3'-Azido-3',5'-dideoxythymidine-5'-methylphosphonic Acid Diphosphate: Synthesis and HIV-1 Reverse Transcriptase Inhibition

G. A. Freeman,*,† J. L. Rideout,† W. H. Miller,‡ and J. E. Reardon‡

Divisions of Organic Chemistry and Experimental Therapy, Burroughs Wellcome Co., 3030 Cornwallis Road, Research Triangle Park, North Carolina 27709. *Received March 23, 1992*

3'-Azido-3'-deoxythymidine-5'-phosphonate was synthesized by a five-step reaction sequence. The 5'-phosphonate was inactive against HIV-1 in MT4 cells. The absence of activity against HIV-1 was at least partially explained by demonstrating that the K_m value for the 5'-deoxy-5'-methylphosphonic acid diphosphate analog with HIV-1 reverse transcriptase (RT) was 320-fold greater than the K_m value for 3'-azido-3'-deoxythymidine-5'-triphosphate (AZTTP), and the k_{cat} value for the 5'-deoxy-5'-methylphosphonic acid diphosphate analog was one-seventh the value for AZTTP. These differences in kinetic constants were due to a change in the rate-determining step from dissociation of the RT chain-terminated template-primer complex to the catalytic step. Thus, substitution of a methylene group for the 5'-oxygen atom of AZTTP resulted in an 1800-fold reduction in the rate constant for RT-catalyzed phosphodiester bond formation.

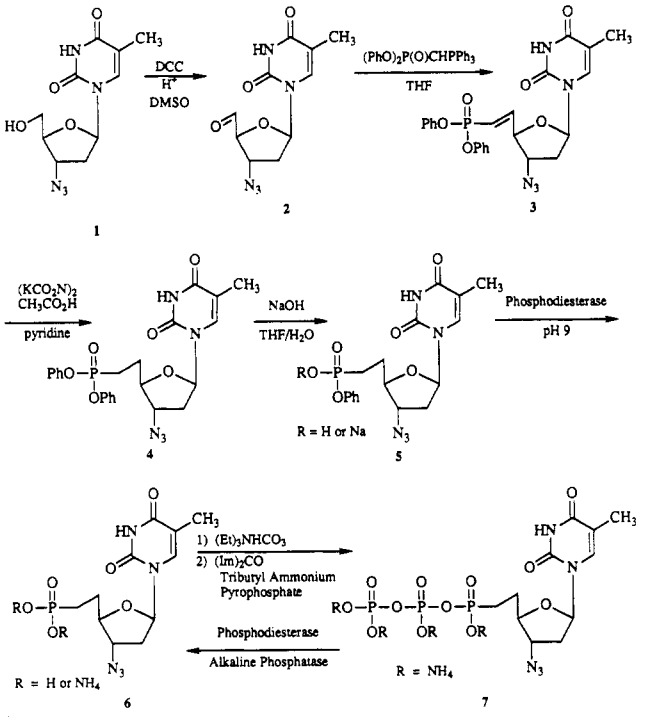
3'-Azido-3'-deoxythymidine (AZT, zidovudine) is one of the most active compounds against the human immunodeficiency virus type 1. The discovery of the antiviral activity of 3'-azido-3'-deoxythymidine[1] has prompted the synthesis and antiviral testing of many nucleoside and nucleotide analogs. 3'-Azido-3'-deoxythymidine is anabolized by thymidine kinase to the monophosphate and by other host cell kinases to the triphosphate analog. The triphosphate metabolite is an alternate substrate for HIV-1 reverse transcriptase (RT) that after incorporation into the growing DNA strand, terminates DNA synthesis. The anabolic activation of 3'-azido-3'-deoxythymidine through the monophosphate to the triphosphate suggested the possibility of using a 5'-phosphonate as a monophosphate mimic that after anabolism to the methylphosphonic acid diphosphate might act as an alternate substrate for HIV-1 RT. The well-characterized inhibition of HIV-1 RT by 3'-azido-3'-deoxythymidine-5'-triphosphate (AZTTP) provides a background for studying the effect of alternate triphosphate analogs on HIV-1 RT.[2,3]

The utility of phosphonate derivatives of adenine as antiviral agents, including activity against HIV, has been demonstrated for (*S*)-9-[(3-hydroxy-2-(phosphonylmethoxy)propyl]adenine (HPMPA) and (*S*)-9-[2-(phosphonylmethoxy)ethyl]adenine (PMEA).[4] An alternate preparation of the 5'-phosphonate analog **6** of 3'-azido-3'-deoxythymidine[5] (see Scheme I), as well as the 5',6'-double bond analog **9** (see Scheme II), has been achieved. However, neither compound was active against HIV-1 in MT4 cells. The methylphosphonic acid diphosphate analog **7** was prepared and its substrate kinetic properties with HIV-1 RT were examined.

