Phosphodiester Amidates of Allenic Nucleoside Analogues: Anti-HIV Activity and Possible Mechanism of Action[1]

Holger Winter,[†] Yosuke Maeda,[‡] Hiroaki Mitsuya,[‡] and Jiri Zemlicka*,[†]

Department of Chemistry, Experimental and Clinical Chemotherapy Program, Barbara Ann Karmanos Cancer Institute, Wayne State University School of Medicine, Detroit, Michigan 48201-1379, and The Experimental Retrovirology Section, Medicine Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892

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Lipophilic phosphodiester amidates **2a**, **2b**, **4a**, **4b**, and **6** derived from anti-HIV agent adenallene **1a**, **3a**, inactive hypoxallene **1b**, **3b**, and 9-(4-hydroxy-2-butyn-1-yl)adenine (**5**) were synthesized and studied as inhibitors of HIV-1 in ATH8 cell system. All phosphodiester amidates were more biologically active than their parent nonphosphorylated compounds. Analogues **2a** and **4a** derived from (±)-adenallene **1a** and (*R*)-enantiomer **3a** are effective anti-HIV agents with EC_{50} approximately 0.88 and 0.21 μM, respectively. Both analogues are 16 and 28 times more effective than parent compounds **1a** and **3a**, respectively. Some anti-HIV activity of hypoxallene derivatives **2b** and **4b** was noted in the range of 0.1−10 μM but the dose−response relationship was poor. Phosphodiester amidate analogue **6** also exhibited anti-HIV activity in the range of 0.1−100 μM, but this effect was accompanied by cytotoxicity. Hydrolytic studies performed at pH 9.8 and with pig liver esterase at pH 7.4 have shown that analogue **2a** gives adenallene 4′-phosphoralaninate (**10a**) as the major product. These results can be interpreted in terms of initial hydrolysis of phosphodiester amidates **2a**, **2b**, **4a**, **4b**, and **6** catalyzed by intracellular esterase(s) to give stable phosphomonoester amidate intermediates with a free carboxyl group. The results obtained with hypoxallene phosphoramidates **2b** and **4b** indicate that the aminosuccinate−fumarate enzyme system responsible for activation of AIDS drug ddIno (didanosine, Videx) can also, albeit less efficiently, activate hypoxallene 4′-phosphate (**9b**) and the respective (*R*)-enantiomer released inside the HIV-infected cells.

Synthesis and biological evaluation of phosphate forms of nucleoside analogues capable of being delivered inside the cells are important facets of current medicinal chemistry.[2−6] Several studies have established that such phosphates can overcome the activating-enzyme (kinase) deficiency[3,6] in cells resistant to antiviral agents such as AZT. Of the reported phosphate delivery forms, phosphodiesters carrying a lipophilic ester moiety and an amino acid as an amidate are particularly promising and have undergone much scrutiny.[3,7] Nevertheless, most of the work in this area has centered on active antiviral agents such as AZT and, very recently[8] d4T, but reports on activation of less active or inactive nucleoside analogues are scant.[9]

In recent years, we have investigated a new class of anti-HIV agents, allenic nucleoside analogues.[10] The antiretroviral effect of two of these analogues, adenallene (**1a**) and cytallene, is similar to the corresponding 2′,3′-dideoxyribonucleosides,[11] 2′,3′-dideoxyadenosine, and 2′,3′-dideoxycytidine, whereas the allene derivatives of hypoxanthine **1b**, guanine, and thymine were inactive. It is therefore of interest to examine lipophilic phosphate derivatives of active and inactive allenic analogues as potential anti-HIV agents. Two questions are of particular importance: (i) Will a lipophilic moiety increase the anti-HIV efficacy of active allenic analogues and (ii) will such a function activate inactive analogues?

For initial experiments, we have selected the phenyl phosphoralaninate **2a** derived from an active anti-HIV agent adenallene **1a** and a similar analogue **2b** obtained from hypoxallene (**1b**) which lacks anti-HIV activity. Because our previous studies[12,13] showed that (*R*)-adenallene (**3a**) and (*R*)-cytallene were responsible for biological effects of the parent racemic compounds, we have included the phosphoralaninates **4a** and **4b** in our investigation. Likewise, analogue **6** derived from 9-(4-hydroxy-2-butyn-1-yl)adenine (**5**), a synthetic precursor[14] of adenallene (**1a**) which is devoid[11] of anti-HIV effect, was also of interest. In this paper, we report on the synthesis and biological evaluation of analogues **2a**, **2b**, **4a**, **4b**, and **6** as well as chemical and enzymatic hydrolysis of **2a**, **2b**.

Synthesis. Analogues **2a**, **2b**, **4a**, **4b**, and **6** were prepared by a method used for synthesis[3] of phenylphosphoralaninates of AZT (Schemes 1−3). Compounds **1a**, **1b**, **3a**, **3b**, and **5** which were described previously[12,14,15] served as starting materials and phosphorochloridate[16] **7** as a phosphorylating agent. The butynol derivative **5** was prepared by alkylation of adenine with 1-bromo-4-(benzoyloxy)-2-butyne[17] as described.[15] The 1,4-butynediol monobenzoate which is a starting material for preparation of the latter alkylating agent was obtained by the known procedure[17] or by reesterification of 2-butyne-1,4-diol dibenzoate with 2-butyne-1,4-diol according to a method described for ethylene glycol monobenzoate.[18] The advantage of this procedure is the utilization of 2-butyne-1,4-diol dibenzoate, a side-product which forms in substantial amounts during preparation of the corresponding monobenzoate.[17] The (*R*)-hypoxallene (**3b**) was obtained by deamination of (*R*)-adenallene (**3a**) with adenosine

