BIOORGANIC CHEMISTRY **24,** 251–261 (1996)
ARTICLE NO. 0023

Synthesis, Characterization, and Inhibitory Activities of Nucleoside α,β-Imido Triphosphate Analogues on Human Immunodeficiency Virus-1 Reverse Transcriptase

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Received February 14, 1996

Six deoxynucleoside triphosphate (dNTP) analogues were synthesized, having the α,β-P—O—P bond replaced with an imido (P—N—P) functionality. They were all shown to be reasonably potent inhibitors of human immunodeficiency virus-1 reverse transcriptase (HIV-1 RT). This has permitted, for the first time, an estimate of the relative binding affinities of the parent triphosphates (dATP, TTP, dGTP, and dCTP) toward the enzyme's active site, in that they can be compared indirectly by correlation with the behavior of their α,β-imido analogues. Other complicating processes such as consecutive incorporation into the growing DNA chain can be excluded because the imido linkage cannot be cleaved by HIV-1 RT. The 5-iodo analog of deoxyuridine triphosphate (IdUMPNPP, **5**) was the most potent inhibitor, having an IC_{50} value of 7 μM. A general route for the phosphorylation of purine and pyrimidine 5′-imidodiphosphates by pyruvate kinase was developed using phosphoenolpyruvate (PEP) as a phosphoryl group donor. Enzymatic phosphorylation was shown to be a more efficient approach than chemical methods.

INTRODUCTION

Human immunodeficiency virus-1 reverse transcriptase (HIV-1 RT)[4] plays a pivotal role in the life cycle of the human immunodeficiency virus-1. It has been a

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[4] Abbreviation used: HIV-1 RT, human immunodeficiency virus-1 reverse transcriptase; AIDS, acquired immune deficiency syndrome; ddN, 2′,3′-dideoxynucleoside; AZT, azidothymidine; DCC, 2′,3′-dideoxycytidine; DDI, 2′,3′-dideoxyinosine; D4T, 2′,3′-dideoxythymidine; 3TC, 2′,3′-dideoxy-3′-thiacytidine; ddU, 2′,3′-dideoxyuridine; LSIMS, liquid secondary ion mass spectrometry; HRFAB MS, high resolution fast atom bombardment mass spectrometry; PEP, phosphoenolpyruvate; TEAB, triethylamine bicarbonate; CDI, carbonyldiimidazole; PEI, polyethylenimine; TIBO, {tetrahydroimidazo[4,5,1-*jk*][1,4]benzodiazepin-2(1*H*)-thione}; dNTP, deoxynucleosidetriphosphate; dNMPNPP, 2′-deoxynucleoside 5′-(α,β-imido)triphosphate; dAMPNP, 2′-deoxyadenosine 5′-(α,β-imido)diphosphate; dCMPNP, 2′-deoxycytidine 5′-(α,β-imido)diphosphate; dGMPNP, 2′-deoxyguanosine 5′-(α,β-imido)diphosphate; TMPNP, thymidine 5′-(α,β-imido)diphosphate; IdUMPNP, 5-iodo-2′-deoxyuridine 5′-(α,β-imido)diphosphate; AZTMPNP, 3′-azido-3′-deoxythymidine 5′-(α,β-imido)diphosphate; dAMPNPP, 2′-deoxyadenosine 5′-(α,β-imido)triphosphate; dCMPNPP, 2′-deoxycytidine 5′-(α,β-imido)triphosphate; dGMPNPP, 2′-deoxyguanosine 5′-(α,β-imido)triphosphate; TMPNPP, thymidine 5′-(α,β-imido)triphosphate; IdUMPNPP, 5-iodo-2′-deoxyuridine 5′-(α,β-imido)triphosphate; AZTMPNPP, 3′-azido-3′-deoxythymidine 5′-(α,β-imido)triphosphate.

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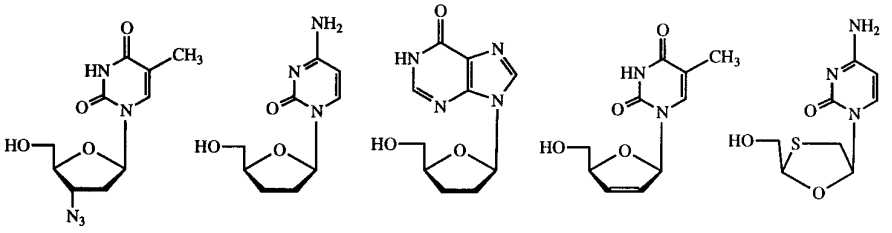


FIG. 1. Nucleoside inhibitors of HIV-1 RT.

