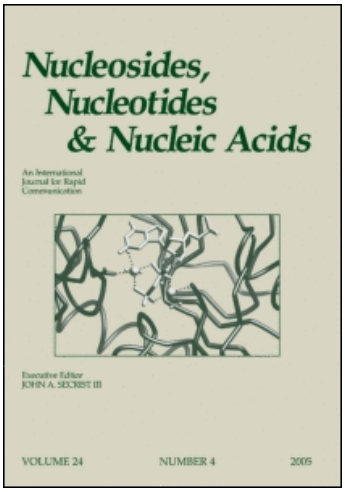

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N^2-(*p*-n-Octylphenyl)dGTP: Synthesis and Inhibitory Activity Against DNA Polymerases

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N^2-(*p*-n-OCTYLPHENYL)dGTP: SYNTHESIS AND INHIBITORY ACTIVITY AGAINST DNA POLYMERASES

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Abstract.

N^2-(*p*-n-Octylphenyl)-2'-deoxyguanosine 5'-triphosphate (OctPdGTP) has been synthesized chemically. OctPdGTP inhibited DNA polymerases (pol) α, δ and ϵ from calf thymus, with moderate selectivity for pol α. Mechanistic studies on pol α and bacteriophage T4 DNA polymerase revealed competitive and mixed kinetics of OctPdGTP with respect to the substrate dGTP when the enzymes were assayed on activated DNA and oligo dT:poly dA, respectively.

We recently identified several 6-(*p*-n-alkylanilino)uracils as selective inhibitors of uracil-DNA glycosylase (UDG) encoded by Herpes simplex type 1 virus.[1] The p-n-hexyl, heptyl and octyl derivatives were progressively more potent as inhibitors of the viral enzyme. However, the n-octyl compound, "OctAU", inhibited [^{3}H]thymidine incorporation by HeLa cells in culture, rendering it unsuitable for study of the role of the UDG in viral replication and latency.

The UDG inhibitors are closely related to 6-(*p*-n-butylanilino)uracil (BuAU), a compound that is a competitive inhibitor of eukaryotic DNA polymerase α.[2] This similarity suggested that OctAU might inhibit cellular DNA synthesis by its effect on DNA polymerase α (pol α) and/or one or both of the other enzymes involved in replicative DNA synthesis, pol δ and pol ϵ.[3] Indeed, OctAU inhibited calf thymus pol α (Table 1) with potency similar to that of BuAU, 65 μM.[2] This weak activity coupled with the poor water solubility of this class of compounds prompted us to synthesize an

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This paper is dedicated to Professor Yoshihisa Mizuno.

Table 1. Inhibition of DNA polymerases by bases.

Compound	Apparent K_i (μM)[1] calf thymus pol α	T4 DNA pol
6-(*p*-n-octylanilino)uracil (OctAU)	50	263
N^2-(*p*-n-octylphenyl)guanine (OctPG)	31	190
N^2-(*p*-n-butylphenyl)guanine (BuPG)	6.5	57

[1] Enzymes were assayed with activated DNA in the presence of dCTP, dATP and [^{3}H]dTTP, i.e. the truncated assay which allows direct measurement of K_i for a competitive inhibitor.

analog that would be more suitable as an inhibitor-probe of the eukaryotic replicative DNA polymerases. Such a compound had been derived conceptually from the selective pol α inhibitor, BuAU, by converting it to its guanine counterpart BuPG and ultimately to the corresponding deoxyribonucleoside 5'-triphosphate, BuPdGTP.[4] In this paper we describe the synthesis of the target compound, N^2-(*p*-n-octylphenyl)-2'-deoxyguanosine 5'-triphosphate (OctPdGTP), and its range of inhibitory activities against DNA polymerases α, δ and ϵ from calf thymus. In addition we have compared its activity and mechanism against pol α with those against bacteriophage T4 DNA polymerase, an enzyme that shares sequence homology with pol α.[5]