* Send correspondence to this author at the Barbara Ann Karmanos Cancer Institute, 110 E. Warren Ave., Detroit, MI 48201-1379. Telephone: (313) 833-0715, ext 312 or 262. Fax: (313) 832-7294. e-mail: zemlicka@kci.wayne.edu.
[†] Wayne State University School of Medicine.
[‡] National Cancer Institute.
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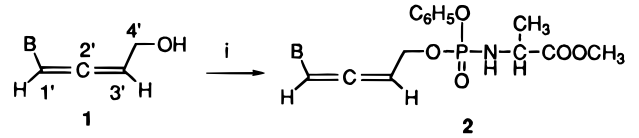
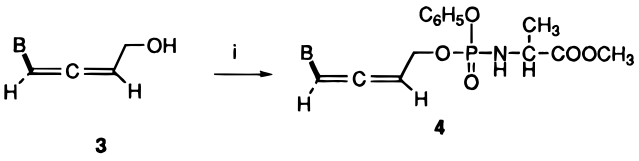
Scheme 1[a]



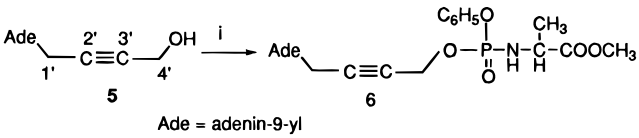
[a] Schemes 1 and 2: Series a: B = adenin-9-yl, series b: B = hypoxanthin-9-yl. Also see legend for Scheme 3.

Scheme 2[a]



[a] See legend for Schemes 1 and 3.

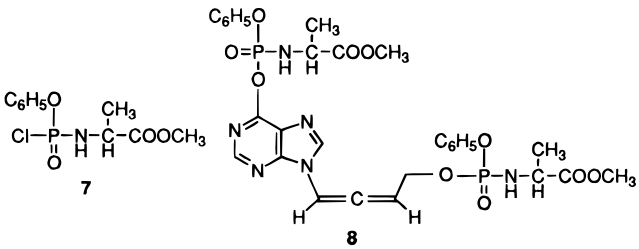
Scheme 3



[a] Schemes 1−3: (i) **7**, *N*-methylimidazole, THF.

deaminase.[12,14] The chromatography was avoided by performing the reaction in water instead of a phosphate buffer, enzyme was denatured by a brief heating, and product **3b** was obtained in 71% yield.

The yields of phosphorylation were dependent on the extent of precipitation of a syrupy material, presumably a complex between phosphorochloridate **7** and *N*-methylimidazole, containing also the starting material as shown by TLC. Thus, allenic phosphates **2a** and **4a** were obtained in 71 and 65% yield, respectively, whereas yields of **2b**, **4b**, and **6** were lower (33−52%). No significant N-phosphorylation was observed in case of adenine derivatives **2a** and **4a**. With hypoxanthine derivatives **1b** and **3b**, formation of an intermediate less polar than **2b** or **4b** was observed. The column chromatography or treatment of the crude reaction product with silica gel and methanol led to disappearance of this compound for which a tentative structure **8** is proposed. It is known that the O^6 of purine nucleosides can be attacked by phosphorylating agents.[19]



Analogues **2a**, **4a** and **2b**, **4b** are hygroscopic and of limited stability in solid form and, therefore, microanalytical data were not obtained. Their frozen solutions in DMSO stored at −70 °C were stable for several months without change in the biological activity. All compounds were obtained as amorphous foams, and they were characterized by ^1H, ^{13}C, ^{31}P NMR, mass, and quantitative UV spectra as well as TLC and HPLC. Phosphodiester amidates **2a**, **2b**, **4a**, **4b**, and **6** contain an L-alanine moiety and an asymmetric phosphorus

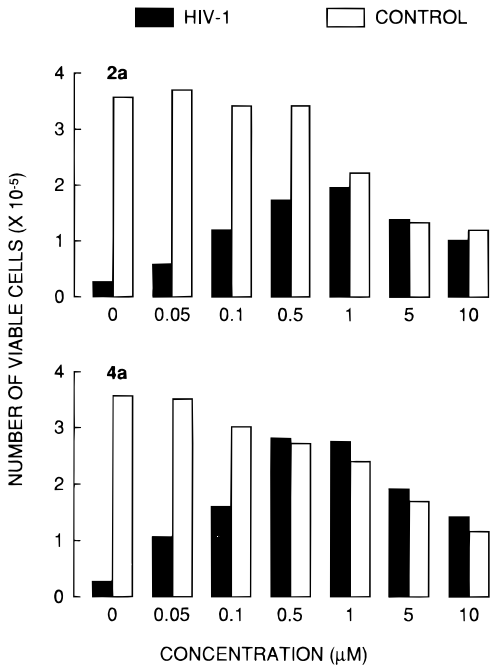


Figure 1. Inhibition of infectivity and cytopathic effect of HIV-1 in ATH8 cells by phosphodiester alaninates **2a** and **4a**. For details see Experimental Section.

atom. Consequently, analogues **2a** and **2b** derived from racemic allenes **1a** and **1b** are the mixtures of four diastereoisomers whereas each of the phosphates **4a**, **4b** derived from (*R*)-allenes **3a**, **3b** and optically inactive butynol **5** contains only two diastereoisomers. This was corroborated by NMR spectra although not all the signals were resolved. The ^{31}P spectra of phosphodiester amidates **2a**, **2b**, **4a**, **4b**, and **6** showed the requisite number of peaks expected for a given diastereoisomeric composition. The HPLC was also indicative of a partial resolution of diastereoisomers. Thus, all phosphodiester amidates examined gave two poorly resolved peaks. A similar behavior was noted in case of the corresponding analogues of AZT[3] which are mixtures of two diastereoisomers.

