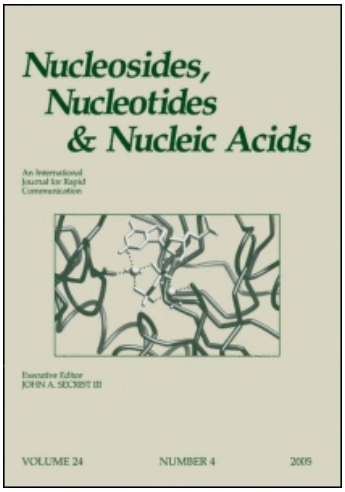

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Synthesis and Biochemical Study of N^2-(*p*-n-Butylphenyl)-2'-deoxyguanosine 5'-(α,β-imido)triphosphate (BuPdGMPNHPP): A Non-Substrate Inhibitor of B Family DNA Polymerases

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SYNTHESIS AND BIOCHEMICAL STUDY OF N^2-(*p*-n-BUTYLPHENYL)-2'-DEOXYGUANOSINE 5'-(α,β-IMIDO)TRIPHOSPHATE (BuPdGMPNHPP): A NON-SUBSTRATE INHIBITOR OF B FAMILY DNA POLYMERASES.

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Abstract: BuPdGMPNHPP was synthesized and assayed as a non-incorporable inhibitor of B family DNA polymerases. The derivative was synthesized by preparation of the imidophosphorane of BuPdG followed by reaction with orthophosphate using the imidazolide method. BuPdGMPNHPP inhibited human DNA polymerase α and T4 DNA polymerase 10 and 3.5-times more potently than BuPdGTP, respectively, and was not a substrate for either enzyme. BuPdGMPNHPP acts as an active site affinity probe that could find use in co-crystallization trials of B family DNA polymerases.

N^2-(*p*-n-Butylphenyl)-2'-deoxyguanosine 5'-triphosphate (BuPdGTP, see structures) is a potent, selective inhibitor of B family DNA polymerases and has been used, in part, to characterize the polymerase (pol) active site of these enzymes. Eukaryotic DNA polymerase α (pol α) from various sources appears to be the most sensitive member.[1] BuPdGTP was found to competitively inhibit immunopurified calf thymus pol α with K_i = 1 nM.[2] Extensive studies of the inhibitory mechanism revealed that pol α inhibition did not involve incorporation of BuPdGMP. A synthetic primer:template designed for dGTP incorporation did not show extension by calf thymus pol α in the presence of 10 μM BuPdGTP when analyzed by polyacrylamide gel electrophoresis (PAGE).[3] BuPdGTP inhibits other members of the B family, such as bacteriophage φ29 pol and herpes simplex virus 1 (HSV1) pol with much lower potencies than found for pol α (competitive K_i = 20 and 52 μM, respectively[4,5]), while bacteriophage T4 pol is intermediate in sensitivity (competitive K_i = 0.82 μM[6]). However, in contrast to pol α, these enzymes incorporate BuPdGTP when dGTP is the required nucleotide. For example, T4 pol efficiently incorporates BuPdGTP into a synthetic primer:template even at concentrations as low as 0.01 μM.[3] These differences in potency and substrate potential are curious considering the sequence conservation[7] and probable similarity in pol site structure between these and pol α-like DNA polymerases.

Because some B family DNA polymerases incorporate BuPdGTP, while others do not, it is difficult to directly compare results from enzyme to enzyme. Inhibition can arise from affinity of the *p*-n-butylphenyl group on the nucleotide with a distinct "receptor" within the pol site, or the modified primer:template resulting from incorporation can act as the actual inhibitory species, as we previously observed in the case of T4 pol.[6] To simplify the study of the kinetics involved in polymerase inhibition, i.e. to prevent incorporation of the "butylphenyl" nucleotides, derivatives of BuPdGTP with stable modifications of the α,β-anhydride oxygen of the triphosphate group have