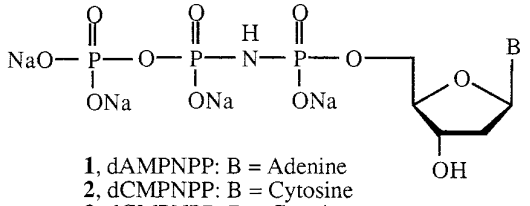
major target for the development of drugs against the acquired immune deficiency syndrome (AIDS) (*1–7*). The vast majority of known HIV-1 RT inhibitors are 2′,3′-dideoxynucleoside (ddN) analogues. The more potent inhibitors include azidothymidine (AZT), 2′,3′-dideoxycytidine (DCC), 2′,3′-dideoxyinosine (DDI), 2′,3′-dideoxythymidine (D4T), and 2′,3′-dideoxy-3′-thiacytidine (3TC) (see Fig. 1). All these substrate analog inhibitors are now in clinical use for the treatment of AIDS.

All of these ddN analogues are prodrugs and therefore require phosphorylation to their triphosphate forms to be catalyzed by cellular host enzymes. Only the triphosphate form is recognized by HIV-1 RT as a substrate so that the corresponding nucleoside monophosphate moieties can be incorporated into the growing DNA chain. Since these ddN analogues lack the 3′-hydroxyl group, their incorporation leads to DNA chain termination (*8*). However, some ddN analogues like ddU (2′,3′-dideoxyuridine) are inefficiently converted to their triphosphate forms due to their low affinity for the cellular kinases (*1*).

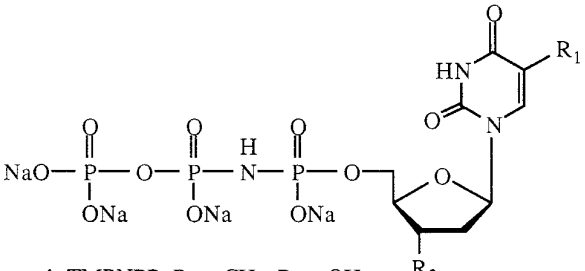
The α,β-imido (P—N—P) linkage of 2′-deoxynucleoside triphosphates closely resembles the α,β-P—O—P bond in bond angles and bond lengths (*9, 10*). Consequently, most enzymes which use dNTP as a substrate can also bind the α,β-imido analogues of dNTP. However, the imido linkage is resistant to nearly all known enzymatic cleavages of α,β-P—O—P bond, with alkaline phosphatase from *Escherichia coli* being the only known exception (*9, 10*). Therefore, these inhibitors hold some promise as potential drugs by binding tightly to the enzyme's active site. They are not prodrugs and consequently do not need to be converted to their inhibitory form by cellular kinases. However, problems associated with their delivery into the target cell will have to be overcome.

This series of inhibitors can also be used as model compounds for kinetic studies of the binding mode of nucleoside triphosphates and can potentially reveal structural information in enzyme–inhibitor complexes in X-ray crystallographic studies. It should be borne in mind that these imido nucleoside triphosphates could find general use in studying *any* enzyme that is involved in the cleavage of the α,β-P—O—P bond.

In our continuing effort to design potential drugs to combat AIDS (*11*), we have synthesized 2′-deoxynucleoside 5′-(α,β-imido)triphosphate analogues **(1–6)** as shown in Chart 1. Among these analogues, compounds **2, 3,** and **5** were synthe-



1, dAMPNPP: B = Adenine
2, dCMPNPP: B = Cytosine
3, dGMPNPP: B = Guanine



4: TMPNPP, $R_1 = CH_3$, $R_2 = OH$
5: IdUMPNPP, $R_1 = I$, $R_2 = OH$
6: AZTMPNPP, $R_1 = CH_3$, $R_2 = N_3$

CHART 1

sized for the first time. In this paper we describe their syntheses, characterization, and inhibitory activities against HIV-1 RT.

EXPERIMENTAL

Thin-layer chromatography using polyethylenimine-cellulose (PEI-cellulose TLC plates, J. T. Baker Chemical Co., Phillisburg, NJ) was used to monitor reactions and check product homogeneity. Fractions from anion exchange column chromatography were monitored at 254 nm with a UV detector (UA6 UV/VIS detector, ISCO Inc., Lincoln, NE). Nuclear magnetic resonance (NMR) spectra were recorded at 121 MHz for ^{31}P (85% H_3PO_4 as external standard) on a General Electric QE-300 spectrometer. LSIMS (liquid secondary ion mass spectrometry) spectra were obtained at the UCSF Mass Spectrometry Facility, A. L. Burlingame, Director. High resolution fast atom bombardment mass spectrometry (HRFAB MS) spectra were obtained on a VG ZAB2-EQ instrument at the Mass Spectrometry Laboratory, University of California at Berkeley. BioRex 5 and AG MP-1 were obtained from Bio-Rad (Hercules, CA). PEI cellulose resin, phosphoenolpyruvate, and pyruvate kinase were all purchased from Sigma. The reverse transcriptase SPA enzyme assay system with HIV-1 reverse transcriptase were obtained from Amersham Life Science Inc. (Arlington Heights, IL) and Worthington Biochemical Corp. (Freehold, NJ), respectively. All other chemicals were purchased from Aldrich Chemical Co. and used directly without further purification unless otherwise noted. Triethyl phosphate was dried over sodium. Thymidine, AZT, and 5-iodo-2′-deoxyuridine were dried in vacuum over P_2O_5 before use. TIBO was a generous gift from Janssen