Biological Activity. Analogues **2a**, **2b**, **4a**, **4b**, and **6** were tested as inhibitors of replication and cytopathic effect of HIV-1 in ATH8 cells.[11,12] Results with phosphoramidates of adenallene **2a** and **4a** are given in Figure 1. Analogue **2a** (EC_{50} 0.88 μM) is 16 times more effective than the parent racemic analogue[12] **1a** (EC_{50} 14 μM). Phosphoramidate **4a** is more active than **2a** (EC_{50} 0.21 μM), and the results then follow the pattern[12] observed in case of adenallene (**1a**) and (*R*)-enantiomer **3a**. The presence of a monophosphate generated from (*S*)-enantiomer of adenallene does not seem to influence the activity profile. A higher activity of **2a** and **4a** was accompanied by an increase of cytotoxicity relative to (±)-adenallene[11] (**1a**) and (*R*)-enantiomer[12] **3a** (IC_{50} 2.1 and 3 μM, respectively). This indicates that an increase of intracellular concentration of the phosphorylated intermediate(s) does not necessarily lead to an improvement of the selectivity index of a given analogue. A toxicity effect related to an intracellular generation of the free 5′-nucleotide of an antiviral agent was reported earlier.[20] Possibly, the higher levels of intracellular phosphorylated intermediates can increase their substrate activity toward mammalian enzymes and, consequently, inhibit the DNA polymerase. It is likely that

all diastereoisomers of the examined phosphodiester amidates are capable of penetrating the cellular membrane by a nonfacilitated transport, but their intrinsic cytotoxicities, if any, may differ.

The data obtained with phosphodiester amidates **2a** and **4a** are also of importance from the viewpoint of mechanism of action of adenallene **1a** and **3a**. It was established that cytallene was phosphorylated by deoxycytidine kinase from leukemic spleen cells[21] although with a decreased efficiency. By contrast, enzyme that phosphorylates adenallene (**1a**) or **3a** remains to be identified[22] although the fact that both analogues inhibit[11] HIV-1 and HIV-2 strongly suggests[23] involvement of phosphate intermediates. Anti-HIV activity of **2a** and **4a** clearly indicates involvement of phosphorylated species in the mechanism of action of adenallene **1a** and **3a**. The results with butyne phosphoramidate **6** derived from an inactive analogue[11] **5** are shown in Figure 2. Although the former analogue was biologically active, no clear separation of antiviral activity and cytotoxicity was observed.

Another inactive analogue, hypoxallene[11] (**1b**) and (*R*)-enantiomer **3b**, became also activated after conversion to phosphodiester amidates **2b** and **4b** (Figure 2). The results indicated in both cases some anti-HIV activity in the range of 0.1−10 μM. However, no clear dose−response pattern was seen with either analogue and cytotoxicity of **2b** was noted at 10 μM and above. Amidate **4b** derived from (*R*)-enantiomer **3b** was significantly less toxic. These results also show that hypoxallene 4′-phosphate (**9b**) (generated from **2b** or **4b** inside the cells) is able to participate in further enzymatic activation by the adenylate succinate−fumarate synthetase/lyase system operating in case of AIDS drug 2′,3′-dideoxyinosine (ddI, didanosine, Videx).[24] The lower potency of **2b** and **4b** relative to **2a** and **4a** reflects probably the less efficient generation of free phosphate **9b** and/or the latter metabolite is not an effective substrate for synthetase/lyase enzyme system necessary to provide adenallene 4′-phosphate (**9a**). Also, the stereochemical (*R* vs *S*) requirements of phosphate **9b** toward synthetase/lyase are not known. They may not be necessarily the same as those for anti-HIV activity. The difference in enantioselectivities between the anti-HIV effect and action of adenosine deaminase[12] toward adenallene **1a** may be seen as a precedent.

Analogues **2a** and **2b** were inactive in a clonogenic assay with murine leukemia L1210 cells (ID_{50} > 300 μM). They were less active than adenallene[25] (**1a**) (ID_{50} 150 μM). It is possible that the metabolism in these cells does not provide for an effective conversion of **2a** and **2b** to the respective phosphorylated intermediates.

Chemical and Enzymatic Hydrolysis of Phosphodiester Amidates 2a and 2b. Although various aspects of biological activity of phosphodiester amino acid amidates of antiviral nucleosides, mostly AZT, were studied earlier[3,7,9,26,27] the mechanism of their intracellular conversion to active phosphorylated species has not been clarified. Hypotheses were advanced that these analogues may be substrates for HIV proteinase[26,27] or an unspecified intracellular enzyme capable of cleaving the amino acid moiety.[26] It was surmised[28] that phosphodiester of AZT resultant from HIV proteinase cleavage may be further digested by

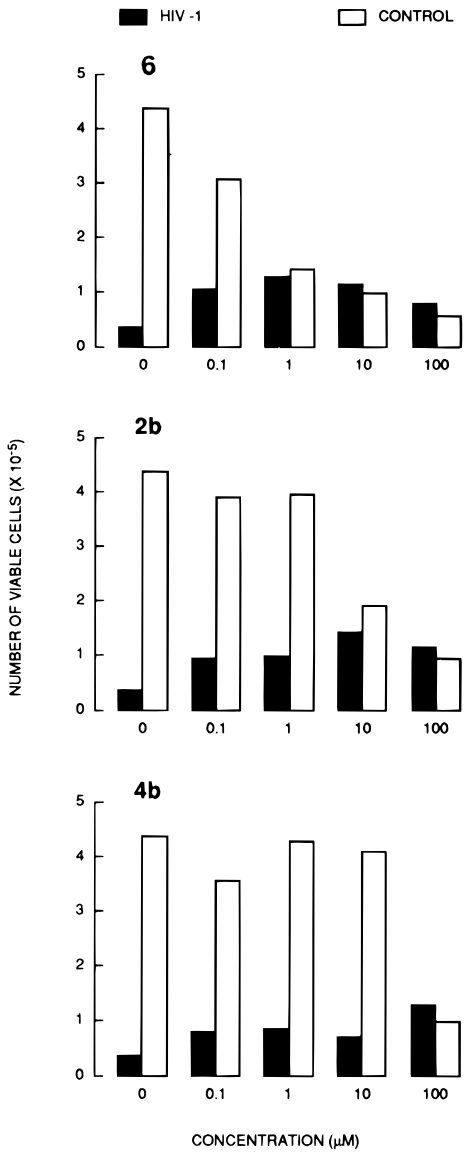


Figure 2. Inhibition of infectivity and cytopathic effect of HIV-1 in ATH8 cells by phosphodiester alaninates **2b**, **4b**, and **6**. For details see Experimental Section.

