical degradation.

Results and Discussion

The synthesis of d4T from thymidine was conducted essentially by the method of Horwitz[24] noting the later comments of Mansuri.[25] (Aryloxy)phosphorochloridates, and (aryloxy)aminophosphorochloridates were prepared entirely as previously noted.[26] Phosphoramidates **8a**−**8i** (Table 1) and **2** were synthesized according to the method described previously.[17] The structure and purity of compounds **8a**−**8i** were determined by extensive multinuclear NMR and analytical HPLC using two distinct eluent gradients. The *p*-aryl substituents in **8a**−**8h** were chosen to reflect a broad cross-section of electron-donating/withdrawing effects (as measured by Hammet σ values) with particular emphasis on the latter (Table 1). Previous work with diaryl phosphates of AZT demonstrated elevated antiviral activity for aryl substituents containing electron-withdrawing substituents.[27] Although the mechanism(s) of degradation of these AZT derivatives and the phosphoramidates synthesized here may be different, it is possible that electron-withdrawing substituents will increase the rate at which the aryloxy group leaves after enzymatic hydrolysis of the amino ester. The choice of substituents

Table 1[a]

structure	X	σ_p	σ_m	π
8a	*p*-NO_2	0.81		−0.28
8b	*p*-CN	0.70		−0.57
8c	*p*-COOMe	0.48		−0.01
8d	*p*-COMe	0.47		−0.55
8e	*p*-Cl	0.24		0.71
8f	*p*-F	0.15		0.14
8g	*p*-Me	−0.14		0.56
8h	*p*-OMe	−0.28		−0.02
8i	*m*-COMe		0.35	
2	H	0	0	0

[a] Data for σ and π constants taken from Hansch and Leo.[28] Quoted π values are for X-C_6H_5 and not for the specific structures **8a**−**8i**.

was also influenced by the desire to examine the influence of compound lipophilicity on antiviral activity. Consequently, the substituents also reflect a broad range of Hansch constants (π). Also, to compare the possible effects of *m*-aryl versus *p*-aryl substituents, the *m*-acyl-substituted analogue of **8d** was synthesized (**8i**). Given the enzymatic nature of the putative initial step in phosphoramidate degradation, specific aryl substitution patterns may favor or disfavor interaction of the phosphoramidate with the active site of the enzyme(s), and substituent polarity and hydrogen-bonding characteristics may also prove to be important in this respect.

Antiviral Activity. All of the synthesized phosphoramidates **8a**−**8i** were evaluated for their ability to inhibit the replication of HIV, as previously described.[26] The results obtained using HIV-1- or HIV-2-infected CEM cells are displayed in Table 2. The tests were also conducted against HIV-2 in thymidine kinase-deficient (TK^-) CEM cells. For the purpose of reference, similar data for d4T and **2** obtained previously[17] are also displayed.

All of the (substituted-aryloxy)phosphoramidates apart from **8a**, **8b**, and **8d** showed a notable elevation in antiviral potency against HIV-1 and HIV-2 compared to the free nucleoside, d4T. Furthermore, whereas d4T showed a pronounced decrease in activity in CEM/TK^- cells, all of the synthesized phosphoramidates appeared to retain full antiviral activity, in common with **2**. The activity displayed by most of the compounds was comparable to that of the lead structure **2**. The notable exception to this is the *p*-Cl derivative **8e**, which displayed an approximately 14-fold greater activity than **2** (100 times greater than d4T). This increased potency was also reflected in CEM/TK^- cells. Significantly enhanced activity was also shown by the *p*-COOMe derivative **8c**.

Compounds containing the most electron-withdrawing substituents (highest σ value) appear to be among the least potent in the series. Also, the compounds showing greatest antiviral activity have σ values in the range 0.24−0.48. The exceptions to this are the *p*- and *m*-acyl-substituted compounds (**8d** and **8i**) which have σ values within this range but do not display a substantially elevated potency compared to **2**. The cytotoxicity data (CC_{50}) show all of the synthesized phosphoramidates to exhibit greater toxicity than **2**. Furthermore, a strong positive correlation exists between toxicity and potency, indicating that toxicity most likely arises from the same pharmacophore in each case. None of the tested compounds displayed significant antiviral activity against Moloney murine sarcoma virus (MSV).