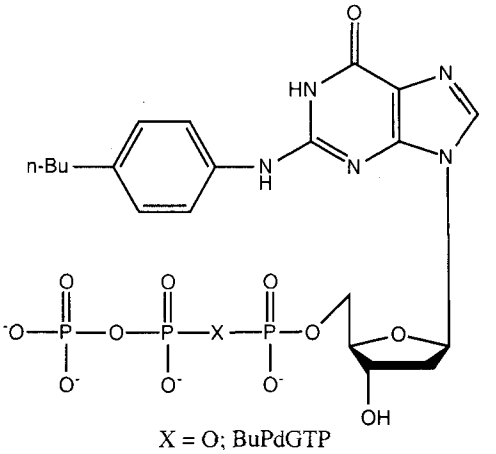
been sought. We first prepared N^2-(*p*-n-butylphenyl)-2'-deoxyguanosine 5'-(α,β-methylene)triphosphate (BuPdGMPCH$_2$PP), and showed that it inhibited both pol α and T4 pol, but was not a substrate for the latter enzyme.[8] In an extension of this work, we now describe the synthesis of the related imido derivative, N^2-(*p*-n-butylphenyl)-2'-deoxyguanosine 5'-(α,β-imido)triphosphate (BuPdGMPNHPP) and its potent activity as a non-substrate inhibitor of human pol α and T4 pol. The recently reported X-ray structure of bacteriophage RB69 DNA polymerase,[9] an enzyme closely related to T4 pol and the first representative of the B family whose structure has been solved, has further stimulated our interest in developing probes of the pol site of this family of DNA polymerases.

Chemistry. By analogy with our successful synthesis of the methylenediphosphate derivative, BuPdGMPCH$_2$P,[8] we attempted to prepare BuPdGMPNHP by a displacement reaction of 5'-mesyl-BuPdG with the tetrabutylammonium salt of imidodiphosphoric acid (PNHP). However, the reaction gave mostly the N^3, 5'-cyclized compound (cBuPdG), and the nucleotide fraction showed a complex composition. Although 5'-sulfonyl-BuPdG derivatives react with pyrophosphate salts prepared at lower pH to give predominantly substituted products, i.e. nucleotides, and to cause minimal cyclization to cBuPdG (unpublished observations), this reaction was not attempted at lower pH in the present case because PNHP is unstable under slightly acidic conditions.[10]

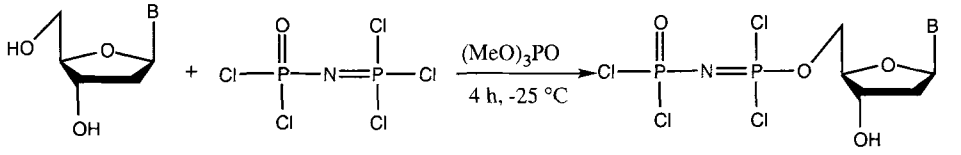
The synthesis of BuPdGMPNHP was accomplished by the method of Tomasz[11] by selective phosphorylation of the 5'-OH of BuPdG with trichloro[(dichlorophosphoryl)imino]-phosphorane (**1**) (Scheme). Compound **1** was prepared from ammonium sulfate and PCl_5 by the method of Emsley et al.[12] and purified by vacuum distillation. The reagent was stored below 0 °C and protected from moisture for a couple of months without deterioration. The reaction of BuPdG with **1** gave an intermediate, the 5'-nucleosyldichloro[dichlorophosphoryl)iminophosphorane (**2**), which was isolated in 81% yield by precipitation from the reaction mixture with water, and characterized by its ^{1}H, ^{31}P and ^{13}C NMR spectra (EXPERIMENTAL PROCEDURES). It was insoluble in water and chloroform, soluble in DMSO, and hydrolyzed quickly in 2 M TEAB buffer to give the final product, BuPdGMPNHP. BuPdGMPNHP was isolated in 47% overall yield by DEAE-Sephadex chromatography at 4 °C and characterized by its ^{1}H, ^{31}P NMR and FAB mass spectra (EXPERIMENTAL PROCEDURES).

Consistent with previous observations of nucleoside imidodiphosphates,[11] BuPdGMPNHP was relatively unstable in aqueous solution as monitored by the ^{31}P NMR spectra (data not shown). The compound hydrolyzed with a half-life ≈ 1 week at r.t., but the products of hydrolysis were not identified. The peak at highest field (δ = 0.75), which did not change upon turning the proton decoupler off, appeared to be orthophosphate. The other two signals (δ = 0.95; 9.96), which are triplets in the proton coupled spectrum (J = 5.6 Hz), might result from BuPdGMP and its amidate. In contrast, a solution of BuPdGMPNHP in 50 mM Tris buffer was stable when stored at -30 °C for three months. The decomposition of BuPdGMPNHP in unbuffered aqueous solution is likely autocatalytic, because its hydrolysis is accompanied by a decrease of pH, and PNHP and its derivatives are most unstable under slightly acidic conditions.[10]