phosphodiesterase to give AZT and the corresponding 5′-phosphate. Other lipophilic but structurally unrelated (non-amidate) phosphodiester analogues were shown to be converted to free phosphate monoesters by esterase[4] or combined esterase−phosphodiesterase action.[6] It was therefore of interest to undertake model experiments which could shed some light on this important problem.

Phosphodiester amidates **2a** and **2b** were found stable at pH 7.5 for at least 24 h at room temperature. Some hydrolysis (ca. 15% after 24 h) was noted at 37 °C. Hydrolysis of both analogues was studied at pH 9.8 and 1.2. At pH 9.8 hypoxallene phosphoramidate **2b** was hydrolyzed significantly slower ($t_{1/2}$ 308 min) than adenallene derivative **2a** ($t_{1/2}$ 151 min). This may be explained by ionization of the hypoxanthine residue (pK ~ 9.5) which has a retardation influence on the rate of hydrolysis. As expected, both amidates were hydrolyzed at pH 1.2 with almost equal half-lives $t_{1/2}$ 24.4 h (**2a**) and 23.4 h (**2b**), respectively.

Pig liver esterase at pH 7.4 was used as a model[4,8] for hydrolysis of the phosphodiester amidates **2a** and

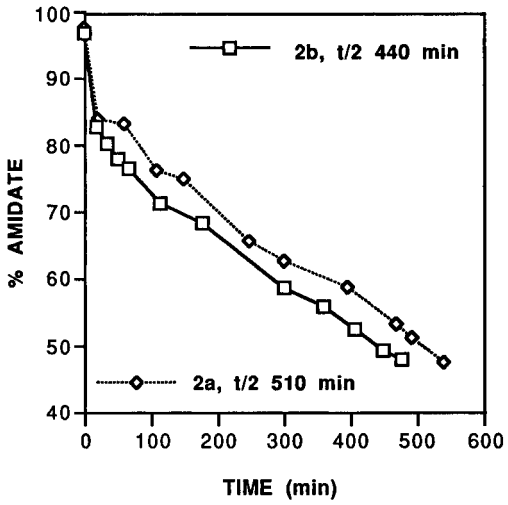
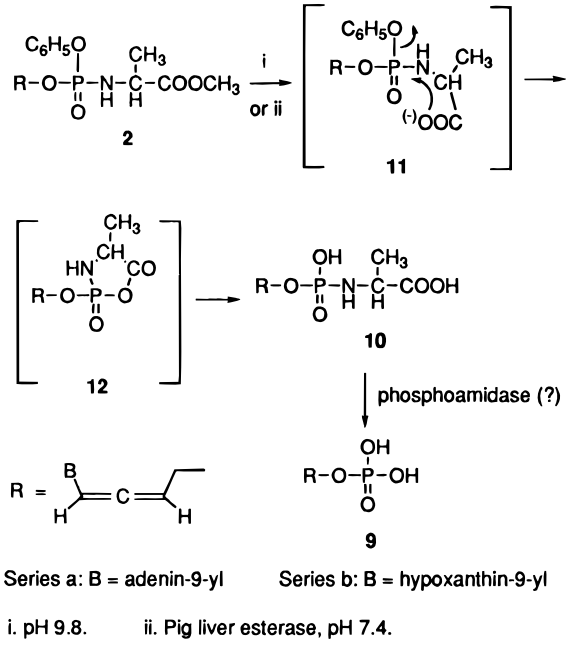


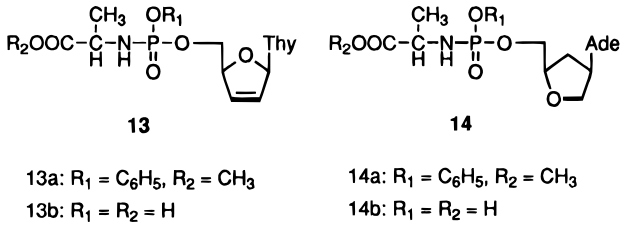
Figure 3. Kinetics of hydrolysis of phosphodiester alaninates **2a** and **2b** catalyzed by pig liver esterase at pH 7.4 and 37 °C. For details see Experimental Section.

Scheme 4



2b by intracellular esterase(s). At room temperature, the cleavage was slow ($t_{1/2} \sim 3$ days). The reaction became faster at 37 °C, but a relatively high concentration of enzyme was required. Both analogues were smoothly hydrolyzed at pH 7.4 by pig liver esterase with $t_{1/2}$ 440 min (**2b**) and 510 min (**2a**), respectively (Figure 3). These model experiments have indicated that hydrolysis of phosphodiester amidates **2a**, **2b**, **4a**, **4b**, and **6** catalyzed by intracellular esterase(s) is plausible. Peptidases capable of hydrolyzing the amino acid (peptide) esters[29] may also be involved.