Compound Partition Coefficients. The partition coefficients of compounds **8a**−**8i** and **2** were determined in 1-octanol/aqueous buffer (pH 7.0). Each compound was partitioned between 1-octanol and aqueous buffer for 5 min. After separation of the two layers the relative proportion of compound in each layer was determined by measuring the UV absorption of the octanol layer compared to a control solution. This method of determining the partition coefficients allows for a qualitative comparison between the compounds in the series. The results are shown in Table 3. The partition coefficients correlated well with the Hansch constants of the respective substituents (Table 1) used initially to predict the general order of lipophilicity. Compounds containing the most polar substituents (**8a**, **8b**, **8d**, and **8i**) gave rise to the lowest Pa values, while those containing the least polar substituents (**8e** and **8g**) gave rise to the highest values. Comparative data for d4T[29] indicate significantly increased lipophilicity for all the phosphoramidates relative to their parent nucleoside. A plot of log Pa versus−log EC_{50} (HIV-1) reveals a close correlation (correlation coefficient, $r = 0.90$) between antiviral potency and compound hydrophobicity (Figure 1), with the most lipophilic compound (**8e**) showing the greatest biological activity. This may reflect an increased membrane transport by passive diffusion for compounds bearing lipophilic substituents. In addition it may also reflect increased preference by cellular hydrolytic enzymes.

Enzymatic Degradation Studies. In an attempt to establish the dependence of drug potency on enzymatic degradation, compounds **8a**−**8i** and **2** were incubated with pig liver esterase (PLE) in pH 7.6 buffer. PLE is a mixture of five isozymes; the two major isozymes, I and V, hydrolyze aromatic/long-chain esters and short-chain esters, respectively. An addition of acetone was made in each case to solubilize the test compound, and it should be noted that acetone has been found to inhibit isozyme V.[30] Evidence suggests that most mammalian esterases are similar but not identical. For example, PLE has a virtually identical subunit weight and similar amino acid sequence as human esterase. They differ in that human esterase contains fewer isozymes.[31] The progress of degradation was followed by ^{31}P NMR spectroscopy, by monitoring the loss of starting material.

In all cases the degradation of the substrate gave rise to a signal in the phosphorus NMR spectra previously determined[32] to correspond to compound **6** (Scheme 1) ($\delta_P(D_2O)$ 8.1 ppm). The results (summarized in Table 3) show the overall half-lives of degradation to be remarkably similar for the majority of compounds, varying between 100 and 150 h, with some notable exceptions. The lead structure **2** and the *p*-fluoro derivative **8f** exhibited the greatest stabilities, both having half-lives of 300 h. The *p*-nitro (**8a**) and *p*-methoxy (**8h**) derivatives were the least stable with half-lives of 60 and 11 h, respectively. Control experiments were conducted to establish the independent rates of chemical hydrolysis, by incubating the compounds in the same buffer in the absence of enzyme (results shown in Table 3). The degradation half-lives in the presence of enzyme are labeled with an asterisk for those com-

Table 2. Anti-HIV-1, Anti-HIV-2, and Cytotoxicity of Compounds **8a**−**8i**, **2**, and d4T in Vitro

	EC_{50}[a] (μM)			CC_{50}[b] (μM)	EC_{50} (μM)	MCC[c] (μM)
compd	CEM/0 (HIV-1)	CEM/0 (HIV-2)	CEM/TK$^-$ (HIV-1)	CEM/0 (HIV-2)	C3H/3T3 (MSV)	C3H/3T3
8a	0.130	0.190	0.09	73	9.3	>100
8b	0.150	0.130	0.09	60	30	>100
8c	0.025	0.022	0.025	31	5.6	>100
8d	0.110	0.100	0.050	40	13	>100
8e	0.005	0.007	0.006	11	2.2	>100
8f	0.053	0.060	0.022	26	7.4	>100
8g	0.040	0.050	0.025	34	10	>100
8h	0.057	0.053	0.047	35	16	>100
8i	0.080	0.090	0.045	50	26	>100
2	0.075	0.075	0.075	>100	15	>100
d4T	0.651	0.770	33	174	*d*	*d*

[a] EC_{50} is the 50% effective compound concentration required to protect CEM cells against the cytopathogenicity of HIV by 50% or to inhibit MSV-induced transformation of C3H/3T3 cells by 50%. Data are the means of 2−4 independent experiments. [b] CC_{50} is the 50% cytotoxic compound concentration required to inhibit CEM cell proliferation. [c] MCC is the minimum cytotoxic concentration that causes a microscopically detectable alteration of cell morphology. EC_{50} and CC_{50} data for d4T are taken from McGuigan et al.[17] [d] Not determined.

Table 3. Partition Coefficient Data and Degradation Half-Lives at pH 7.6 and 37 °C in the Presence and Absence of Pig Liver Esterase (Principle Product: **6**, Scheme 1)

		$t_{1/2}$ (h)	
compd	log Pa	enzymatic	chemical
8a	0.90	*60 ± 6	151 ± 15
8b	0.91	*100 ± 10	151 ± 15
8c	1.01	*100 ± 10	301 ± 30
8d	0.90	*150 ± 15	301 ± 30
8e	1.43	151 ± 15	*a*
8f	1.10	301 ± 30	*a*
8g	1.19	151 ± 15	*a*
8h	1.09	11 ± 1	*a*
8i	0.92	*151 ± 15	301 ± 30
2	1.04	301 ± 30	*a*

*Chemical hydrolysis observed. [a] Stable to chemical hydrolysis.