Results

Chemistry. An alternate synthesis of the 5'-phosphonate of 3'-azido-3'-deoxythymidine is outlined in Scheme I. Moffatt oxidation of the 5'-hydroxyl of 3'-azido-3'-deoxythymidine (**1**) with 1,3-dicyclohexylcarbodiimide and pyridinium trifluoroacetate in DMSO gave 3'-azido-3'-deoxy-5'-oxothymidine (**2**) in 65% yield.[6] Reaction of 3'-azido-3'-deoxy-5'-oxothymidine with 1 equiv of diphenyl (triphenylphosphoranylidenemethyl)-phosphonate in tetrahydrofuran gave the trans isomer **3**.[7] A small amount of a second component was noted in the NMR and could have been the cis isomer. However, the second component was not isolated or clearly identified. Selective reduction of the 5',6'-double bond of compound

Scheme I



3 (in the presence of the azido group) was achieved with diimide in pyridine, giving compound **4**. Diimide was

(1) Furman, P. A.; Fyfe, J. A.; St. Clair, M. H.; Weinhold, K.; Rideout, J. L.; Freeman, G. A.; Lehrman, S. N.; Bolognesi, D. P.; Broder, S.; Mitsuya, H.; Barry, D. W. Phosphorylation of 3'-Azido-3'-deoxythymidine and Selective Interaction of the 5'-Triphosphate with Human Immunodeficiency Virus Reverse Transcriptase. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 8333-8337.

(2) Reardon, J. E.; Miller, W. H. Human Immunodeficiency Virus Reverse Transcriptase: Substrate and Inhibitor Kinetics with Thymidine 5'-Triphosphate and 3'-Azido-3'-deoxythymidine 5'-Triphosphate. *J. Biol. Chem.* **1990**, *265*, 20302-20307.

(3) Reardon, J. E. Human Immunodeficiency Virus Reverse Transcriptase: Steady-State and Pre-Steady-State Kinetics of Nucleotide Incorporation. *Biochemistry* **1992**, *31*, 4473-4479.

(4) (a) De Clercq, E.; Holy, A.; Rosenburg, I.; Sakuma, T.; Balzarini, J.; Maudgal, P. C. A Novel Selective Broad-Spectrum Anti-DNA Virus Agent. *Nature (London)* **1986**, *323*, 464-467. (b) Pauwels, R.; Balzarini, J.; Schols, D.; Baba, M.; Desmyter, J.; Rosenberg, I.; Holy, A.; De Clercq, E. Phosphonylmethoxyethyl Purine Derivatives, a New Class of Anti-Human Immunodeficiency Virus Agents. *Antimicrob. Agents Chemother.* **1988**, *32*, 1025-1030.

† Division of Organic Chemistry.
‡ Division of Experimental Therapy.

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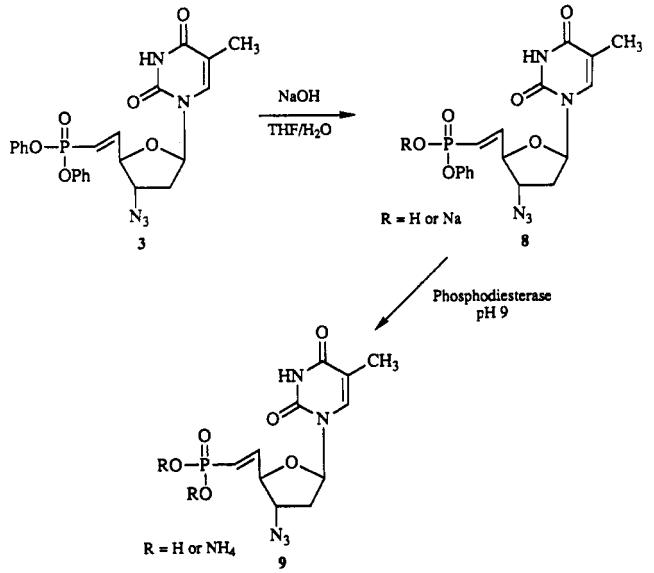
Scheme II



Scheme III. Sequence of Defined Template Primer. The Nomenclature Refers to the Type (RNA vs DNA) and Length of the Template Primer

3'-CCC CUA GGA GAU CUC AGC UGG ACG UCC GUA CGU UCG AAC AGA GG-5'
5'-GGG GAT CCT CTA GAG TCG ACC-3'
r44:d21mer

generated by the reaction of 5 equiv of potassium azodicarboxylate with 7.5 equiv of acetic acid in pyridine.[8] Treatment of the diphenyl phosphonate ester **4** with sodium hydroxide at pH 11–11.5 in tetrahydrofuran/water (3:2 v/v) cleaved one of the phenyl groups and gave compound **5**, the monophenyl ester.[7]

Removal of the second phenyl group was accomplished enzymatically with *Crotalus atrox* phosphodiesterase at pH 9, giving the target compound **6** after neutralization.[7] Synthesis of the 5',6'-double bond analog **9** was readily accomplished by deblocking compound **3** with sodium hydroxide to give **8**, followed by deesterification with phosphodiesterase to give **9** (see Scheme II). NMR indicated both **8** and **9** were pure trans isomers.