Analysis of the hydrolysis products of phosphodiester amidate **2a** showed the following. Phosphomonoester amidate **10a** was identified as a major component after hydrolysis of **2a** at pH 9.8 and with pig liver esterase. It is then likely that chemical or enzymatic hydrolysis of the carboxylic ester function of **2a** to give intermediate **11a** is the first step of the process in both cases (Scheme 4). A nucleophilic attack of the phosphorus atom by the carboxylate function of **11a** leads to an expulsion of phenolate and formation of cyclic anhydride **12a**. Hydrolysis of the latter product then gives phosphoramidate **10a**. A similar mechanism was proposed for hydrolysis of simple phosphodiester amino acid amidates.[30] The 5′-phosphoramidates similar to **10a** derived from nucleosides were a subject of much scrutiny.[31,32] A similar course of chemical and enzymatic hydrolysis was recently observed[8] for phenyl phosphoralaninate of d4T **13a** where phosphoramidate **13b** was identified as a major metabolite.



13a: $R_1 = C_6H_5$, $R_2 = CH_3$
13b: $R_1 = R_2 = H$

14a: $R_1 = C_6H_5$, $R_2 = CH_3$
14b: $R_1 = R_2 = H$

It is possible that phosphoramidate monoesters **10a** and **10b** are intracellular metabolic products of phosphodiester amidates **2a** and **2b**. As already mentioned, product **13b**, similar to phosphoramidate monoester **10a**, was found[8] among the metabolites of phosphoralaninate **13a**. It was suggested that **13b** can contribute to the overall antiviral activity by intracellular release of d4TMP but no details were given. Very recently, it was shown that phosphoralaninate of anti-HIV analogue isoddA **14a** is metabolized in CEM cell extract to a similar phosphomonoester amidate[33] **14b**. A hypothesis was proffered that further metabolic disposition of **14b** includes phosphodiesterase-catalyzed hydrolysis to isoddAMP. However, it is known[34] that the P−N bond of nucleotide phosphomonoester amidates derived from amino acids is resistant to phosphodiesterases. Allenic amidate **10a** was also not hydrolyzed by phosphodiesterase from snake venom. The question of metabolic fate of **10a**, **10b**, **13b**, and **14b** thus remains open. It cannot be excluded that such intermediates can directly interfere with reverse transcriptase. To our knowledge, there is no indication that nucleotide amino acid amidates themselves can participate in a phosphorylation pathway catalyzed by nucleotide kinases to obtain triphosphates necessary for inhibition of HIV reverse transcriptase. Hydrolysis of benzylphosphoramidate in dioxane led to a formation of the corresponding symmetrical pyrophosphate,[35] but it is doubtful whether such a transformation can mimic some process inside the cells. Alternately, an enzyme different from phosphodiesterase may be involved in releasing free phosphate monoesters **9a**, **9b** from phosphomonoester amidates **10a**, **10b**. In this context, it is worthwhile to note that a ribonucleoside phosphoamidase capable of hydrolyzing ribonucleoside 5′-phosphoramidates was isolated from bacterial and mammalian sources.[31] The substrates for this enzyme include phosphoramidates with a free amino group, L- or D-amino acid residue. However, 2′-deoxyribonucleoside 5′-phosphoramidates are either weak substrates or they are inactive. It is then not clear whether structurally more distant phosphoramidates such as **10a** and **10b** can be effectively converted to phosphates **9a** or **9b** with this enzyme. Intermediates such as **10a** and **10b** may also, at least in some cases, represent a potential source of cytotoxicity. Additional studies are needed to clarify all these points.

Experimental Section

General Methods. See ref 13. High-performance liquid chromatography (HPLC) was performed using Synchropak RP-P column (C18, 6.6 μm, 2.1 × 250 mm, SynChrom, Inc., Lafayette, IN) in water−CH_3CN (9:1, 0−10 min; 85:15, 10−15 min and 4:1, 15−30 min, flow-rate 1 mL/min, detection at 260 nm). The following solvents were used for TLC and column chromatography: S_1 (CH_2Cl_2−MeOH, 95:5) and S_2 (CH_2Cl_2−MeOH, 9:1). The NMR spectra were determined in $CDCl_3$ at 300.095 (^{1}H), 74.57 (^{13}C), and 121.57 (^{31}P) unless stated otherwise. Adenosine deaminase (EC 3.5.4.4, Type II, 1.5 units/mg solid) and pig liver esterase (esterase from porcine liver, EC 3.1.1.1, 210 units/mg protein) were obtained from Sigma Chemical Co., St. Louis, MO. Phosphodiesterase from *Crotalus durissus* (EC 3.1.4.1, 1.5 units/mg) was the product of Boehringer Mannheim Biochemicals, Indianapolis, IN.

2-Butyne-1,4-diol Monobenzoate. A mixture of crude 2-butyne-1,4-diol dibenzoate (recovered from benzoylation of 2-butyne-1,4-diol,[17] 604.1 g, 2.05 mol), 2-butyne-1,4-diol (176.5 g, 2.05 mol), and chloroform (1 L) was refluxed for 7 h. After cooling, the dark solution was washed with water (2 × 1 L), the organic phase was dried (Na_2SO_4) and it was evaporated. The crude product was distilled in vacuo and then redistilled to give a yellow oil, bp 160−185 °C/0.08 mmHg, 324.8 g (41.7 %), containing, according to TLC (CH_2Cl_2), a trace of dibenzoate. This product was suitable for a preparation of 1-bromo-4-(benzoyloxy)-2-butyne.