Research Foundation (Division of Janssen Pharmaceutica N.V. B-2340, Beerse, Belgium).

Syntheses of dAMPNPP **(*1*),** *dCMPNPP* **(*2*),** *and dGMPNPP* **(*3*)**

2′-Deoxynucleoside 5′-(α,β-imido)diphosphaes **(8a–8c** and **10a–10c)** were synthesized based on the procedures described by Tomasz *et al.* (*12*) by directly coupling either the 2′-deoxynucleoside or its derivatives with trichloro[(dichlorophosphoryl)-imino]phosphorane. 2′-Deoxynucleoside 5′-(α,β-imido)triphosphates **1–3** were obtained by phosphoryl transfer from phosphoenolpyruvate (PEP) catalyzed by pyruvate kinase (Scheme 1). Although we previously reported that we were unsuccessful in synthesizing TMPNPP from TMPNP enzymatically (*11*), we have successfully phosphorylated the pyrimidine diphosphate (dCMPNP, **8a**) to form dCMPNPP **(2)** in this study. Trichloro[(dichlorophosphoryl)imino]phosphorane **(11)** was synthesized using the published procedure by Emsly *et al.* (*13*).

dAMPNP **(*8a*),** *dCMPNPP* **(*8b*),** *and dGMPNP* **(*8c*).** In a 10-ml flask, compound **11** (300 mg, 1.12 mmol) was dissolved in triethyl phosphate (5 ml) and cooled to about −15°C. Solid 2′-deoxyguanosine hydrate (160 mg, 0.56 mmol) was added with stirring after 1.5 h at −15°C by which time all of the solid was dissolved. The reaction mixture was poured into anhydrous ether (200 ml). The precipitate was separated by decantation and washed with ether (3 × 50 ml) and dried. The dried precipitate was dissolved in water (3 ml), and 0.5 N NaOH (20 ml) was added to this solution, which was stirred for 1 h. The mixture was neutralized to pH 9–10 with Dowex-50 (H^+ form) at 4°C and filtered. The solution was then loaded onto a DEAE-Sephadex A-25 column (3 × 50 cm^2, bicarbonate form) and separated with a 2-liter linear gradient of 0.1–0.6 M triethylamine/bicarbonate (TEAB), pH 8.5. Compound **8c** (dGMPNP) was eluted at ~0.5 M buffer concentration. Fractions containing the desired product were pooled, and solvents were removed under reduced pressure at a bath temperature no higher than 30°C to give a white solid (250 mg, 51% yield). The same procedure was followed for the syntheses of both dAMPNP (75% yield) and dCMPNPP (67% yield). For dAMPNP, **8a:** R_f = 0.40 (PEI-cellulose plates, 0.4 M TEAB, pH 8.5). LSIMS (M − H) for $C_{10}H_{15}N_6O_8P_2$; calcd, 409.0; found, 409.0. For dCMPNP, **8b:** R_f = 0.37 (PEI-cellulose plates, 0.4 M TEAB, pH 8.5). HRFAB MS (M + H) for $C_9H_{17}N_4O_9P_2$: calcd, 387.0471; found, 387.0461. For dGMPNP, **8c:** R_f = 0.42 (PEI-cellulose plates, 0.4 M TEAB, pH 8.5). LSIMS (M − H) for $C_{10}H_{15}N_6O_9P_2$: calcd, 425.0; found, 425.0.

dAMPNPP **(*1*),** *dCMPNPP* **(*2*),** *and dGMPNPP* **(*3*).** Compounds **1–3** were prepared enzymatically using pyruvate kinase. dGMPNP (100 mg, 20 mmol), for example, was incubated in Hepes buffer (10 ml, 50 mM, pH 10) containing PEP (40 mM), KCl (100 mM), $MgCl_2$ (30 mM), and pyruvate kinase (2 mg/ml) at 25°C for 12 h. Protein was removed by filtration using ultrafiltration membrane YM30 from Amicon (Beverly, MA). The filtrate was loaded onto DEAE-Sephadex A-25 (bicarbonate form) using a 2-liter linear gradient of 0–0.6 M TEAB, pH 8.5. The fractions were determined by PEI-cellulose thin layer chromatography (*14*) using UV detection. Fractions containing dGMPNPP **(3)** were combined, and solvents were removed under reduced pressure. The residue was passed through SP-Sepha-