The triphosphate derivative, BuPdGMPNHPP was synthesized from BuPdGMPNHP and orthophosphate by the imidazolide method (Scheme), and was isolated in 29% yield after DEAE-



X = O; BuPdGTP
X = CH_2; BuPdGMPCH$_2$PP
X = NH; BuPdGMPNHPP



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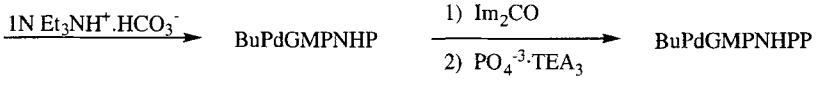

B = N^2-(*p*-n-butylphenyl)guan-9-yl

SCHEME.

Sephadex chromatography and ion-exchange HPLC. The product was identified by its ^1H and ^{31}P NMR and FAB mass spectra (EXPERIMENTAL PROCEDURES). Formation of BuPdGDP along with BuPdGMP and other by-products was observed by thin layer chromatography during the reaction (results not shown). In fact, phosphoroamidates are phosphorylating agents, and the imidazolide of BuPdGMPNHP can be attacked by orthophosphate both at P^α and P^β. The attack on P^α would generate the nucleoside diphosphate.

The possibility of preparing BuPdGMPNHPP by enzymatic phosphorylation of BuPdGMPNHP was studied. The diphosphate analog was not a substrate for baker's yeast nucleoside diphosphate kinase nor for rabbit muscle creatine kinase. In addition, the inverse imidazolide method[13] in which the orthophosphate is activated with carbonyldiimidazole and coupled with the nucleoside diphosphate, was attempted without success.

Inhibition of pol α and T4 pol by BuPdGMPNHPP. The effect of the imido modification of the α,β-phosphoanhydride group of BuPdGTP on inhibition was tested on

recombinant human pol α and recombinant T4 pol. The pol α used in these studies, generously provided by Dr. Teresa S.-F. Wang (Stanford University), was a recombinant single polypeptide from human cells.[14] Although the recombinant pol α was reported to be inhibited by BuPdGTP, no detailed comparisons of inhibitor affinity and substrate potential have been reported. BuPdGMPNHPP was a potent inhibitor of pol α with K_i = 0.037 nM, almost ten-fold more potent than BuPdGTP itself (Table 1). Interestingly, this recombinant enzyme appeared to be more sensitive to BuPdGTP than previous multiprotein forms of pol α.[1] The analogous methylene derivative, BuPdGMPCH$_2$PP, was about four-fold less potent as an inhibitor of pol α than the parent nucleotide, consistent with our previous results for this compound on immunopurified calf thymus pol α.[8] The difference in potency of the derivatives could reflect changes in proton and divalent cation affinity between the parent triphosphate and the substituted derivatives. Blackburn, et al.,[15] for example, found that AMPPNHP had pK values for proton and Ca^{2+} similar to those of ATP, while the values for AMPPCH$_2$P were higher. Both BuPdGMPNHPP and BuPdGMPCH$_2$PP inhibited pol α competitively with dGTP as revealed by variable substrate assays (data not shown). Thus, both derivatives employ the same inhibitory mechanism as BuPdGTP, differing only in relative affinities for the active site of pol α.

Table 1 also summarizes inhibitor potencies for T4 pol, an enzyme that utilizes BuPdGTP as a terminating substrate.[3] BuPdGMPNHPP inhibited T4 pol with a K_i value slightly lower than the apparent K_i of BuPdGTP, whereas BuPdGMPCH$_2$PP inhibited the enzyme with a K_i several-fold higher than the parent inhibitor (Table 1). Because the modified compounds were not substrates for T4 pol (see below), these values reflect true affinities. In fact, BuPdGMPNHPP is about ten-fold more potent than BuPdGMPCH$_2$PP, implying a similar differential in affinities as found for pol α. However, the compounds bind at least one hundred-fold weaker to T4 pol than to pol α. Both the imido and methylene derivatives exhibited competitive inhibition with respect to dGTP as determined by variable substrate analysis (data not shown), suggesting that these compounds function at the pol domain. In contrast, BuPdGTP exhibited mixed inhibition kinetics,[6] likely a result of the modified 3'-BuPdG-primer:template being a contributing inhibitory species of T4 pol.