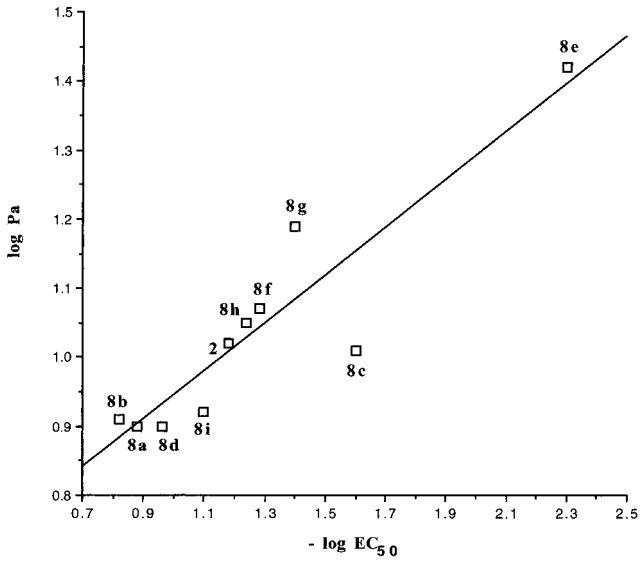


Figure 1. Antiviral potency in CEM/0 cells (−log EC_{50} HIV-1 in μM) plotted against partition coefficient (log Pa). Correlation coefficient (r) = 0.90.

pounds where independent chemical hydrolysis was observed. Of the compounds containing electron-withdrawing substituents, all exhibited independent chemical hydrolysis apart from the *p*-fluoro (**8f**) and *p*-chloro (**8g**) derivatives. Such chemical hydrolysis may be problematic in terms of drug delivery, as substantial hydrolysis in the extracellular environment may lead to the phosphate monoester, which, carrying a charge, may have a diminished rate of membrane transport by passive diffusion compared to the phosphate diester.

Showing no independent chemical hydrolysis over the time scale of the enzyme experiments, the rapid rate of degradation shown by the *p*-methoxy derivative **8h** in the presence of enzyme must reflect the rate of its enzymatic activation. *p*-Methoxyphenol is an inferior chemical leaving group compared to any of the phenols having electron-withdrawing substituents. Furthermore, the lipophilicity of **8h** is similar to that of **2** (log Pa **8h** = 1.09, log Pa **2** = 1.04); therefore, specific structural characteristics of **8h** most likely serve to enhance its recognition as a substrate compared to **2**.

No apparent correlations were noted between the degradation half-lives in the presence of enzyme and the antiviral activity of the different compounds. An obvious trend is also absent when the half-lives of those compounds shown not to undergo independent chemical hydrolysis (over the time scale of the enzyme experiments) are compared to biological activity. In these cases (compounds **8e**−**8h** and **2**), there would be a direct correlation between the biological activity and the rate of enzymatic degradation. These data show that quantitative kinetic comparisons between the enzymatic assay and the in vitro antiviral potency are not evident for this series of compounds. However, they demonstrate the formation of **6** from all compounds in the series **8a**−**8i**, via esterase-mediated degradation. These results are consistent with earlier observations that **6** is the major metabolite of **2** in six discrete cell lines and is presumed to degrade by chemical or enzymatic means to the corresponding nucleoside monophosphate.[22]

A simple correlation between substituent σ values and overall degradation half-life is not apparent, since the least stable compounds (**8a** and **8h**) have the highest and lowest σ values, respectively. The relatively rapid degradation of the *p*-nitro derivative **8a** cannot be entirely explained in terms of additional chemical hydrolysis during the course of the enzyme experiment, since the *p*-cyano derivative **8b**, although chemically hydrolyzed at the same rate as **8a**, was almost twice as stable in the presence of enzyme. Also, the lipophilicities of compounds **8a** and **8b** are similar (log Pa **8a** = 0.90, log Pa **8b** = 0.91). Therefore, improved molecular recognition of the *p*-nitro derivative by the enzyme is the probable explanation. This is in contrast to the conclusion drawn by Imbach and co-workers[21] regarding

the rate of enzyme-induced activation of the (*p*-nitrophenyloxy)phosphoramidate of isoddA.

Differences between meta- and para-substitution appear to be minimal in the present series. The partition coefficients of the *p*- and *m*-acyl derivatives **8d** and **8i** are similar (**8d** log Pa = 0.90, **8i** log Pa = 0.92), as are their respective rates of degradation in the presence and absence of carboxyesterase. These similarities are reflected well in their respective in vitro antiviral potencies (EC_{50}(HIV-1): **8d** = 0.11, **8i** = 0.08; EC_{50}(HIV-2): **8d** = 0.10, **8i** = 0.09).