(*R*)-(−)-9-(4-Hydroxy-1,2-butadien-1-yl)hypoxanthine (3b). A mixture of (*R*)-(−)-adenallene[12] (**3a**, 310 mg, 1.51 mmol) and adenosine deaminase (Type II, 50 mg, 90 units) was stirred in water (32 mL) at room temperature for 36 h. The solution was then refluxed for 5 min to denature the enzyme which was then filtered off. The solvent was evaporated, and the solid residue was washed several times with warm CH_2Cl_2−MeOH (4:1, 700 mL total). The combined organic phase was evaporated, and the product was recrystallized from 90% EtOH to give (*R*)-(−)-hypoxallene (**3b**) as colorless crystals (229 mg, 71.4%), mp 228−229 °C after another recrystallization from EtOH. UV (pH 7.0) λ_{max} 224 nm (ϵ 24 300). $[\alpha]^{25}_D$ −178° (MeOH, *c* 0.021), lit.[12] for (*S*)-(+)-enantiomer $[\alpha]^{25}_D$ 175° (MeOH, *c* 0.04). ^{1}H NMR (CD_3SOCD_3) δ 12.41 (s, 1H, NH), 8.12 and 8.07 (2s, 2H, H_8 and H_2), 7.3 (m, 1H, $H_{1'}$), 6.20 (m, 1H, $H_{3'}$), 5.15 (t, 1H, OH), 4.10 (m, 2H, $H_{4'}$). ^{13}C NMR 196.26 ($C_{2'}$), 156.50 (C_6), 147.60 and 146.68 (C_4 and C_2), 138.37 (C_8), 124.78 (C_5), 106.63 ($C_{3'}$), 94.27 ($C_{1'}$), 59.18 ($C_{4'}$). Anal. $C_9H_8N_4O_2$ (C, H, N).

Methyl (*R,S*)-9-(4-Hydroxy-1,2-butadien-1-yl)adenine (*R,S*)-4′-Phenylphosphoryl-/P→N/-(*S*)-alaninate (2a). (*R,S*)-Adenallene[14] (**1a**) (200 mg, 0.99 mmol) was added to a solution of methyl chlorophenylphosphoryl-/P→N/-(*L*)-alaninate[16] (**7**, 600 mg, 2.15 mmol) in dry THF (12 mL). After addition of *N*-methylimidazole (0.34 mL, 4.15 mmol), the reaction mixture was stirred at room temperature for 3 h. The solvent was evaporated, and the residue was dissolved in CH_2Cl_2 (50 mL). The solution was washed with water (3 × 15 mL), and it was dried (Na_2SO_4) and evaporated. The crude product was purified by chromatography on a silica gel column using solvent system S_1 to yield a colorless foam **2a** (312 mg, 70.8%) as a mixture of four diastereoisomers. R_f (S_2) 0.5; HPLC, retention time: 23.26 and 22.46 min, purity 98.5%. UV (EtOH) λ_{max} 261 nm (ϵ 14 000), 211 (ϵ 27 400). ^{1}H NMR δ 8.343, 8.341, 8.336 and 8.00, 7.99, 7.98 (6s, 2H, H_8 and H_2), 7.4−7.0 (m, 6H, phenyl and $H_{1'}$), 6.21−6.15 (m, 3H, $H_{3'}$ and NH_2), 4.75 (m, 2H, $H_{4'}$), 4.40 (q), 4.25 (q), and 4.05 (m, 2H, CHNH of Ala), 3.71, 3.69, 3.67 and 3.66 (4s, 3H, OCH_3), 1.37 (m, 3H, Ala-CH_3). ^{31}P NMR 2.84, 2.89, 3.00 and 3.03. ^{13}C NMR 197.57 ($C_{2'}$), 101.54 ($C_{3'}$), 95.9, 94.95 ($C_{1'}$), 63.58, 63.29 ($C_{4'}$); alanine: 173.98 (CO, ester), 52.51 (OCH_3), 50.25, 50.16 (CH), 20.90 (CH_3); phenyl: 150.48 (C-ipso), 129.64, 129.62, 129.51 (C-para), 125.00 (C-ortho), 120.20, 120.17 (C-meta); adenine: 155.53 (C_6), 153.28 (C_2), 148.83 (C_4), 138.56, 138.47 (C_8), 119.60 (C_5). FAB-MS 445 (M + H, 6.6), 260 (19.6), 200 (75.7), 136 (adenine + H, 100.0).

Methyl (*R*)-9-(4-Hydroxy-1,2-butadien-1-yl)adenine (*R,S*)-4′-Phenylphosphoryl-/P→N/-(*S*)-alaninate (4a). The reaction with (*R*)-(−)-adenallene[12] (**3a**) was performed as in the case of racemic compound **1a** on 0.5 mmol scale using 4.26 mmol of *N*-methylimidazole. Yield 156 mg (65.1%) of **4a** as a mixture of two diastereoisomers. R_f (S_2) 0.5; HPLC, retention time: 24.62 and 23.62 min, purity 99.5%. UV (EtOH) λ_{max} 261 nm (ϵ 14 100 nm), 218 (ϵ 24 800). ^{1}H NMR δ 8.35, 8.34 and 8.02 (3s, 2H, H_8 and H_2), 7.4−7.0 (m, 6H, phenyl and $H_{1'}$), 6.19 (m, 3H, $H_{3'}$ and NH_2); 4.78 (m, 2H, $H_{4'}$), 4.55 (t), 4.44 (t) and 4.05 (m, 2H, CHNH of Ala), 3.71, 3.66 (2s, 3H, OCH_3), 1.37 (apparent t, 3H, Ala-CH_3). ^{31}P NMR 3.28 and 3.20. ^{13}C NMR 197.53, 197.23 ($C_{2'}$), 101.57, 101.48, 101.38 ($C_{3'}$), 95.09, 94.96 ($C_{1'}$), 63.51, 63.28, 63.24 ($C_{4'}$); alanine: 174.17, 174.09 (CO, ester), 52.47 (OCH_3), 50.23, 50.13 (CH); 20.79, 20.73 (CH_3); phenyl: 150.42 (C-ipso), 129.60, 129.48 (C-para), 124.94 (C-ortho); 120.15, 119.48 (C-meta); adenine: 155.79 (C_6), 153.30 (C_2), 148.70 (C_4), 138.47, 138.35 (C_8), 119.48 (C_5). FAB MS 445 (M + H, 49.3), 186 (100.0), 136 (adenine + H, 44.9).