dex (sodium form) to generate the dGMPNPP tetrasodium salt as a white powder ($dGMPNPPNa_4$, 96 mg, 80% yield). Overall yield from 2′-deoxyguanosine hydrate **(7c)** was 41%. The same procedure was followed for the syntheses and purification of both dAMPNPP **(1)** (overall yield 54%) and dCMPNPP **(2)** (overall yield 36%). For dAMPNPP, **1:** proton decoupled ^{31}P NMR (D_2O) δ 0.56 (d, J = 6.0 Hz, 1 $^{\alpha}P$), −5.65 (d, J = 20.1 Hz, 1 $^{\gamma}P$), −11.05 (dd, J = 6.1 Hz; J = 20.0 Hz, 1 $^{\beta}P$); proton-coupled ^{31}P NMR, only $^{\alpha}P$ changed from doublet to one broad singlet; HRFAB MS (M + H) for $C_{10}H_{14}N_6O_{11}P_3Na_4$ calcd, 578.9525; found, 578.9560. For dCMPNPP, **2:** proton decoupled ^{31}P NMR (D_2O) δ 0.85 (d, J = 6.3 Hz, 1 $^{\alpha}P$), −5.95 (d, J = 20.5 Hz, 1 $^{\gamma}P$), −10.99 (dd, J = 6.4 and 20.5 Hz, 1 $^{\beta}P$); HRFAB MS (M + H) for $C_9H_{14}N_4O_{12}P_3Na_4$ calcd, 554.9412; found, 554.9433. For dGMPNPP, **3:** proton decoupled ^{31}P NMR (D_2O) δ 0.21 (d, J = 6.4 Hz, 1 $^{\alpha}P$), −10.24 (d, J = 20.5 Hz, 1 $^{\gamma}P$), −11.59 (dd, J = 6.3 Hz; J = 20.6 Hz, 1 $^{\beta}P$); HRFAB MS (M + H) for $C_{10}H_{14}N_6O_{12}P_3Na_4$ calcd, 594.9474; found, 594.9451.

Synthesis of AZTMPNPP **(6),** *IdUMPNPP* **(5),** *and TMPNPP* **(4)**

Published procedures by Ma *et al.* (*11*) and Michelson (*15*) were modified to attach the γ-phosphate to the diphosphate analogues by chemical methods using either carbonyldiimidazole (CDI) or diphenylchlorophoshate as activating agents, respectively (Scheme 2).

AZTMPNP **(10c).** AZT (250 mg, 0.94 mmol) was dissolved in dry triethyl phosphate (5 ml) and cooled to −15°C. Compound **11** (378 mg, 1.404 mmol) was dissolved in dry triethyl phosphate (0.5 ml) and added dropwise. The reaction was allowed to stir for 1.5 h at −15°C and subsequently quenched with chilled 0.1 N NaOH (30 ml). The mixture was allowed to react for 25 min at 4°C, and the pH was frequently readjusted to pH 9–10 with 1 N NaOH. The mixture was extracted with ethyl acetate (3 × 25 ml), and the pH was readjusted after each extraction. The aqueous fractions were concentrated to ~5 ml. The crude product was loaded onto an AG MP-1 column (2.5 × 70 cm^2) and eluted with a 2-liter linear gradient of 0 to 0.9 M TEAB, pH 8.5, followed by a linear gradient of 0.5 liter of 0.9 to 1.0 M TEAB, pH 8.5, at a flow rate of 2 ml/min. The product began to elute at 0.9 M TEAB concentration. The fractions containing the product were combined, and tributylamine (5 ml) was added. The solution was taken to dryness and coevaporated with MeOH (6 × 50 ml) until only a clear film remained in the flask. The tributylammonium salt was converted to its sodium salt by dissolving the residue in MeOH (2 ml) and adding 1 M NaI (12 ml) in acetone. A white precipitate formed immediately. The mixture was centrifuged after an incubation at room temperature for 20 min. The supernatant was carefully removed, and dry acetone (8 ml) was added. The suspension was vortexed, followed by centrifugation and removal of the supernatant. This operation was repeated six times. Then the sodium salt was converted to its monotris-*N*-butylammonium salt form. The sodium salt was dissolved in water (0.5 ml) and passed through a SP-Sephadex (H^+ form) column (1 × 10 cm^2). The UV absorbing fractions were combined, and one equivalent of tributylamine was added. The solution was taken to dryness and coevaporated with MeOH (6 × 3 ml) and acetonitrile (5 × 3 ml), followed by dry DMF (2 × 3 ml). The residue was dried