Substrate potential of BuPdGMPNHPP. The substrate potential of BuPdGMPNHPP was tested using a synthetic primer:template system that required dGTP as the first substrate (see sequence in EXPERIMENTAL PROCEDURES). Under conditions in which almost complete extension of primer:template occurred with 25 μM dGTP, pol α did not incorporate 1 μM BuPdGMPNHPP (Figure 1, lanes 2 and 5, respectively). In similar assays of T4 pol, BuPdGMPNHPP did not extend the primer:template under conditions where both dGTP and BuPdGTP were efficiently incorporated (data not shown). Furthermore, BuPdGMPCH$_2$PP was not a substrate for pol α (Figure 1, lane 4) or for T4 pol (data not shown), results consistent with our earlier observations.[8] A surprising result of the primer extension assays of pol α was the incorporation of BuPdGTP (Figure 1, lane 3). Previous studies showed that immunopurified calf thymus pol α could not utilize BuPdGTP as a substrate,[2,3] and pol α has been regarded as unable to incorporate "butylphenyl" nucleotides.[1] In contrast to the recombinant single polypeptide form of pol α used for this study,[14] all previous forms of pol α have been multiprotein complexes isolated from eukaryotic cells and tissues by conventional purification methods,[2,16,17,18] or by

TABLE 1. Inhibitor sensitivities of recombinant human DNA polymerase α (pol α) and T4 DNA polymerase (T4 pol). Enzyme assays utilized activated calf thymus DNA under "truncated" conditions lacking dGTP as described in EXPERIMENTAL PROCEDURES.[1]

	Apparent K_i	
	pol α	T4 pol
BuPdGMPNHPP:	0.037 ± 0.031 nM	7.1 ± 3.1 nM
BuPdGTP:	0.31 ± 0.03 nM	26 ± 8.4 nM
$BuPdGMPCH_2PP$:	1.19 ± 0.45 nM	127 ± 50 nM

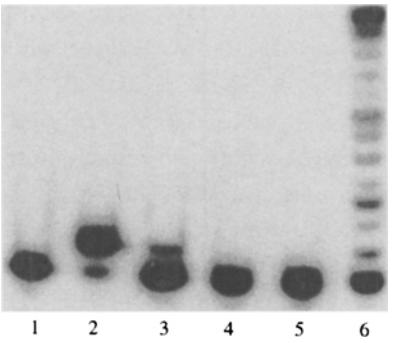

FIG. 1. 12% PAGE analysis of nucleotide incorporation by pol α into 17:29-mer primer:template (p:t) system. Reactions were performed as described in EXPERIMENTAL PROCEDURES. Lane contents: 1: p:t; 2: p:t, pol α, 25 μM dGTP; 3: p:t, pol α, 1 μM BuPdGTP; 4: p:t, pol α, 1 μM $BuPdGMPCH_2PP$; 5: p:t, pol α, 1 μM BuPdGMPNHPP; 6: p:t, pol α, 25 μM dNTPs.

immunopurification.[3,8] The pol α "replisome" immunopurified from calf thymus, for example, exists as a four protein complex, containing both polymerase and primase activities.[19] The presence of additional proteins associated with pol α after immunopurification could possibly prevent BuPdGTP incorporation or reduce its rate below assayed levels. The absence of accessory proteins may allow recombinant pol α to incorporate BuPdGTP as do other B family pols. Preparations of ϕ29 pol, HSV1 pol and T4 pol as single recombinant polypeptides have been found to be capable of incorporating BuPdGTP.[4,5,6] The recombinant pol α used in this study was not different in other regards from conventionally or immunopurified pol α, e.g. in its K_m for dGTP and sensitivity to aphidicolin (data not shown). Further study is needed to determine which, if any, pol α replisome proteins may modulate the incorporation of BuPdGTP.