Synthesis of the 5'-deoxy-5'-methylphosphonic acid diphosphate analog **7** was accomplished by converting **6** to the triethylammonium salt followed by a reaction with 1,1'-carbonyldiimidazole and tributylammonium pyrophosphate (see Scheme I).[9] Confirmation of the triphosphate structure was obtained by observing the cleavage of **7** with alkaline phosphatase first to a diphosphate analog then to the monophosphonate **6**.

Table I. Comparison of Kinetic Constants Determined by Steady-State and Pre-Steady-State Methods

	nucleotide substrate		
parameter[a]	dTTP[b]	AZTTP[b]	**7**
steady state			
k_{cat} (s^{-1})	0.025 ± 0.002	0.015 ● 0.002	0.002
K_m (μM)	0.035 ± 0.004	0.050 ● 0.004	16 ● 2
k_{cat}/K_m (μM^{-1} s^{-1})	0.71	0.30	0.0001
pre steady state			
k_p (s^{-1})	14 ± 2	5.4 ± 0.4	0.003 ● 0.0001
K_d (μM)	9 ± 1	11 ● 1	17 ± 2
k_p/K_d (μM^{-1} s^{-1})	1.55	0.49	0.0002
K_m^{calc} (μM)	0.017	0.031	17

[a] Values for K_m^{calc} were determined from K_d, k_p, and k_{cat} values according to eq 2a. The ratio k_p/K_d is equal to k_{cat}/K_m based on the kinetic model in eq 1. [b] Data from ref 3.

Cleavage of **7** with phosphodiesterase led directly to the monophosphonate **6**.

Antiviral Testing. Compounds **1**, **5**, **6**, **8**, and **9** were screened for antiviral activity against HIV-1 in MT4 cells in a cell-protection assay.[10] Only 3'-azido-3'-deoxythymidine (**1**), with an average IC_{50} of 60 nM, demonstrated the ability to protect MT4 cells from the cytopathic effect of HIV. None of the other compounds exhibited any cell protection at concentrations as high as 200 μM.

Reverse Transcriptase Kinetics. Steady-state kinetic constants for dTTP and AZTTP incorporation into the homopolymeric template primer, poly(rA)-oligo(dT), and the defined sequence template primer, r44:d21 mer (Scheme III), have been determined previously.[2,3,11] AZTTP competitively inhibits incorporation of dTMP into the r44:d21 mer. The K_i value for AZTTP is equal to its K_m value as a substrate with this template primer.[2] Similarly, dTTP is a competitive inhibitor of AZTMP incorporation into the r44:d21 mer with a K_i value equal to its K_m value as a substrate. Thus, dTTP and AZTTP behave as classical competitive substrates with the r44:d21 mer template primer. Therefore, determination of K_i values with a defined sequence template primer provides a simple method for obtaining K_m values for nonradiolabeled nucleoside triphosphate analogs. Once the K_m value is known, k_{cat} can be determined using a gel electrophoresis primer extention assay. This approach was used previously to determine the substrate kinetic constants for a series of obligate chain-terminating nucleotide analogs.[3] The k_{cat} and K_m values for obligate chain-terminating nucleotide analogs determined by this method are in agreement with the values obtained directly using a radiolabeled obligate chain-terminating nucleotide analog. In the present study this method was used to determine the steady-state kinetic constants for **7** (Table I). Values of k_{cat} and K_m for AZTTP were similar to those of dTTP, indicating that AZTTP was almost as efficient a substrate for the enzyme. In contrast, k_{cat}/K_m for **7** was $^1/_{7100}$ of the value for dTTP.

A simplified kinetic model for reverse transcriptase, where the concentration of template primer is saturating and dissociation of the template primer occurs after every nucleotide incorporation event, is

$$E{\cdot}TP \overset{K_d}{\rightleftharpoons} E{\cdot}TP{\cdot}dNTP \xrightarrow{k_p} E{\cdot}TP_{n+1} \xrightarrow{k_{off}} E + TP_{n+1} \quad (1)$$

(5) (a) Tanaka, H.; Fukui, M.; Haraguchi, K.; Masaki, M.; Miyasaka, T. Cleavage of a Nucleosidic Oxetane with Carbanions: Synthesis of a Highly Promising Candidate for Anti HIV Agents-A Phosphonate Isostere of AZT 5'-Phosphate. *Tetrahedron Lett.* **1989**, *30*, 2567–2570. (b) Barton, D. H. R.; Gero, S. D.; Quiclet-Sire, B.; Samadi, M. Stereoselectivity in Radical Reactions of 2'-Deoxynucleosides. A Synthesis of an Isostere of 3'-Azido-3'-Deoxythymidine-5'-Monophosphate (AZT-5' Monophosphate). *Tetrahedron Lett.* **1989**, *30*, 4969–4972.