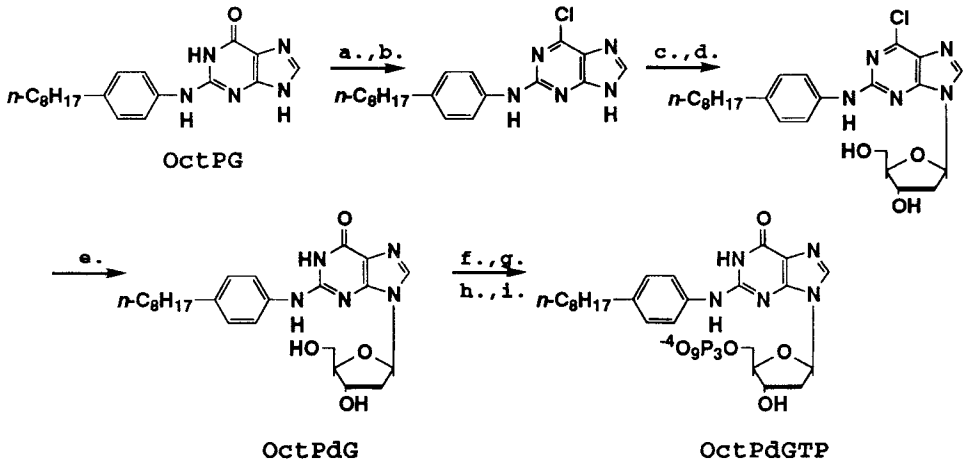
Conversion of OctAU to its guanine counterpart, i.e. N^2-(*p*-n-octylphenyl)guanine (OctPG), was based on the expectation that the latter compound would inhibit DNA pol α competitively with dGTP.[2,6] OctPG was prepared by reaction of 2-bromohypoxanthine with *p*-n-octylaniline as described.[7] OctPG inhibited calf thymus pol α with potency similar to that of OctAU but more weakly than that of BuPG (Table 1).

OctPG was converted to the desired nucleotide, OctPdGTP, by a multistep method that we have employed for synthesis of related compounds (Scheme).[7,8] First, OctPG was converted to 2-(*p*-n-octylphenyl)-6-chloropurine by treatment with thionyl chloride in DMF. Sodium salt glycosylation[9] of the chloro compound in acetonitrile with 1-α-chloro-2-deoxy-3,5-di-(*p*-toluyl)-β-D-ribofuranose gave predominantly the 9-β isomer (78%) with minor amounts of other isomers (uncharacterized).

Identification of the product was based on 1D and 2D (NOESY) ^1H NMR spectra. Assignment of the N^9 regioisomeric structure was apparent from the similarity of chemical shifts to those of the corresponding N^2-(*p*-n-butylphenyl) analog,[7] and the β anomeric structure on the presence in the NOESY spectrum of expected crosspeaks between H8-H3′ and H8-H2′.[10] After removal of *p*-toluyl groups with sodium methoxide in methanol at rt, hydrolysis of the 6-chloro nucleoside with sodium methoxide/2-mercaptoethanol in ethanol at reflux gave N^2-(*p*-n-octylphenyl)-2′-deoxyguanosine (OctPdG, Scheme) in 88% yield.

N^2-(*p*-n-Octylphenyl)-2′-deoxyguanosine 5′-phosphate (OctPdGMP) was obtained in 75% yield by treatment of the nucleoside with phosphoryl chloride in trimethyl phosphate and purification by HPLC on a Synchropak anion exchange column with a gradient of aqueous ammonium bicarbonate and acetonitrile. Activation of the monophosphate with carbonyldiimidazole in hexamethylphosphoric triamide, followed by addition of tri-(n-butyl)-ammonium pyrophosphate and purification on a Synchropak HPLC column, gave the 5′-triphosphate, OctPdGTP, as the ammonium salt in 81% yield. Structure confirmation of nucleotides was done by means of ^1H (not shown) and ^{31}P NMR spectra. OctPdGMP showed a triplet in the ^{31}P spectrum with J_{PH} = 4.6 Hz from coupling with 5′,5" protons. OctPdGTP showed three P resonances, at positions and with coupling constants consistent with those found for related nucleoside 5′-triphosphates.[11]