Finally, it is worth comparing the *p*-Cl and *p*-Me derivatives (**8e** and **8g**) directly against each other. Medicinally, the methyl group is considered to be a good isostere for the chlorine atom. There is no discernible difference between the two compounds' respective rates of degradation in the presence of enzyme. The approximately 10-fold greater activity of **8e** over **8g** may be associated with the electron-withdrawing characteristics of the former compared to the electron-donating character of the latter and/or the superior lipophilicity of **8e**.

In conclusion, we have been able to demonstrate a structure−activity relationship between substituted (aryloxy)phosphoramidates and their in vitro antiviral activity. Optimum activity appears to correlate with aryloxy substituents which have the following properties: exhibit a mild electron-withdrawing effect corresponding to values of σ between 0.15 and 0.48 and give rise to high lipophilicity as evidenced by octanol/water partition coefficients. To corroborate these conclusions further, the synthesis of a series of compounds possessing these attributes is currently underway in our laboratories.

Experimental Section

All experiments involving water-sensitive compounds were conducted under scrupulously dry conditions. Triethylamine was dried by refluxing over calcium hydride. Anhydrous tetrahydrofuran and dichloromethane were purchased from Aldrich. *N*-Methylimidazole was purified by distillation. Nucleosides were dried by storage at elevated temperature over P_2O_5 in vacuo. Proton, carbon, and phosphorus nuclear magnetic resonance (^{1}H, ^{13}C, and ^{31}P NMR) spectra were recorded on a Bruker Avance DPX spectrometer operating at 300, 75.5, and 121.5 MHz, respectively. All ^{13}C and ^{31}P NMR spectra were recorded proton-decoupled. All NMR spectra were recorded in $CDCl_3$ at room temperature (20 ± 3 °C). Chemical shifts for ^{1}H and ^{13}C NMR spectra are quoted in parts per million downfield from tetramethylsilane. Coupling constants are referred to as *J* values in hertz (Hz). Signal splitting patterns are described as singlet (s), doublet (d), triplet (t), quartet (q), or multiplet (m). Chemical shifts for ^{31}P NMR spectra are quoted in parts per million relative to an external phosphoric acid standard. Many proton and carbon NMR signals were split due to the presence of (phosphate) diastereoisomers in the samples. The mode of ionization for mass spectrometry was fast atom bombardment (FAB) using MNOBA as matrix. Column chromatography refers to flash column chromatography carried out using Merck silica gel 60 (40−60 μm) as stationary phase. HPLC (Shimadzu) was conducted on an SSODS2 reverse-phase column using a water/acetonitrile (Fisher, HPLC grade) eluent; gradient I (standard gradient): 0−80% CH_3CN (0−60 min), 80−0% CH_3CN (60−65 min), flow rate 1 mL/min, UV detection at 265 nm; gradient II: 0−10% CH_3CN (0−5 min), 10−70% CH_3CN (5−55 min), 70−0% CH_3CN (55−60 min), flow rate 1 mL/min, UV detection at 265 nm. Final products showed purities exceeding 99% with undetectable levels (<0.02) of parent nucleosides in every case. Partition coefficients were determined using 1-octanol and water (Fisher, HPLC grade); UV absorptions were determined using a Kontron Uvikon 860 UV spectrometer.

General Procedure. Aryl (methoxyalaninyl)phosphorochloridate (5 mmol) was added to a stirred solution of d4T (1.7 mmol) and *N*-methylimidazole (5 mmol) in tetrahydrofuran (THF) (20 mL) at ambient temperature. After 16 h, the solvent was removed under reduced pressure. The residual gum was dissolved in chloroform (50 mL) and washed with 1 M HCl (50 mL), sodium bicarbonate solution (50 mL), and water (50 mL). The organic phase was dried ($MgSO_4$) and the solvent removed under reduced pressure. The residue was purified by column chromatography on silica with elution by dichloromethane−methanol (97:3). Pooling of appropriate fractions followed by removal of solvent under reduced pressure gave the product as a brittle white foam.

2′,3′-Didehydro-2′,3′-dideoxythymidine 5′-(4-nitrophenyl (methoxyalaninyl)phosphate) (8a): yield 53%; δ_P 4.72, 4.06; δ_H 1.40 (3H, m, Ala-Me), 1.88 (3H, d, 5-Me), 3.77 (3H, s, OMe), 4.12 (2H, m, Ala-NH, Ala-CH), 4.40 (2H, m, H-5′), 5.10 (1H, m, H-4′), 5.96 (1H, m, H-3′), 6.42 (1H, m, H-2′), 7.07 (1H, m, H-1′), 7.28 (1H, m, H-6), 7.61 (2H, m, ortho-Ph), 8.09 (2H, m, meta-Ph), 9.10 (1H, d, NH); δ_C 12.78, 12.87 (5-Me), 21.33, 21.40 (d, *J* = 5.2, Ala-Me), 50.55, 50.62 (Ala-CH), 53.24 (OMe), 67.36, 68.08 (d, *J* = 4.5, *J* = 6.0, C-5′), 84.81, 84.93 (C-4′), 90.11, 90.41 (C-1′), 113.52, 113.70 (C-5), 116.10, 116.17 (d, *J* = 5.7, Ph), 120.51, 120.62 (Ph), 127.19, 127.26 (C-2′), 133.22, 133.57 (C-3′), 135.92, 136.11 (C-6), 137.78, 137.97 (Ph), 151.16 (C-2), 153.04, 153.19 (Ph), 165.95, 165.99 (C-4), 175.99, 176.17 (d, *J* = 7.5, *J* = 6.8, Ala-CO); MS *m/e* FAB 511.1224 (MH^+, $C_{20}H_{24}N_4O_{10}P$ requires 511.1230); HPLC t_R 35.56, 36.13 min, t_R 39.72, 40.27 min (gradient II).