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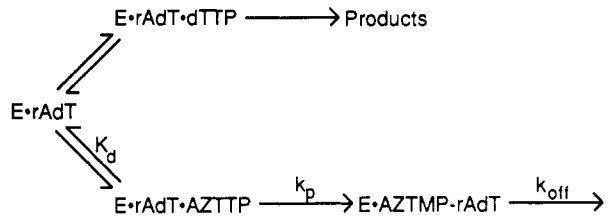
(11) Reardon, J. E.; Cheng, N.; Furfine, E. S. Human Immunodeficiency Virus Reverse Transcriptase: Effect of Primer Length on Template-Primer Binding. *J. Biol. Chem.* **1991**, *266*, 14128–14134.

Scheme IV



where E•TP is the reverse transcriptase•template-primer complex, dNTP is the nucleotide encoded by the template, and TP_{n+1} is the template primer after incorporation of the required dNMP. The rate determining step, k_{cat}, for incorporation of either dTPP or AZTTP into the r44:d21 mer, is dissociation of the enzyme•template-primer complex (i.e. $k_{cat} = k_{off}$).[2,3] Under these conditions, the Michaelis constant for dNTP and k_{cat}/K_m are

$$K_m = \frac{k_{off}K_d}{k_p} \quad (2a)$$

$$k_{cat}/K_m = \frac{k_p}{K_d} \quad (2b)$$

Interestingly, the k_{cat} value for **7** was one-seventh the value for AZTTP. This result suggested that either the rate constant for dissociation of the chain-terminated template primer from the enzyme was reduced or another step in the reaction mechanism was rate determining.

It was unlikely that the 7-fold lower k_{cat} value for **7** compared to AZTTP was due to a slower rate constant for dissociation of the enzyme•template-primer complex. This was confirmed by examining the rate of approach to steady-state inhibition using the homopolymeric template primer, poly(rA)-oligo$(dT)_{20}$. A kinetic model describing AZTTP inhibition of dTMP incorporation into poly(rA)-oligo$(dT)_{20}$ in the presence of saturating template primer is shown in Scheme IV. When the concentration of dTTP is much lower than K_m (4 μM with poly(rA)-oligo$(dT)_{20}$), the rate of approach to the steady state is given by $k_{obs} = k_{on} + k_{off}$, where k_{on} is

$$k_{on} = \frac{k_p[\text{AZTTP}]}{K_d + [\text{AZTTP}]} \quad (3)$$

and in the steady state, % activity = 100 (k_{off}/k_{on}). AZTTP inhibition of dTMP incorporation exhibits a slow approach to the steady state (Figure 1). The value for k_{off} of 0.016 s^{-1} determined by this method is close to the value of 0.01 s^{-1} determined from polymer-trapping studies.[11] In contrast, **7** did not exhibit a slow rate of approach to steady-state inhibition. If the 7-fold lower k_{cat} value for **7** compared to AZTTP was due to a slower rate constant for dissociation of the enzyme•chain-terminated template-primer complex, then the rate of approach to steady-state inhibition would have been slower than that observed for AZTTP. Thus, the high K_m value and low k_{cat} value for **7** was most likely due to a decrease in the rate constant, k_p (eq 1), for incorporation of the phosphonate analog onto the 3′-primer terminus.

The possibility that the high K_m value and low k_{cat} value for **7** was due to a decrease in the rate constant k_p (eq 1) was confirmed by analysis of the pre-steady-state kinetics of nucleotide incorporation. The pre-steady-state kinetics of incorporation of dTTP and AZTTP into the r44:d21 mer are biphasic.[3] A burst of product formation is observed, stoichiometric with the enzyme concentration, followed by a slow steady-state rate. This result is consistent with the kinetic model in eq 1 in which phosphodiester bond formation is rapid relative to the rate of dissociation of the

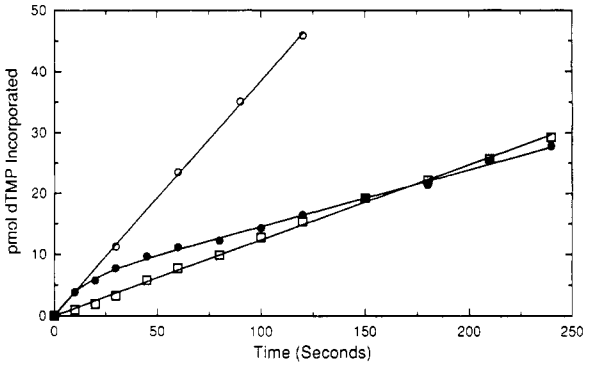


Figure 1. Time course for approach to steady-state inhibition. Reaction mixtures contained 50 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$, 1 μM poly(rA)-oligo$(dT)_{20}$, 1 μM [^{3}H]dTTP, 1 nM RT, and the following: ○, no addition; ●, 100 nM AZTTP; □, 40 μM **7**. Reactions (25 °C) were initiated with enzyme, and aliquots (15 μL) were removed at the indicated times and quenched by addition to 5 μL of 0.5 M EDTA, pH 7.5. The amount of product formed was determined as described previously.[3] The data for AZTTP were fit to a single exponential plus steady-state equation $(y = A(1 - e^{-k_{obs}t}) + k_{ss}t)$ giving $k_{obs} = 0.08 \pm 0.01$ s^{-1}. In the steady state, the enzyme was 75% inhibited at 100 nM AZTTP, thus $k_{off}/k_{on} = 0.25$, giving $k_{off} = 0.016$ s^{-1}.