Inhibition of eukaryotic DNA polymerases. Assays of OctPdGTP with the calf thymus DNA polymerases α, δ and ϵ revealed that pol α was most sensitive, followed by pol δ and then pol ϵ (Table 2). The enzymes were assayed with a primer:template, poly dA:oligo dT, that is non-complementary to the inhibitor in order to permit expression of pol δ activity.[3] Both potency and degree of selectivity of the nucleotide for pol α, however, were considerably different than those properties of the prototype inhibitor, BuPdGTP, assayed under the same conditions. The latter compound inhibited calf thymus pols α, δ and ϵ with IC_{50}s of 0.026, 100 and 87 μM, respectively.[12] Potency of OctPdGTP against pol α was 200-fold lower than that of BuPdGTP, and the degree of selectivity of OctPdGTP against pol α relative to pols δ and ϵ (threefold and twentyfold, respectively) was much less than the several thousand-fold selectivity of BuPdGTP for pol α.[6,12] Interestingly, OctPdGTP inhibited the related bacteriophage T4 DNA polymerase (T4 pol) twofold more potently than it inhibited pol α



a. $SOCl_2$/DMF. b. conc. NH_4OH. c. NaH/MeCN; 1-α-chloro-3,5-di-(*p*-toluyl)-β-D-ribofuranose. d. NaOMe/MeOH. e. $HOCH_2CH_2SH$/NaOMe/MeOH. f. $POCl_3$/$(MeO)_3PO$. g. H_2O/NEt_3. h. Im_2CO/$(Me_2N)_3PO$. i. $P_2O_7 \cdot (NBu_3)_4$/$(Me_2N)_3PO$.

Scheme

Table 2. Inhibition of DNA polymerases by N^2-(*p*-n-octylphenyl)-2'-deoxyguanosine 5'-triphosphate, OctPdGTP.

DNA polymerase	IC_{50} (μM)[1]
pol α	5
pol δ	17
pol ε	100
T4 pol	2.8

[1] Assayed with oligo dT:poly dA and 5 μM [^{3}H]dTTP as described in the Experimental Procedures.

(Table 2), in stark contrast to the much greater potency of BuPdGTP against pol α compared to T4 pol.[13]

Mechanisms of inhibition of pol α and T4 pol by OctPdGTP. OctPdGTP inhibited both pol α and T4 pol competitively with dGTP when assayed with activated DNA as primer:template. Lineweaver-Burk plots of the results of assays with fixed concentrations of dATP, dCTP and [^{3}H]dTTP and varying concentrations of dGTP (Figure 1, A and C) clearly indicate competitive kinetics, with K_i values of 0.046 and 1.51 μM for inhibition of pol α and T4 pol, respectively. The significant difference in relative potencies in the competitive (Figure 1, A and C) and non-competitive assays (Table 2) suggests differences in mode of inhibitor binding in each case. In fact, assays of OctPdGTP with the primer:template, oligo dT:poly dA, that is not complementary to the inhibitor, and [^{3}H]dTTP as variable substrate revealed non-competitive or mixed kinetics for inhibition of both DNA polymerases (Figure 1, B and D).

OctPdGTP is a substrate for T4 pol. Although BuPdGTP is a potent, non-substrate inhibitor of pol α,[14] the nucleotide is a terminating substrate for T4 pol.[13] When T4 pol was incubated with a defined 17:29mer primer:template requiring dGTP as the first substrate and the products were analyzed by denaturing polyacrylamide gel electrophoresis, extension of the primer to an 18mer was observed in the presence of OctPdGTP, and the extent of conversion depended on enzyme concentration (Figure 2). The appearance of product in the presence of OctPdGTP was not due to residual dGTP in the enzyme preparation, because incubation of T4 pol with primer:template alone causes complete degradation of primer (by 3′ to 5′ exonuclease activity) under these conditions. At equal enzyme concentrations dGTP was more efficiently incorporated than OctPdGTP (Figure 2; compare lanes 3 and 6). As found previously for BuPdGTP,[14] OctPdGTP did not completely protect primers from the 3′ to 5′ exonuclease activity of T4 pol (Figure 2).