2′,3′-Didehydro-2′,3′-dideoxythymidine 5′-(4-cyanophenyl (methoxyalaninyl)phosphate) (8b): yield 61%; δ_P 4.31, 3.71; δ_H 1.38 (3H, m, Ala-Me), 1.88 (3H, d, 5-Me), 3.73 (3H, s, OMe), 4.02 (2H, m, Ala-NH, Ala-CH), 4.30 (2H, m, H-5′), 5.08 (1H, m, H-4′), 5.96 (1H, m, H-3′), 6.35 (1H, m, H-2′), 7.08 (1H, m, H-1′), 7.33 (3H, m, H-6, ortho Ph), 7.69 (2H, m, meta-Ph), 9.56 (1H, d, NH); δ_C 12.84, 12.88 (5-Me), 21.18, 21.26 (Ala-Me), 50.52, 50.62 (Ala-CH), 53.18 (OMe), 67.32, 68.04 (C-5′), 84.81, 84.93 (C-4′), 90.05, 90.34 (C-1′), 109.34, 109.46 (C-5), 111.64, 111.80 (C-5), 118.51 (CN), 121.49, 121.69 (d, *J* = 5.3, Ph), 128.02, 128.13 (C-2′), 133.23, 133.53 (C-3′), 134.50, 134.54 (Ph), 135.93, 136.16 (C-6), 151.34 (Ph), 154.05 (C-2), 160.64 (Ph), 164.31, 164.36 (C-4), 174.11, 174.33 (d, *J* = 7.5, *J* = 6.8, Ala-CO); MS *m/e* FAB 491.1320 (MH^+, $C_{21}H_{24}N_4O_8P$ requires 491.1331); HPLC t_R 35.02, 35.44 min, t_R 37.39, 37.83 min (gradient II).

2′,3′-Didehydro-2′,3′-dideoxythymidine 5′-(4-(methylcarboxy)phenyl (methoxyalaninyl)phosphate) (8c): yield 91%; δ_P 3.40, 4.04; δ_H 1.28 (3H, m, Ala-Me), 1.75 (3H, d, 5-Me), 3.60 (3H, s, CH_3CO), 3.83 (3H, s, ester-OCH_3), 3.92 (1H, m, Ala-CH), 4.29 (3H, m, H-5′, Ala-NH), 4.97 (1H, m, H-4′), 5.83 (1H, m, H-3′), 6.26 (1H, m, H-2′), 6.94 (1H, m, H-1′), 7.22 (3H, m, H-6, ortho-Ph), 7.92 (2H, m, meta-Ph), 9.86 (1H, d, NH); δ_C 13.51, 13.55 (5-Me), 21.86, 21.92 (d, *J* = 3.6, *J* = 2.9, Ala-Me), 51.22, 51.33 (Ala-CH), 53.39 (ester-OMe), 53.78 (Ala-OMe), 67.82, 67.88 (C-5′), 85.64, 85.76 (d, *J* = 2.5, C-4′), 90.70, 90.97 (C-1′), 112.40, 112.54 (C-5), 121.07, 121.26 (d, *J* = 5.2, *J* = 5.1, Ph), 128.06, 128.12 (C-2′), 128.62, 132.67, 132.73 (Ph), 134.08 (C-3′), 136.75, 136.97 (C-6), 152.25 (Ph), 155.10, 155.18 (C-2), 165.29, 165.30 (C-4), 175.03, 175.16 (d, *J* = 7.1, *J* = 6.3, Ala-CO); MS *m/e* FAB 524.1421 (MH^+, $C_{22}H_{27}N_3O_{10}P$ requires 524.1434); HPLC t_R 37.84, 38.10 min, t_R 41.38, 41.65 min (gradient II).