enzyme•template-primer complex. Values for K_d and k_p can be obtained from an analysis of the nucleotide concentration dependence of the burst-rate constant.[3] The pre-steady-state kinetic constants for **7** are shown in Table I. The pre-steady-state kinetic constants for dTTP and AZTTP were determined previously[3] and are included for comparison. In contrast to the results with dTTP and AZTTP, the pre-steady-state kinetics for **7** were not biphasic. At a saturating concentration of **7**, the rate constant, k_p, for incorporation of **7** into the r44:d21 mer, was 0.003 s^{-1}, in agreement with the k_{cat} value of 0.002 s^{-1} determined by steady-state kinetic analysis. Thus, the rate-determining step for incorporation of **7** was the chemical step and not dissociation of the chain-terminated template primer from the enzyme. The K_m value for **7** was equal to the K_d value for **7** binding to the enzyme as predicted by the kinetic model in eq 1 when k_p is the rate-determining step. On the basis of the kinetic mechanism for reverse transcriptase shown in eq 1, the kinetic constants determined by pre-steady-state kinetic analysis were in good agreement with the steady-state values. Further, the finding that all three nucleotide substrates bound similarly to reverse transcriptase is consistent with their structural similarity. The pre-steady-state data clearly demonstrate that the relative efficiency of steady-state incorporation of nucleotides into DNA is most influenced by the rate of phosphodiester bond formation. Finally, these results demonstrate that substitution of a methylene group for the 5′-oxygen atom of AZTTP reduces the rate constant for the catalytic step by a factor of at least 1800.

Discussion

The 5′-phosphonate analog **6** of 3′-azido-3′-deoxythymidine was synthesized and found to be inactive against HIV in vitro. Effective antiviral activity requires transport of the phosphonate analog across cell membranes, phosphorylation to the diphosphate analog, and efficient utilization of the triphosphate analog by the viral DNA polymerase as an alternate substrate. The possibility of transport of phosphonates, at least into some cells, anabolism to triphosphate analogs, and utilization of the triphosphate analogs by viral DNA polymerases is indicated by the antiviral activity of PMEA and HPMPA.[4]

The poor antiviral activity of 6 was explained, at least in part, by analysis of the substrate kinetics of the methylphosphonic acid diphosphate analog 7 with HIV-1 RT. The results indicate that substitution of a methylene group for the 5'-oxygen atom of AZTTP reduced the rate constant for RT-catalyzed phosphodiester bond formation by a factor of at least 1800. This reduction in catalytic efficiency resulted in a change in rate-determining step in the enzymatic reaction mechanism from dissociation of the enzyme-chain-terminated template-primer complex to the catalytic step. The change in rate-determining step was manifested as a 320-fold increase in K_m and a 7-fold decrease in k_{cat} relative to AZTTP.

Two other 5'-methylene phosphonate analogs of 3'-azido-3'-deoxythymidine have been prepared and tested against HIV-1 and have been evaluated as inhibitors of HIV-1 RT.[12] Balzarini and co-workers prepared the 5'-α,β-methylene and the 5'-β,γ-methylene triphosphate analogs of 3'-azido-3'-deoxythymidine. The 5'-α,β-methylene triphosphate analog was reported to be >400-fold less protective than 3'-azido-3'-deoxythymidine against the cytopathic effect of HIV-1 on ATH8 cells. The 5'-β,γ-methylene analog was found to be 10–40-fold less protective than 3'-azido-3'-deoxythymidine in the same assay. Both compounds were markedly less inhibitory against HIV-1 RT than AZTTP. The 50% inhibitory concentrations for the 5'-α,β-methylene compound and the 5'-β,γ-methylene compound against HIV-1 RT were reported as 4.6 ± 1.1 μM and 7.9 ± 0.2 μM, respectively, compared to 0.023 ± 0.004 μM for AZTTP. These data indicate weak inhibition of HIV-1 RT by other nucleoside phosphonate analogs, as was found for 7.

In conclusion, 3'-azido-3',5'-dideoxythymidine-5'-methylphosphonic acid was synthesized. The compound was inactive against HIV in vitro. This poor antiviral activity was partially explained by the very inefficient use of the diphosphate analog as a substrate by HIV-1 RT. Questions concerning the transport of 5'-deoxy-5'-phosphonates through cellular membranes and cellular anabolism remain unanswered. However, the inability of 7 to serve as an effective substrate for HIV-1 RT restricts the utility of the 5'-deoxy-5'-phosphonate analog of 3'-azido-3'-deoxythymidine as an antiviral agent.