DISCUSSION

OctPdGTP has been shown to inhibit the calf thymus DNA polymerases implicated in DNA replication, pols α, δ and ϵ, but with lower potency and selectivity compared to the prototype inhibitor, BuPdGTP. Mechanistic studies with the most sensitive enzyme, pol α, indicate that OctPdGTP is a competitor of dGTP when the latter is required as a substrate, but

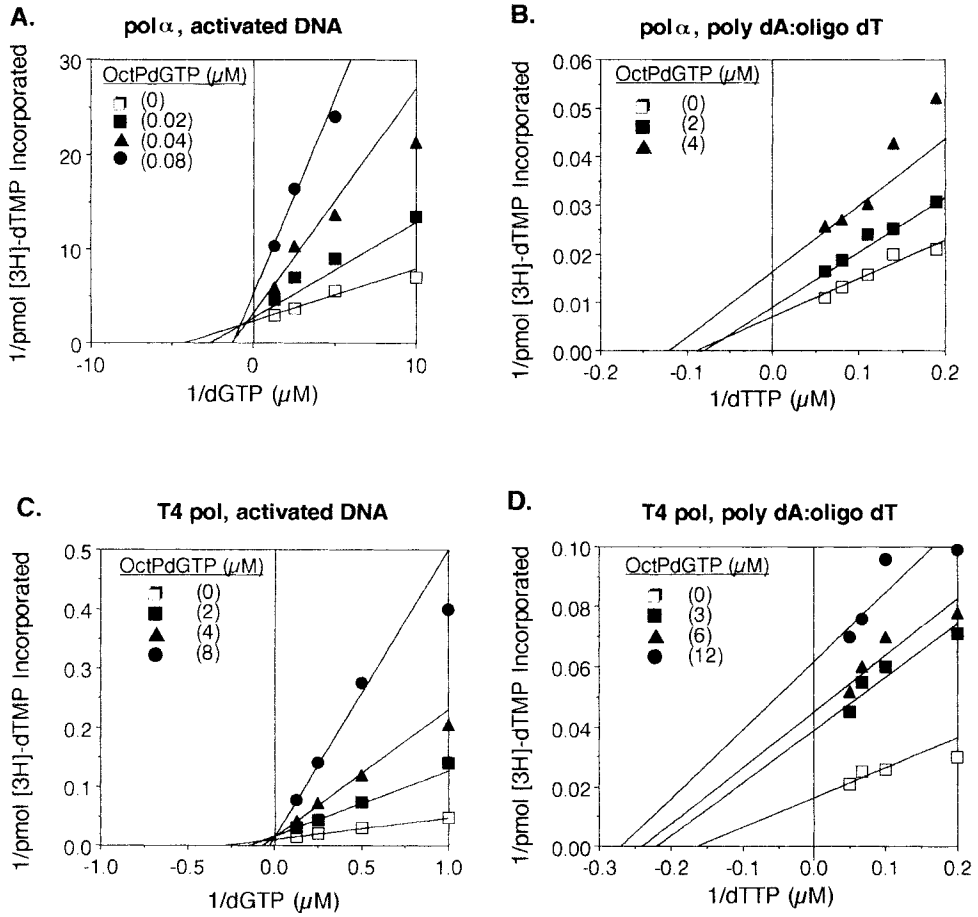


Figure 1. Kinetics of DNA polymerase inhibition by OctPdGTP. Enzymes were assayed as described in the absence and presence of OctPdGTP, and the results are presented as double reciprocal (Lineweaver-Burk) plots. A. Calf thymus pol α assayed on activated DNA. K_m for dGTP is 0.23 μM. B. Calf thymus pol α assayed on oligo dT:poly dA. K_m for dTTP is 11.4 μM. C. T4 pol assayed on activated DNA. K_m for dGTP is 3.5 μM. D. T4 pol assayed on oligo dT:poly dA. K_m for dTTP is 2.5 μM.