BuPdGMPNHPP and $BuPdGMPCH_2PP$ were potent, non-substrate inhibitors of pol α and T4 pol. Thus, both compounds are excellent polymerase active site probes of B family DNA polymerases. These compounds could be used in co-crystallization studies with enzyme and primer:template to generate "catalytically relevant" but stable complexes. Indeed, both BuPdGMPNHPP and $BuPdGMPCH_2PP$ are potent inhibitors of RB69 pol (results to be published), the first member of the B family whose structure has been solved.[9]

EXPERIMENTAL PROCEDURES

General. Melting points were determined on a Mel-temp apparatus and are uncorrected. ^1H and ^{31}P NMR spectra were recorded on a Varian Unity 300 instrument Chemical shifts are referenced as follows: ^1H in DMSO-d_6 and $CDCl_3$, internal TMS; in D_2O, internal DSS; ^{31}P, external 1% phosphoric acid. Preparative HPLC was done with a Waters model 600 gradient system and a Lambda-max model 481 detector using a Synchropak AX-100 column (Rainin). Thin layer chromatography (TLC) was done with Merck Kieselgel 60F-254 analytical plates, and column chromatography employed silica gel 230-400 mesh from EM Science. Pyridine was refluxed and distilled over calcium hydride. Hexamethylphosphoric triamide (HMPA) was distilled over calcium hydride under reduced pressure. Acetonitrile (MeCN) was "Sureseal" obtained from Aldrich Chemical Co. All dry solvents were stored in the dark over molecular sieves (4 Å). DEAE-Sephadex was from Pharmacia. Aphidicolin was a gift from the National Cancer Institute. dNTPs were from New England Biolabs. Dithiothreitol (DTT) was from Bio-Rad, calf thymus DNA from Worthington, and buffers and other reagents from Sigma.

N^2-(*p*-n-Butylphenyl)-2'-deoxyguanosine 5'-imidodiphosphate (BuPdGMPNHP). Trichloro[(dichlorophosphoryl)imino]phosphorane ($Cl_3PNPOCl_2$)[12] (108 mg, 0.4 mmol) was dissolved in trimethyl phosphate (100 µL) under N_2, and the solution was cooled to -20 °C. BuPdG (80 mg, 0.2 mmol) was dissolved in trimethyl phosphate (400 µL) under N_2, and added to the stirred solution of $Cl_3PHPOCl_2$ at -25 °C during 1-2 min. After 4 h. at -25 °C to -30 °C the reaction mixture was diluted with ice cold 0.1 M triethylamine (TEA) (40 mL). The precipitate was separated by centrifugation, washed with 0.1 M TEA (8 mL), and lyophilized to give 99.6 mg of the 5'-nucleosyl dichloro[(dichlorophosphoryl)imino]phosphorane. An aliquot of this intermediate (14 mg) was purified by dissolving it in DMF (0.5 mL) and precipitating with CH_2Cl_2 (5 mL). ^1H NMR (DMSO-d_6): 10.86 (bs, 1H, H-1), 9.15 (s, 1H, N^2-H), 7.96 (s, 1H, H-8), 7.51 (m, 2H, Ph-H), 7.18 (m, 2H, Ph-H), 6.26 (t, 1H, H-1', $J_{1'2'} = J_{1'2''} = 6.96$), 4.45 (m, 1H, H-3'), 4.06 (m, 1H, H-4'), 4.01 (m, 2H, H-5',5"), 2.63 (m, 1H, H-2'), 2.55 (t, 2H, Bu-1), 2.34 (m, 1H, H-2"), 1.55 (m, 2H, Bu-2), 1.31 (m, 2H, Bu-3), 0.90 (t, 3H, CH3); ^{31}P (^1H dec., D_2O): 0.86 (d, J_{P1P2} = 11.0 Hz; ^1H dec. off, dt, J_{PH} = 6.3 H; P^1), -0.86 (d, P^2); ^{13}C (DMSO): 158.2, 151.8, 145.2, 132.6, 131.9, 131.7, 124.4 (C-3", 5"), 115.9, 115.2 (C-2", 6"), 81.4 (d, C-4', $^3J_{CP}$ = 8.7 Hz), 79.1 (C-1'), 66.8 (C-3'), 61.0 (d, C-5, $^2J_{CP}$ = 4.0 Hz), 41.5 (C-2'), 30.1 (Bu-1), 29.1 (Bu-2), 17.7 (Bu-3), 9.7 (Bu-4).