Experimental Section

General. ^{1}H-NMR spectra were recorded with a Varian 300-MHz spectrometer. UV absorption spectra were recorded with a Beckman DU-70 spectrophotometer. TLC was performed with Merck HPTLC 60 F_{254} plates. Flash chromatography was performed on Merck silica gel G60 0.040–0.063-mm mesh. Ion-exchange chromatography was performed with DEAE Sephadex A-25 resin from Aldrich Chemical Co. Tributylammonium pyrophosphate was from Sigma Chemical Co. Alkaline phosphatase was purchased from Boehringer Mannheim. Snake venom phosphodiesterase I was from Pharmacia. PEI-cellulose plates were from Brinkman.

Reverse Transcriptase Kinetics. Preparation of the homopolymeric template primer, poly(rA)-oligo(dT) and the defined sequence r44:d21 mer template primer has been described previously.[2,3,11] Steady-state and pre-steady-state kinetic studies were performed as described previously.[3]

Antiviral Testing. The human T-cell lymphotropic virus type 1 transformed cell line MT4 was infected with HIV-1 (strain IIIB) at 100 times the amount necessary to cause a 50% reduction in cell growth. The infected cells were incubated for 5 days in the presence of various concentrations of each test compound. HIV-mediated cytopathic effect (CPE) was expressed as inhibition of MT4 cell growth. A compound that expressed anti-HIV activity reversed the CPE. The extent of CPE reversal was monitored by a propidium iodide stain for DNA.[10]

(*E*,2*R*,4*S*,5*R*)-1-[4-Azidotetrahydro-5-[2-(di-*O*-phenylphosphono)vinyl]-2-furyl]thymine (3). Compound 2 (3.2 g, 12.1 mmol) was azeotroped with toluene (350 mL) for 5 min. The toluene was removed in vacuo and THF (120 mL) was added. Diphenyl(triphenylphosphoranylidenemethyl)phosphonate (7.6 g, 15.3 mmol, 1.25 equiv) was added, and the reaction was stirred at room temperature overnight. Thin-layer chromatography on silica gel eluted with chloroform/methanol (95:5 v/v) indicated a complete reaction. The solvent was removed in vacuo, and the residue was chromatographed on silica gel (135 g) eluted with ethyl acetate/chloroform (2:3 v/v). The product-containing fractions were combined, and the solvents were removed in vacuo to give 4.82 g (80% yield) of the title compound: ^{1}H-NMR (DMSO) 11.4 (s, 1 H, NH), 8.3 (s, 0.2 H, $CHCl_3$), 7.5–7.1 (m, 11 H, 2-Ph and H6), 6.4 (m, 1 H, H5'), 6.12 (dd, $J_{1',2'H_a}$ = 4.9 Hz, $J_{1',2'H_b}$ = 7.5 Hz, 1 H, H1'), 6.02 (t, $J_{5',6'}$ = 16.3 Hz, 1 H, H6'), 4.4–4.25 (m, 1 H, H3'), 4.2–4.05 (m, 1.8 H, H4' and $CH_3COCH_2CH_3$), 2.5–2.25 (m, 2 H, H2'), 1.99 (s, 1.2 H, $CH_3COCH_2CH_3$), 1.79 (s, 3 H, 5-CH_3), 1.18 (t, 1.2 H, $CH_3COCH_2CH_3$); UV (EtOH) λ_{max} 265 (ϵ = 11200), λ_{min} 233 (ϵ = 2300). Anal. ($C_{23}H_{22}N_5O_6P$) C, H, N.

(2*R*,4*S*,5*R*)-1-[4-Azidotetrahydro-5-[2-(di-*O*-phenylphosphono)ethyl]-2-furyl]thymine (4). Compound 3 (0.5 g, 1 mmol) was dissolved in pyridine (20 mL) and potassium azodicarboxylate (1 g, 5 mmol, 5 equiv) was added. Acetic acid (433 mL, 7.5 mmol) was added last, and the reaction was stirred at room temperature overnight. The reaction was filtered, and the pyridine was removed in vacuo. The residual pyridine was removed by coevaporation with toluene. Residual toluene was removed by coevaporation with nitromethane. The residue was chromatographed on 35 g of silica gel eluted with ethyl acetate/chloroform (3:2 v/v). The product-containing fractions were combined, and the solvents were removed in vacuo to give 0.35 g (70% yield) of the title compound: ^{1}H-NMR (DMSO) 11.3 (s, 1 H, NH), 7.5–7.1 (m, 11 H, 2-Ph and H6), 6.08 (t, $J_{1',2'}$ = 6.9 Hz, 1 H, H1'), 4.45–4.25 (m, 1 H, H3'), 3.9–3.75 (m, 1 H, H4'), 2.8–2.0 (m, H6, H2', H5', and H6'), 1.76 (s, 3 H, 5-CH_3).