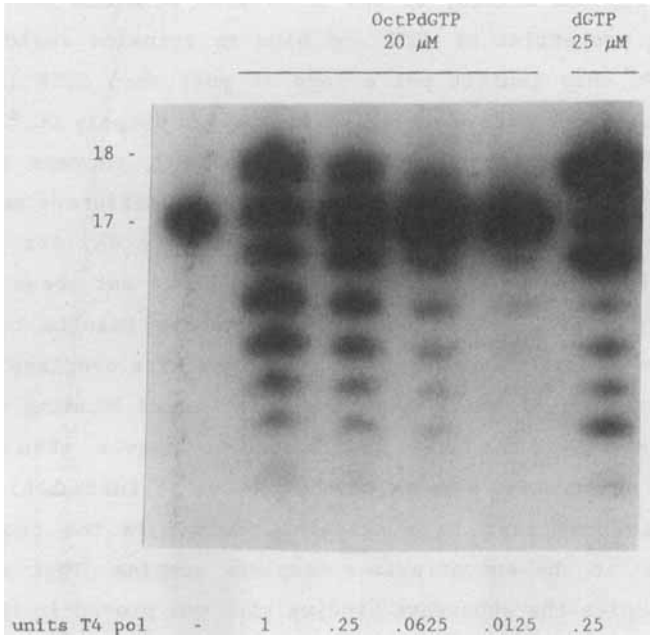


Figure 2. Incorporation of OctPdGTP by T4 DNA polymerase. Reactions of 5'-[^{32}P]-GTAAAACGACCGGCCAGT:3'-CATTTTGCTGCCGGTCACATGCCGATCCC) and T4 DNA polymerase were carried out with the indicated additions as described in Experimental Procedures.

inhibits with mixed kinetics with a non-homologous substrate such as dTTP (Figure 1). BuPdGTP inhibition of pol α was also competitive with dGTP when activated DNA or the inhibitor-complementary oligo dG:poly dC were assayed, but was demonstrably uncompetitive with dTTP when assayed on oligo dT:poly dA.[15] The competitive mechanism for both nucleotides suggests that, like BuPdGTP, OctPdGTP binds the enzyme:primer:template complex in place of a required dGTP, but with lower affinity than BuPdGTP. Mixed kinetics in the homopolymer assays (Figure 1, B and D) suggest that OctPdGTP may bind to both free enzyme and enzyme:primer:template complex when a non-complementary template is used, but with considerably lower affinities than with the complementary activated DNA template.

Inhibition of pol α by the simple bases, BuAU and BuPG, involves competition of inhibitor with dGTP for binding to the enzyme:DNA complex when the template base to be copied is cytosine.[6] This mechanism is

consistent with the ability of the inhibitors to mimic the Watson-Crick base pairing properties of dGTP and bind to cytosine residues. Indeed, BuAU and BuPG only inhibit pol α (and T4 pol) when dGTP is a required substrate, i.e. with activated DNA or with oligo dG:poly dC.[6] In contrast, the nucleotide inhibitor BuPdGTP inhibits both enzymes on non-dGTP-requiring primer:templates, but by an apparently different mechanism than on a dGTP-requiring template. With oligo dT:poly dA, for example, the effect of BuPdGTP on both pol α[15] and T4 pol (data not shown) is strictly uncompetitive with [^{3}H]dTTP. We interpret these results to imply that BuPdGTP binds to free enzyme in both cases at a site overlapping a nascent "substrate binding site" in a manner that blocks binding of enzyme to primer:template. This mechanism is overridden, however, when dGTP is among the required substrates, because of the greater affinity achieved when the inhibitor can base pair to a cytosine residue in the true "substrate binding site" in the enzyme:primer:template complex. That the inhibitor actually occupies the substrate binding site was proved in the case of T4 pol by the ability of BuPdGTP to be a bona fide substrate for incorporation into a primer:template by that enzyme.[13]

OctPdGTP is a potent, competitive inhibitor of calf thymus pol α when activated DNA is the primer:template, but considerably weaker as an inhibitor of T4 pol. Its mechanism of inhibition with a non-complementary template is more complex than that of BuPdGTP, however. Mixed kinetics for both pol α and T4 pol (Figure 1, B and D) may indicate that OctPdGTP binds multiple sites on the free enzymes, or binds to both free and primer:template-bound enzyme with different affinities. The long hydrophobic side chain of OctPdGTP could participate in non-specific binding with both polymerases, a process that would be masked by specific, i.e. competitive, active site binding when complementary templates are present (Figure 1, A and C). Indeed, OctPdGTP must occupy the active site in the latter case based on the finding that it is a substrate for T4 pol (Figure 2). The significant inhibition of pol α and T4 pol by OctPdGTP under both competitive and mixed competitive conditions suggests that the effects of mutations of putative active site residues may reveal more about these mode(s) of interaction.