Methyl (*R,S*)-9-(4-Hydroxy-1,2-butadien-1-yl)hypoxanthine (*R,S*)-4′-Phenylphosphoryl-/P→N/-(*S*)-alaninate (2b). The procedure described for compound **2a** was followed on 0.83 mmol scale of (*R,S*)-hypoxallene[14] (**1b**). The crude product was purified by chromatography on a silica gel column using solvent system S_2 to afford colorless foam **2b** (192 mg, 51.9%) as a mixture of four diastereoisomers. R_f (S_2) 0.45; HPLC, retention time: 19.17 and 18.48 min, purity 98.0%. UV (EtOH) λ_{max} 222 nm (ϵ 24 700). ^{1}H NMR δ 8.23, 7.96 and 7.94 (3s, 2H, H_8 and H_2), 7.4−7.0 (m, 6H, phenyl and $H_{1'}$), 6.19 (qt, 1H, $H_{3'}$), 4.77 (m, 2H, $H_{4'}$), 4.33 (q), 4.16 (t) and 4.02 (m, 2H, CHNH of Ala), 3.68 and 3.64 (2s, 3H, OCH_3), 1.36 (apparent t, 3H, Ala-CH_3). ^{13}P NMR 2.94, 3.01, 3.07 and 3.09. ^{13}C NMR 197.69, 197.48 ($C_{2'}$), 101.78, 101.66 ($C_{3'}$), 95.04 ($C_{1'}$), 63.30 ($C_{4'}$); alanine: 173.93 (CO, ester), 52.53, 52.51 (OCH_3), 50.27, 50.18 (CH), 20.78 (CH_3); phenyl: 150.50 (C-ipso), 129.63, 129.58 (C-para), 125.02 (C-ortho), 120.21, 120.17 (C-meta); hypoxanthine: 158.54 (C_6), 147.79 (C_2), 145.93 (C_4), 137.92 (C_8), 124.70 (C_5). FAB MS 446 (M + H, 28.4), 260 (55.8), 200 (86.0), 137 (hypoxanthine + H, 43.7).

Methyl (*R*)-9-(4-Hydroxy-1,2-butadien-1-yl)hypoxanthine (*R,S*)-4′-Phenylphosphoryl-/P→N/-(*S*)-alaninate (4b). *N*-Methylimidazole (0.35 mL, 4.25 mmol) was added to a solution of phosphorochloridate **7** (600 mg, 1.98 mmol) in dry THF (6 mL). After 5 min, (R)-(−)-hypoxallene (**3b**) (100 mg, 0.49 mmol) was added in portions, and the reaction mixture was stirred for 24 h at room temperature. The solvent was removed, and the residue was dissolved in CH_2Cl_2 (50 mL). The solution was washed with 5% aqueous HCl (2 × 15 mL) and saturated $NaHCO_3$ (2 × 15 mL), dried (Na_2SO_4), and evaporated. The residue was dissolved in MeOH (10 mL), and silica gel (1 g) was added. The suspension was stirred for 5 h at room temperature, it was evaporated to dryness, and the residue was put on the top of a silica gel column which was eluted with solvent S_2 to give a colorless foam **4b** (71 mg, 32.5%) as a mixture of two diastereoisomers. R_f (S_2) 0.45; HPLC, retention time: 17.76 and 20.21 min, purity 97.2%. UV (EtOH) λ_{max} 213 nm (ϵ 24 600). ^{1}H NMR δ 8.20, 7.97 and 7.96 (3s, 2H, H_8 and H_2), 7.4−7.0 (m, 6H, phenyl and $H_{1'}$), 6.20 (qt, 1H, $H_{3'}$), 4.79 (m, 2H, $H_{4'}$), 4.12−3.87 (m, 2H, CHNH of Ala), 3.70 and 3.67 (2s, 3H, OCH_3), 1.40 (s, 3H, Ala-CH_3). ^{13}P NMR 3.06 and 3.03. ^{13}C NMR 197.70, 197.46 ($C_{2'}$), 101.78, 101.66 ($C_{3'}$), 95.04, 94.97 ($C_{1'}$), 63.40, 63.23, 63.18 ($C_{4'}$); alanine: 173.89 (CO, ester), 52.52 (OCH_3), 50.16 (CH), 20.79, 20.73 (CH_3); phenyl: 150.49 (C-ipso), 129.58 (C-para), 125.04 (C-ortho), 120.17 (C-meta); hypoxanthine: 158.66 (C_6), 147.82 (C_2), 145.79 (C_4), 137.99 (C_8), 124.83 (C_5). FAB MS 446 (M + H, 27.6), 260 (28.4), 200.0 (100.0), 137 (hypoxanthine + H, 35.1).

Methyl 9-(4-Hydroxy-2-butyn-1-yl)adenine 4′-Phenylphosphoryl-/P→N/-(*S*)-alaninate (6). The procedure described for analogue **4b** was applied for phosphorylation of butynol[15] **5** on a 0.99 mmol scale. The reaction mixture was stirred at room temperature for 3 h. The solvent was evaporated, and the residue was dissolved in CH_2Cl_2 (50 mL). The solution was washed three times with water (3 × 10 mL), dried (Na_2SO_4), and evaporated. The residue was purified by chromatography on silica gel column using solvent S_2 to give two diastereoisomers of **6** as a colorless foam (188 mg, 43.2%). R_f (S_2) 0.4; HPLC, retention time: 22.20 and 22.77 min, purity