(2*R*,4*S*,5*R*)-1-[4-Azidotetrahydro-5-[2-(*O*-phenylphosphono)ethyl]-2-furyl]thymine (5). Compound 4 (0.39 g, 0.78 mmol) was dissolved in THF (20 mL), and water (15 mL) was added. The pH was adjusted between 11 and 11.5 with 1 N sodium hydroxide. The reaction was allowed to stir at room temperature 24–36 h. Thin-layer chromatography, on silica gel developed with ethyl acetate, indicated the disappearance of starting material from the reaction. The reaction was neutralized with 1 N HCl and the solvents were removed in vacuo. The residue was chromatographed on 35 g of silica gel eluted with chloroform/methanol/water (10:3:0.5 v/v/v). The product-containing fractions were combined, and the solvents were removed in vacuo to give 0.275 g (79% yield) of the title compound: ^{1}H-NMR (DMSO) 11.3 (s, 1 H, NH), 7.4–7.1 (m, 6 H, 2-Ph and H6), 6.01 (t, $J_{1',2'}$ = 6.2 Hz, 1 H, H1'), 4.2–4.0 (m, 1 H, H3'); 3.7–3.5 (m, 1 H, H4'), 2.5–2.1 (m, 2 H, H2'), 2.0–1.4 (m, 7 H, H5', H6', and 5-CH_3).

(2*R*,4*S*,5*R*)-1-[4-Azidotetrahydro-5-(2-phosphonoethyl)-2-furyl]thymine (6). Compound 5 (0.15 g, 0.34 mmol) was dissolved in water (15 mL). The pH was adjusted to 9 with 0.1 N sodium hydroxide. Phosphodiesterase (375 units) was added as a powder. The reaction was allowed to stir at room temperature overnight. Thin-layer chromatography on silica gel eluted with chloroform/methanol/water (5:4:1 v/v/v) indicated a complete reaction. The reaction solution was extracted with ethyl acetate to remove phenol. The pH was adjusted to 7. A column of DEAE Sephadex in the bicarbonate form was prepared by washing 120 mL of resin with 400 mL of 50 mM ammonium bicarbonate. The reaction solution was diluted to a volume of 30 mL with water and added to the column. The column was eluted with 400 mL of 50 mM ammonium bicarbonate followed by 200 mL of 100 mM ammonium bicarbonate. The product was collected in three fractions. Thin-layer chromatography on silica gel eluted with chloroform/methanol/water (5:4:1 v/v/v) indicated equal purity

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for all fractions. The fractions were combined, and the solvent was removed in vacuo. Water was added several times and reevaporated to remove excess ammonium bicarbonate. The residue was dissolved in water and freeze-dried, giving 0.102 g, 79% yield, of the title compound: ^{1}H-NMR (DMSO) 7.40 (s, 1 H, H6), 6.05 (dd, $J_{1',2'H_a}$ = 2.6 Hz, $J_{1',2'H_b}$ = 6.6 Hz, 1 H, H1'), 4.3–4.15 (m, 1 H, H3'), 3.8–3.6 (m, 1 H, H4'), 2.45–2.15 (m, 4 H, H2' and H6'), 1.78 (s, 3 H, 5-CH_3), 1.6–1.25 (m, 2 H, H5'). Anal. ($C_{11}H_{16}N_5O_6P$) C, H, N.

(2*R*,4*S*,5*R*)-1-[4-Azidotetrahydro-5-(2-triphosphonoethyl)-2-furyl]thymine (7). Compound 6 (0.071 g, 0.17 mmol) was converted to the triethylammonium salt by evaporation from 35 mL of 100 mM triethylammonium bicarbonate two times. Residual water was removed by coevaporation with acetonitrile. The triethylammonium salt was dissolved in 3.5 mL of 1,3-dimethyl-3,4,5,6-tetrahydropyrimidin-2(1*H*)-one that had been dried over calcium hydride. 1,1'-Carbonyldiimidazole (0.143, 0.88 mmol) was added, and the reaction was stirred at room temperature for 45 min. Methanol (0.06 mL, 1.5 mmol) was added, and stirring was continued for 35 min. Tributylammonium pyrophosphate (0.404 g, 0.87 mmol) was added and stirring continued for an additional 65 min. The reaction was terminated by the addition of 30 mL of cold water. The entire mixture was diluted to 100 mL with water and applied to a 2.5 × 15 cm column of DEAE Sephadex A-25 that had been equilibrated in 50 mM ammonium bicarbonate. The column was eluted with 300 mL of 50 mM ammonium bicarbonate followed by 1100 mL of 100 mM ammonium bicarbonate. A linear gradient of 1 L of ammonium bicarbonate (200–600 mM) was used to elute compound 7. The fractions containing 7 were combined, dried in vacuo, redissolved in water, and then dried and dissolved twice more to give 0.125 mmol (73% yield) as the tetraammonium salt. Ammonia was determined by a previously published method.[13] The purity of 7 was determined to be 99.7% by analytical HPLC (strong anion-exchange column eluted with a gradient of 10 mM to 1 M ammonium phosphate, pH 5.5) monitored at 266 nm: UV pH1 HCl λ_{max} 266 nm, λ_{min} 234 nm, pH 7.0 potassium phosphate λ_{max} 266 nm, λ_{min} 234 nm, pH 13 NaOH λ_{max} 266 nm, λ_{min} 244 nm; ^{1}H NMR (D_2O) δ 7.4 (1 H, s, H6), 6.1 (1 H, t, H1), 4.1 (1 H, m, H3'), 3.8 (1 H, m, H4'), 2.4 (2 H, t, H6'), 1.8 (5 H, m, H2', H5', CH_3); ^{31}P NMR (D_2O, 1 mM EDTA) δ 18.5 (d, α-P), -21.9 (dd, β-P), -6.3 (d, λ-P).