The intermediate was hydrolyzed by treating its solution in DMF (2 mL) with 2 M TEA (20 mL) and TEA (1 mL). After stirring for 0.5 h. the undissolved solid was separated by centrifugation, dissolved in DMF, and treated with 2 M TEA and TEA as above, which, after stirring for 0.5 h. caused it to dissolve. The combined solutions were evaporated under high vacuum at 20 °C, and the residue was dissolved in 0.1 M TEA and loaded on a DEAE-Sephadex column (2x20 cm). The products were eluted at 4 °C with a linear gradient of 0.1-1.0 M TEA during 16 h. at a flow rate of 2.67 mL/min. Fractions 70-84 (15 mg each) were co-evaporated with n-BuOH at 20 °C, and the residue was lyophilized to give 71.6 mg (47%) of N^2-(*p*-n-butylphenyl)-2'-deoxyguanosine 5'-(imido)diphosphate, BuPdGMPNHP, as the bis(TEA) salt. An aqueous solution of this compound was passed through a Chelex 100 column (0.5x5 cm) in the Na^+ form, and the product was eluted with water (2 mL). Lyophilization of the eluent gave

BuPdGMPNHP as the disodium salt. ^{1}H NMR (D_2O): 8.44 (s, 1H, 8-H), 7.30, 7.16 (d, 2H ea, Ph), 6.23 (t, 1H, H-1'; $J_{1'2'} = J_{1'2''} = 6.5$ Hz), 4.52 (m, 1H, 3'-H), 4.11 (m, 1H, 4'-H), 3.93 (m, 2H, 5', 5"-H), 2.67 (m, 1H, 2'-H), 2.51 (t, 2H, Bu-1), 2.43 (m, 1H, 2"-H), 1.48 (m, 2H, Bu-2), 1.21 (m, 2H, Bu-3), 0.79 (t, 3H, Bu-4). ^{31}P NMR (D_2O): 0.56 (m, P^1, ^{1}H dec. on, d, J_{P1P2} = 5.4 Hz), -0.20 (d, P^2; J_{P1P2} = 5.4 Hz). FAB MS (of TEA salt): $[M+H]^+$ = 559, $[M+TEA+H]^+$ = 660, $[M+2TEA+H]^+$ = 761, $[M+3TEA+H]^+$ = 862, $[2M+H]^+$ = 1117.

N^2-(*p*-n-butylphenyl)-2'-deoxyguanosine 5'-(P^1,P^2-imido)triphosphate (BuPdGMPNHPP).

a) TBA salt of BuPdGMPNHPP. The TEA salt of BuPdGMPNHP (15 mg, 0.02 mmol) was dissolved in water (0.5 mL) and the solution was passed through a Dowex 8X50 column (5x0.5 cm) in the pyridinium form. The nucleotide was eluted with 5% pyridine in water, and the eluent was treated with tributylamine (TBA) (9.5 µL, 0.04 mmol) and evaporated to dryness under vacuum. The residue was dried by evaporation form anhydrous pyridine (2x) and anhydrous DMF (2x).

b) Tributylammonium orthophosphate. A mixture of 85% H_3PO_4 (13.5 µL, 0.2 mmol) in pyridine: water (1:1) was treated with TBA (48 µL, 0.2 mmol), and the solution was evaporated and dried by repeated evaporation from dry pyridine and dry DMF.

A solution of the TBA salt of BuPdGMPNHP in HMPA (0.2 mL) was treated with carbonyldiimidazole (16.2 mg, 0.1 mmol), and the mixture was stirred overnight under N_2. Tributylammonium orthophosphate, dissolved in a minimal amount of HMPA, was added to the reaction mixture, and stirring was continued for an additional 48 h. The mixture was diluted with 10 mL ice cold 0.1 M TEA, and purified on a DEAE-Sephadex column as described above for BuPdGMPNHP. The fractions corresponding to BuPdGMPNHPP were co-evaporated with n-BuOH at 20 °C to give 4.5 mg of product as the TEA salt. The UV absorption corresponded to a yield of 5.78 µmol (29%) of BuPdGMPNHPP. This product was purified by HPLC on a Synchropak AX-100 column (1x25 cm) in a gradient of 30% MeCN in water to 30% MeCN in 0.2 M ammonium bicarbonate over 60 min. at a flow rate of 8 mL/min. Fractions containing the product (at 0.08-0.1 M ammonium bicarbonate) were lyophilized. An aliquot was converted to the sodium salt by passing through a Chelex column in the Na^+ form and eluting with H_2O. ^{1}H NMR: (D_2O): 8.04 (s, 1H, 8-H), 7.32, 7.16 (m, 2H ea, Ph), 6.22 (t, 1H, H-1'; $J_{1'2'} \approx J_{1'2''} = 6.9$ Hz), 4.58 (m, 1H, 3'-H), 4.11 (m, 1H, 4'-H), 3.94 (m, 2H, 5'5"-H), 2.66 (m, 1H, 2'-H, $J_{2'3'} = 6.7$ Hz), 2.49 (t, 2H, Bu-1, J = 7.7 Hz), 2.35 (m, 1H, 2"-H, $J_{2'3'} = 3.7$ Hz, $J_{2'1'} = 6.6$ Hz, $J_{2'2''} = 14.1$ Hz), 1.46 (m, 2H, Bu-2), 1.20 (m, Bu-3), 0.78 (t, 3H, CH3, J = 7.2 Hz). ^{31}P NMR (D_2O): 0.58 (^{1}H dec. on, d, P^1, J_{P1P2} = 6.2 Hz; ^{1}H dec. off, q, J_{PH} = 5.5 Hz), -9.60 (d, P^3; J_{P2P3} = 20.9 Hz), -11.36 (dd, P^2; J_{P2P1} = 6.2 Hz, J_{P2P3} = 20.9 Hz). FAB MS: $[M+H]^+$ = 639, $[M+Na]^+$ = 661, $[M-H+Na]^+$ = 683.