2′,3′-Didehydro-2′,3′-dideoxythymidine 5′-(4-acetylphenyl (methoxyalaninyl)phosphate) (8d): yield 91%; δ_P 3.56, 4.20; δ_H 1.38 (3H, m, Ala-Me), 1.89 (3H, d, 5-Me), 2.60 (3H, s, CH_3CO), 3.77 (3H, s, OMe), 4.02 (2H, m, Ala-NH, Ala-CH), 4.41 (2H, m, H-5′), 5.10 (1H, m, H-4′), 5.98 (1H, m, H-3′), 6.39 (1H, m, H-2′), 7.08 (1H, m, H-1′), 7.32 (3H, m, H-6, ortho-Ph), 7.98 (2H, m, meta-Ph), 9.11 (1H, d, NH); δ_C 12.79, 12.84 (5-Me), 21.32 (Ala-Me), 27.00 (COMe), 50.49, 50.61 (Ala-CH), 53.14 (OMe), 66.16, 67.78 (C-5′), 84.88, 84.99 (d, *J* = 5.3, C-4′),

90.03, 90.30 (C-1′), 111.65, 111.81 (C-5), 120.43, 120.65 (d, J = 5.3, J = 3.8, Ph), 127.93, 128.08 (C-2′), 130.78, 130.84 (Ph), 133.32, 133.65 (C-3′), 135.96, 136.20 (C-6), 151.23 (Ph), 153.34, 153.50 (C-2), 164.17 (C-4), 174.84 (Ala-CO), 197.15 (CO); MS *m/e* FAB 508.1495 (MH^+, $C_{22}H_{27}N_3O_9P$ requires 508.1485); HPLC t_R 35.42, 35.65 min, t_R 39.54, 40.28 min (gradient II).

2′,3′-Didehydro-2′,3′-dideoxythymidine 5′-(4-chlorophenyl (methoxyalaninyl)phosphate) (8e): yield 72%; δ_P 3.97, 4.59; δ_H 1.39 (3H, m, Ala-Me), 1.91 (3H, d, 5-Me), 3.77 (3H, s, OMe), 3.98 (2H, m, Ala-NH, Ala-CH), 4.36 (2H, m, H-5′), 5.09 (1H, m, H-4′), 5.93 (1H, m, H-3′), 6.40 (1H, m, H-2′), 7.10 (1H, m, H-1′), 7.26 (5H, m, H-6, Ph), 9.11 (1H, d, NH); δ_C 12.79, 12.84 (5-Me), 21.34, 21.41 (d, J = 5.3, Ala-Me), 50.49, 50.61 (Ala-CH), 53.11 (OMe), 67.04, 67.72 (d, J = 0, J = 5.3, C-5′), 84.92, 85.01 (C-4′), 90.01, 90.27 (C-1′), 111.68, 111.83 (C-5), 121.88, 122.05 (d, J = 4.5, Ph), 127.89, 128.03 (C-2′), 130.78, 130.84 (Ph), 133.35, 133.69 (C-3′), 135.95, 136.24 (C-6), 149.19, 149.34 (d, J = 6.8, J = 6.0, Ph), 151.23, 151.25 (C-2), 164.15, 164.20 (C-4), 174.21, 174.40 (d, J = 7.5, J = 6.8, Ala-CO); MS *m/e* FAB 500.1003 (MH^+, $C_{20}H_{24}N_3O_8PCl$ requires 500.0990); HPLC t_R 44.43, 44.79 min, t_R 41.82, 42.32 min (gradient II).

2′,3′-Didehydro-2′,3′-dideoxythymidine 5′-(4-fluorophenyl (methoxyalaninyl)phosphate) (8f): yield 68%; δ_P 4.15, 4.77; δ_H 1.36 (3H, m, Ala-Me), 1.91 (3H, d, 5-Me), 3.79 (3H, s, OMe), 4.05 (2H, m, Ala-NH, Ala-CH), 4.37 (2H, m, H-5′), 5.10 (1H, m, H-4′), 5.99 (1H, m, H-3′), 6.37 (1H, m, H-2′), 7.08 (3H, m, H-1′, ortho-Ph), 7.29 (3H, m, H-6, meta-Ph), 8.88 (1H, d, NH); δ_C 12.78, 12.84 (5-Me), 21.40, 21.47 (d, J = 5.3, Ala-Me), 50.48, 50.62 (Ala-CH), 53.12 (OMe), 66.95, 67.70 (d, J = 4.5, C-5′), 84.92, 85.06 (C-4′), 90.01, 90.27 (C-1′), 111.68, 111.83 (C-5), 116.65, 116.94 (Ph), 121.99, 122.10 (d, J = 4.5, J = 3.7, Ph), 127.83, 128.00 (C-2′), 133.42, 133.76 (C-3′), 135.99, 136.29 (C-6), 146.50 (Ph), 151.15 (C-2), 163.99, 164.06 (C-4), 174.17, 174.22, 174.40 (d, J = 7.5, J = 6.8, Ala-CO); MS *m/e* FAB 484.1285 (MH^+, $C_{20}H_{24}N_3O_8FP$ requires 484.1285); HPLC t_R 39.15 min, t_R 42.05 min (gradient II).