99.6%. UV (EtOH) λ_{max} 261 nm (ϵ 14 700), 209 (ϵ 24 700). ^{1}H NMR δ 8.370, 8.365 and 8.00 (3s, 2H, H_8 and H_2), 7.4−7.0 (2m, 5H, phenyl), 5.88 (s, 2H, NH_2), 5.01 (q, 2H, $H_{1'}$), 4.81 (m, 2H, $H_{4'}$), 4.07 (m, 2H, CHNH of Ala), 3.71 and 3.69 (2s, 3H, OCH_3), 1.38 (apparent t, 3H, Ala-CH_3). ^{31}P NMR 2.97 and 3.06. ^{13}C NMR 80.63, 80.52, 80.06, 79.98 ($C_{2'}$ and $C_{3'}$), 54.45, 54.38, 54.32 ($C_{4'}$), 33.11 ($C_{1'}$); alanine: 173.97 (CO, ester), 52.34 (OCH_3), 50.04 (CH), 20.70 (CH_3); phenyl: 150.47, 150.37 (C-ipso), 129.51 (C-para), 124.83 (C-ortho), 120.03, 120.01 (C-meta); adenine: 155.75 (C_6), 153.06 (C_2), 149.28 (C_4), 139.54 (C_8), 119.16 (C_5). FAB MS 445 (M + H, 100.0), 186 (68.3), 136 (adenine + H, 46.3). Anal. $C_{19}H_{21}N_6O_5P \times 0.2\ H_2O$ (C, H, N).

9-(4-Hydroxy-1,2-butadien-1-yl)adenine 4′-Phosphoryl-/P→N/-(*S*)-alaninate (10a). Analogue **2a** (270 mg, 0.60 mmol) was stirred in 0.08 M glycine buffer (pH 9.8, 100 mL) at room temperature for 38 h. The solution was brought to pH 7 with 80% acetic acid, and then it was applied on a DEAE Sephadex A25 column (18 × 2 cm). The column was washed with water (500 mL), and then it was eluted with a linear gradient of water (500 mL) and 0.3 M NH_4HCO_3 (500 mL). The major UV absorbing peak was lyophilized to give the title compound **10a** as an ammonium salt (132 mg, 59%), positive with ninhydrin.[36] Electrophoretic mobility of **10a** at pH 7 was 1.04 of adenosine 5′-phosphate or adenallene 4′-phosphate[37,38] (**9a**). HPLC, water (0−9 min, 0.2 mL/min), and then water−CH_3CN, 9:1 (9−35 min, 1 mL/min), retention time: 5.9 min (**10a**), 7.9 min (**9a**), and 16.6 min (**1a**). UV (pH 7) λ_{max} 260 nm (ϵ 12 000), 218 (ϵ 19 800) corresponds to that of adenallene 4′-phosphate[37] **9a**. ^{1}H NMR[39] (D_2O) δ 8.02 and 8.00 (2 split[40] s, 2H, H_8 and H_2), 7.12 (m, 1H, $H_{1'}$), 6.13 (m, 1H, $H_{3'}$), 4.32 (bs, 2H, $H_{4'}$), 3.39 (m, 1H, CH-Ala), 1.07 (d, 3H, CH_3-Ala). ^{13}P NMR 7.78.

Hydrolysis of **10a** with 0.05 M HCl gave adenallene 4′-phosphate (**9a**), adenallene (**1a**), and alanine as the only products. Product **10a** was also formed during hydrolysis of analogue **2a** at pH 7.4 catalyzed by PLE as shown by HPLC and paper electrophoresis.

Hydrolysis Studies. Aliquots of stock solutions of analogues **2a** and **2b** in water (10^{-3} M) were diluted with appropriate buffer: 0.08 M glycine buffer pH 9.8; aqueous HCl, pH 1.2, and 0.02 M Na_2HPO_4, pH 7.0) to a final concentration of 1.1×10^{-4} M. These solutions were stirred at room temperature. At selected time intervals aliquots (20 μL) were removed and analyzed by HPLC using water−CH_3CN (9:1, 0−4 min) followed by water−CH_3CN (7:3, 4−15 min). In case of acid hydrolysis (pH 1.2), the solution was neutralized by adding saturated aqueous $NaHCO_3$ (20 μL) and then analyzed.

Enzyme Studies. A. Pig Liver Esterase. Analogues **2a** and **2b** were dissolved in 0.02 M Na_2HPO_4 (pH 7.4) at a concentration of 2.2×10^{-3} M. To each solution (1 mL) was added pig liver esterase (200 units), and the mixtures were stirred at 37 °C. At selected time intervals aliquots (20 μL) were analyzed by HPLC as described above using water−CH_3CN (9:1, 0−3 min) and water−CH_3CN (7:3, 3−15 min). The results are shown in Figure 3.

B. Phosphodiesterase. Compound **10a** was incubated with phosphodiesterase from *Crotalus durissus* (55 μg, 0.082 units) in 0.1 M Tris-HCl (pH 8.6, 1 mL) at a concentration of 5.6×10^{-4} M for 72 h at 37 °C, and aliquots were analyzed by HPLC as described above using water (0.2 mL/min) as eluent. No degradation was observed. Adenylyl-/3′-5′/-uridine which was used as a positive control was cleaved within 30 min.

Inhibition of HIV-1 Cytopathic Effect. The assay was performed with CD_4^+ ATH8 cells as described.[11,12,41] The ATH8 cells (2×10^5) were exposed to HIV-1/HIV_{LAI} (500 50% tissue culture infectious dose) for 45 min, and they were cultured in the presence of compounds **2a**, **2b**, **4a**, **4b**, and **6**. Total viable cells were counted on day 7. Percent protective effect of a compound on survival and growth of ATH8 cells exposed to the virus was determined by the following formula: 100 × [(number of viable cells exposed to HIV-1 and cultured in the presence of the compound) − (number of viable cells exposed to HIV-1 and cultured in the absence of the compound)]/[(number of viable cells cultured alone) − (number of viable cells exposed to HIV-1 and cultured in the absence of the compound)]. Percent cytotoxicity of a compound was determined as follows: 100 × [1 − number of total viable cells cultured in the presence of the compound)/(number of total viable cells cultured alone)]. The data are summarized in Figures 1 and 2. Positive controls performed with ddI and adenallene **1a** are not shown.

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