An aliquot of 7 (12 mM) was treated with alkaline phosphatase (30 IU/mL) or snake venom phosphodiesterase I (30 IU/mL), and 33 mM 2-amino-2-methylpropanol (pH 9.5) at room temperature. Samples were withdrawn at various times and chromatography was performed on thin-layer PEI-cellulose (Brinkman) in 0.8 M LiCl/0.8 M formic acid 1:1 (R_f = 0.06 for 7, 0.37 for the diphosphate analog, and 0.76 for 6). Over a 4-h period, sequential conversion of triphosphate to diphosphate to monophosphonate was observed in the alkaline phosphatase-treated samples. In the phosphodiesterase-treated samples, the triphosphate was cleaved directly to the monophosphonate.

(*E*,2*R*,4*S*,5*R*)-1-[4-Azidotetrahydro-5-[2-(*O*-phenylphosphono)vinyl]-2-furyl]thymine (8). Compound 3 (0.25 g, 0.51 mmol) was converted to the title compound in a manner analogous to the conversion of compound 4 to compound 5 (60% yield, 0.140 g): ^{1}H-NMR (DMSO) 7.38 (s, 1 H, H6), 7.2–6.85 (m, 5 H, phenyl), 6.5–6.2 (m, 1 H, H5'), 6.1–5.85 (m, 2 H, H6' and H1'), 4.3–4.15 (m, 1 H, H3'), 4.15–4.05 (m, 1 H, H4'), 2.4–2.2 (m, 2 H, H2'), 1.75 (s, 3 H, 5-CH_3). Anal. ($C_{17}H_{18}N_5O_6P$) C, H, N.

(*E*,2*R*,4*S*,5*R*)-1-[4-Azidotetrahydro-5-[2-phosphonovinyl]-2-furyl]thymine (9). Compound 8 was converted to the title compound in a manner analogous to the conversion of compound 5 to compound 6 (79% yield): ^{1}H-NMR (DMSO) 7.48 (s, 1 H, H6), 6.2–5.9 (m, 3 H, H1', H6', and H5'), 4.5–4.3 (m, 1 H, H3'), 4.2–4.1 (m, 1 H, H4'), 2.4–2.2 (m, 2 H, H2'), 1.8 (s, 3 H, 5-CH_3). Anal. ($C_{11}H_{14}N_5O_6P$) C, H, N.

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S-(5'-Deoxy-5'-adenosyl)-1-ammonio-4-(methylsulfonio)-2-cyclopentene: A Potent, Enzyme-Activated Irreversible Inhibitor of *S*-Adenosylmethionine Decarboxylase[1]

Yongqian Wu and Patrick M. Woster*

Department of Pharmaceutical Sciences, College of Pharmacy and Allied Health Professions, Wayne State University, Detroit, Michigan 48202. *Received April 3, 1992*

The compound *S*-(5'-deoxy-5'-adenosyl)-1-ammonio-4-(methylsulfonio)-2-cyclopentene (AdoMac) was prepared and evaluated as an irreversible inhibitor of *S*-adenosylmethionine decarboxylase (AdoMet-DC). AdoMac was shown to inhibit AdoMet-DC in a time-dependent manner with a K_I of 18.3 μM and a k_{inact} of 0.133 min^{-1}. In addition, AdoMet-DC activity could not be restored following extensive dialysis of the enzyme–inhibitor complex, and the enzyme was protected from irreversible inactivation by the known competitive inhibitor methylglyoxal bis(guanylhydrazone). HPLC analysis of the enzymatic reaction products revealed a time-dependent decrease in the peak coeluting with AdoMac, and a corresponding increase in the peak coeluting with (methylthio)adenosine (MTA), a byproduct of the irreversible binding of AdoMac to the enzyme. Thus, AdoMac appears to function as an enzyme-activated, irreversible inhibitor of AdoMet-DC.

Introduction

The polyamine pathway represents a logical target for chemotherapeutic intervention, since depletion of polyamines results in the disruption of a variety of cellular functions, and ultimately in cell death.[2] Inhibitors of the polyamine pathway, therefore, have traditionally been developed as potential antitumor or antiparasitic agents. Such inhibitors also play a critical role as research tools

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