2′,3′-Didehydro-2′,3′-dideoxythymidine 5′-(4-methylphenyl (methoxyalaninyl)phosphate) (8g): yield 65%; δ_P 4.02, 4.67; δ_H 1.36 (3H, m, Ala-Me), 1.87 (3H, d, 5-Me), 2.34 (3H, s, Ph-Me), 3.75 (3H, d, OMe), 3.95 (2H, m, Ala-NH, Ala-CH), 4.36 (2H, m, H-5′), 5.05 (1H, m, H-4′), 5.92 (1H, m, H-3′), 6.36 (1H, m, H-2′), 7.12 (4H, m, H-1′, H-6, meta-Ph), 7.35 (2H, m, ortho-Ph), 9.40 (1H, d, NH); δ_C 12.75, 12.79 (5-Me), 21.13, 21.29 (Ph-Me), 21.20, 21.38 (d, J = 9.8, J = 3.8, Ala-Me), 50.45, 50.58 (Ala-CH), 53.01 (OMe), 66.80, 67.45 (d, J = 4.5, J = 5.3, C-5′), 85.02, 85.05 (d, J = 5.3, C-4′), 89.93, 90.18 (C-1′), 111.75 (C-5), 120.19, 120.37 (d, J = 5.3, J = 4.5, Ph), 127.72, 127.90 (C-2′), 130.57, 130.63 (Ph), 133.48, 133.83 (C-3′), 136.07, 136.38 (C-6), 148.39 (Ph), 151.37 (C-2), 164.37 (C-4), 174.52 (Ala-CO); MS *m/e* FAB 480.1541 (MH^+, $C_{21}H_{27}N_3O_8P$ requires 480.1536); HPLC t_R 40.10, 40.62 min, t_R 43.53, 44.05 min (gradient II).

2′,3′-Didehydro-2′,3′-dideoxythymidine 5′-(4-methoxyphenyl (methoxyalaninyl)phosphate) (8h): yield 64%; δ_P 4.34, 4.97; δ_H 1.39 (3H, m, Ala-Me), 1.89 (3H, d, 5-Me), 3.76 (3H, d, OMe), 3.82 (3H, d, OMe), 3.89 (1H, m, Ala-NH), 4.02 (1H, m, Ala-CH), 4.39 (2H, m, H-5′), 5.07 (1H, m, H-4′), 5.97 (1H, m, H-3′), 6.38 (1H, m, H-2′), 6.87 (2H, m, H-1′, H-6, meta-Ph), 7.12 (3H, m, H-1′, ortho-Ph), 7.37 (1H, m, H-6), 9.23 (1H, d, NH); δ_C 12.75, 12.80 (5-Me), 21.36, 21.43 (d, J = 4.5, Ala-Me), 50.46, 50.61 (Ala-CH), 53.04 (OMe), 56.03 (Ph-OMe), 66.81, 67.48 (d, J = 4.5, J = 5.3, C-5′), 85.02, 85.13 (d, J = 3.0, J = 4.5, C-4′), 89.95, 90.21 (C-1′), 111.69, 111.82 (C-5), 115.03, 115.10 (Ph), 121.40, 121.59 (d, J = 5.3, J = 4.5, Ph), 127.71, 127.90 (C-2′), 133.50, 133.86 (C-3′), 136.10, 136.43 (C-6), 144.09, 144.28 (d, J = 6.0, J = 6.8, Ph), 151.23, 151.28 (C-2), 157.21 (Ph), 164.16, 164.24 (C-4), 174.34, 174.52 (d, J = 7.5, Ala-CO); MS *m/e* FAB 496.1480 (MH^+, $C_{21}H_{27}N_3O_9P$ requires 496.1485); HPLC t_R 36.67, 37.03 min, t_R 40.94 min (gradient II).

2′,3′-Didehydro-2′,3′-dideoxythymidine 5′-(3-acetylphenyl (methoxyalaninyl)(phosphate) (8i): yield 72%; δ_P 4.05, 4.70; δ_H 1.42 (3H, m, Ala-Me), 1.88 (3H, d, 5-Me), 2.67 (3H, s, CH_3CO), 3.79 (3H, s, OMe), 4.01 (2H, m, Ala-NH, Ala-CH), 4.40 (2H, m, H-5′), 5.10 (1H, m, H-4′), 5.99 (1H, m, H-3′), 6.40 (1H, m, H-2′), 7.10 (1H, m, H-1′), 7.32 (1H, m, H-6), 7.50 (2H, m, Ph), 7.80 (2H, m, Ph) 8.83 (1H, d, NH); δ_C 12.70, 12.80 (5-Me), 21.28, 21.35 (d, J = 4.5, Ala-Me), 27.13 (COMe), 50.50, 50.59 (Ala-CH), 53.10 (OMe), 67.08, 67.75 (d, J = 4.5, C-5′), 84.91, 85.02 (d J = 2.3, J = 3.0, C-4′), 90.01, 90.27 (C-1′), 111.65, 111.80 (C-5), 120.18, 120.26 (d, J = 5.2, Ph), 125.27, 125.53 (d, J = 4.5, Ph) 127.92, 128.02 (C-2′), 130.49 (Ph), 133.34, 133.73 (C-3′), 136.02, 136.28 (C-6), 139.12 (Ph), 150.96 (Ph), 151.32, 151.07 (C-2), 164.32 (C-4), 174.32, 174.22 (Ala-CO), 197.35, 197.45 (CO); MS *m/e* FAB 508.1477 (MH^+, $C_{22}H_{27}N_3O_9P$ requires 508.1485); HPLC t_R 35.74, 36.01 min, t_R 39.59, 39.83 min (gradient II).