Enzymes. Recombinant human DNA polymerase α, prepared as a single polypeptide, was a generous gift from Dr. Teresa Wang, Stanford University. T4 DNA polymerase was a generous gift from Dr. Linda Reha-Krantz, University of Alberta. T4 polynucleotide kinase was from New England Biolabs.

DNA polymerase assays. Assays of DNA polymerase activity on activated DNA were done in 25 µL volumes containing 20 mM Tris-Cl (pH 8.0), 2 mM DTT, 10 mM $MgCl_2$, 0.4 mg/mL

activated calf thymus DNA, 25 μM dATP, dCTP and dGTP and 10 μM [^{3}H]-dTTP (New England Nuclear) at 1250 cpm/pmol. [Reactions with pol α also included 0.1 mg/mL bovine serum albumin (BSA)]. Reactions were initiated by the addition of polymerase (0.5-2.0 units) and incubated for 10 min. at 30 °C. Reaction mixtures were quenched by addition of 1 mL 10% trichloroacetic acid (TCA)/100 mM sodium pyrophosphate ($NaPP_i$). Acid-insoluble product was collected on Whatman GF/A filters, washed three times with 0.1 M HCl/100 mM $NaPP_i$ and counted in 1 mL Optifluor.

Inhibitor assays. Compounds were tested in triplicate by the addition of ten-fold serial dilutions of inhibitors stored in 50 mM Tris-Cl (pH 7.5). For determinations of apparent K_i values, 5 concentrations of inhibitor were used in the absence of dGTP ("truncated" assay conditions[1]); the results of three independent experiments are plotted as % inhibition vs. log inhibitor concentration. For Lineweaver-Burke K_i determinations, 5 concentrations of dGTP were used at a constant inhibitor concentration, while dATP, dCTP and [^{3}H]-dTTP (1250 cpm/pmol) concentrations were 50 μM; results were plotted (SigmaPlot) as 1/pmol dGMP incorporated vs. 1/dGTP concentration. Regression lines were obtained using the Macintosh (HyperCard) program Enzyme Kinetics (D.G. Gilbert, Indiana University).

Primer Extension assays. A synthetic 17-mer primer (3'-GTAAAACGACGGCCAGT-5') was labeled with T4 polynucleotide kinase and [^{32}P]ATP, and annealed to a template 29-mer (5'-CATTTTGCTGCCGGTCACATGCCGATCCC-3') as described.[3] Assays were performed in 10 μL volumes containing 30 mM Tris-Cl (pH 7.5), 5% glycerol, 4 mM DTT, 10 mM $MgCl_2$, 1.0 pmol [^{32}P]-17:29-mer and nucleotides/inhibitors where stated. Reactions were initiated by the addition of 0.6 unit of enzyme, and quenched after 10 min. by addition of 3 μL 90% formamide/0.01% bromophenol blue. Samples were loaded onto a 12% polyacrylamide gel and electrophoresed at 1250 v. for approximately 3 hr. Gels were dried under vacuum and exposed to X-ray film (Kodak) at 4 °C for 30 min.

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