Determination of Compound Partition Coefficients. The following method was used to determine the Pa values for compounds **8a**−**8i** and **2**. A sample of each compound (2 μM) was dissolved in 1-octanol (10 mL) by rapid magnetic stirring for 10 min. Aqueous buffer (pH 7.0) (Fisons) (5 mL) was added to an aliquot of the octanol solution (5 mL), and the two phases were mixed by rapid magnetic stirring for 5 min at 25 °C. Following separation of the two layers by standing for a further 5 min, aliquots of the octanol layer and of the original octanol solution were removed and their UV absorptions determined at 265 nm. The Pa values were calculated from the ratio of the absorptions of the two octanol aliquots. Each experiment was repeated at least three times.

Pig Liver Esterase Hydrolysis: Standard Assay. The enzymatic reactions were performed in all cases using pig liver esterase (PLE) (E.C. 3.1.1.1; Sigma) (19 units/mg). Typically 9 μmol of the substrate and 10 mg of PLE were dissolved in a mixture of acetone (0.1 mL) and 0.05 M pH 7.6 Trizma buffer (1 mL, made up in D_2O). The mixture was transferred to an NMR tube and maintained at 37 °C. The reactions were monitored periodically by ^{31}P NMR spectroscopy over 48 h. The reactions were followed by measuring the decrease of starting material, and the data were graphically visualized by plotting the percent of starting material against time. From the logarithmic plot, half-lives were determined for comparison of substrate activity.

Chemical Hydrolysis: Standard Assay. Chemical hydrolyses were evaluated under the same conditions used for the PLE hydrolysis: 9 μmol of substrate was dissolved in a mixture of acetone (0.1 mL) and 0.05 M pH 7.6 Trizma buffer (1 mL, made up in D_2O) and maintained at 37 °C. The rate of hydrolysis was determined periodically by monitoring the decrease of starting material by ^{31}P NMR spectroscopy for 6 days. From a logarithmic plot, half-lives were determined for comparison of substrate stability.

Antiviral Activity Determinations. 1. Viruses. HIV-1(strain III_B) was kindly provided by Dr. R. C. Gallo (at that time at the National Institutes of Health, Bethesda, MD). Virus stocks were prepared from the supernatants of HIV-1-infected MT-4 cells. HIV-2 (strain ROD) was a gift from Dr. L. Montagnier (Pasteur Institute, Paris, France), and virus stocks were prepared from the supernatants of HIV-2-infected MT-4 cells. MSV was prepared from tumors induced following intramuscular inoculation of 3-day-old NMRI mice with MSV.

2. Anti-retrovirus Assays. CEM/0 and CEM/TK$^-$ cells were suspended at 250 000 cells/mL of culture medium and infected with HIV-1 or HIV-2 at ∼20 and ∼100 $CCID_{50}$/mL, respectively. Then, 100 μL of infected cell suspension was added to 200-μL microtiter plate wells containing 100 μL of an appropriate dilution of the test compound. After 4 days of incubation at 37 °C, cell cultures were examined for giant cell formation.

C3H/3T3 cells were seeded at 20 000 cells/mL into wells of tissue culture cluster plates (48 wells/plate). Following a 24-h incubation period, cell cultures were infected with 80 focus forming units of MSV during 120 min, whereafter the culture medium was replaced by 1 mL of fresh medium containing appropriate concentrations of the test compound. After 6 days, transformation of the cells was examined microscopically.

The EC_{50} was defined as the compound concentration required to inhibit HIV-induced cytopathicity (giant cell formation) in CEM cell cultures by 50% or as the compound

concentration required to inhibit MSV-induced C3H/3T3 cell transformation by 50%. The CC_{50} was defined as the compound concentration required to inhibit CEM cell proliferation by 50%. The MCC was defined as the compound concentration required to cause a microscopically visible morphological alteration of the C3H/T3T3 cell cultures.

Acknowledgment. We thank the AIDS Directed Programme of the MRC, the Biomedical Research Programme of the European Commision, and the Belgian Geconcerteerde Onderzoeksacties (Project 95/5) for financial support. We would also like to thank Mrs. A. Absillis for excellent technical assistance, Ms. Helen Murphy for secretarial support, and Stephen Moses for the synthesis of **8c**.

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JM9803931