EXPERIMENTAL PROCEDURES

General. ^{1}H and ^{31}P NMR spectra were recorded at 300 and 202 MHz, respectively, on a Varian Unity 300 instrument. Chemical shifts are

referenced as follows: ^1H in Me_2SO-d_6 and $CDCl_3$, internal TMS; in D_2O, internal DSS; ^{31}P, external 1% phosphoric acid. ^1H NMR spectra of all intermediates and final products were consistent with assigned structures. 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 was done with Kieselgel 60F-254 analytical plates, and column chromatography employed Kieselgel 60 (37-70 mesh), both from Merck. Pyridine was refluxed and distilled over calcium hydride. Hexamethylphosphoric triamide (HMPA) was distilled over calcium hydride under reduced pressure. Acetonitrile 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.

N^2-(*p*-n-Octylphenyl)guanine. 2-Bromohypoxanthine[7] (0.36 g, 1.67 mmol) was suspended in 95% 2-methoxyethanol (10 mL) and *p*-n-octylaniline (0.6 g, 2.95 mmol) was added. The reaction mixture was refluxed under nitrogen for 5 h, then left at rt overnight. The precipitate was filtered, washed twice with MeOH and acetone, and crystallized from DMF to give pure product (0.33 g, 59%), mp 284-286°C. *Anal.* Calcd. for $C_{19}H_{25}N_5O$: C, 67.23; N, 7.42; N, 20.63. Found: C, 67.19; H, 7.43; N, 20.65.

2-(*p*-n-Octylanilino)-6-chloropurine. N^2-(*p*-n-Octylphenyl)guanine (0.373 g, 1.1 mmol) was suspended in dry DMF (7.7 mL) and thionyl chloride (0.29 mL, 4.03 mmol) was added. The reaction mixture was stirred at 70 °C for 5 h and left at rt overnight. The precipitate was filtered and washed with ethyl acetate (fraction 1). The filtrate was neutralized to ca. pH 7 by addition of aqueous sodium bicarbonate. Water (20 mL) was added and the precipitated material was filtered and washed with water (fraction 2). The fractions were combined and stirred with conc. ammonium hydroxide (20 mL) for 24 h at rt. The solid was filtered and washed with water to give 0.332 g (84.4%) of product, mp 178-180°C, used in the following step without further purification. *Anal.* Calcd. for $C_{19}H_{24}N_5Cl$: C, 63.77; H, 6.76; N, 19.57. Found: C, 64.13; H, 7.07; N, 20.06.

2-(*p*-n-Octylanilino)-6-chloro-9-(2-deoxy-ß-D-ribofuranosyl)purine. 2-(*p*-n-Octylanilino)-6-chloropurine (0.2 g, 0.56 mmol) was suspended in dry acetonitrile (24.4 mL), and sodium hydride (25.3 mg, 0.63 mmol, 60% suspension in mineral oil) was added. The reaction mixture was stirred at rt for 20 min, and 1-chloro-2-deoxy-3,5-di-O-(p-toluyl)-α-D-ribofuranose[16] (0.21 g, 0.54 mmol) was added in small portions during 20 min. After

stirring at rt for 1 h the reaction mixture was diluted with chloroform (40 mL). The resulting solution was evaporated to dryness and twice co-evaporated with toluene (20 mL). The residual oil was dissolved in toluene:acetone (19:1) and purified by HPLC (silica gel, 5x20 cm) in the same solvent. Fractions containing the major product were collected and evaporated to dryness yielding 310 mg (78%) of 2-(*p*-n-octylanilino)-6-chloro-[2-deoxy-3,5-di-O-(p-toluyl)-β-D-ribofuranosyl]purine; minor products were not isolated. This intermediate (0.196 g, 0.28 mmol) was deblocked by stirring in 10 mL of methanol containing 0.19 g of sodium for 4 h at rt. The reaction mixture was neutralized with glacial acetic acid and evaporated to dryness; the solid residue was dissolved in methanol (20 mL), silica gel was added, and the solvent was evaporated. The resulting material was purified on a silica gel column (5x20 cm) eluted with a linear gradient of chloroform to chloroform:methanol (9:1) at a flow rate 3 mL/min. Fractions containing the product were collected and evaporated to dryness yielding 0.109 g (83.3%) of 2-(*p*-n-octylanilino)-6-chloro-9-(2-deoxy-β-D-ribofuranosyl)purine as a glass. *Anal.* Calcd for $C_{24}H_{32}N_4O_2Cl$: C, 60.81; N, 6.80; N, 14.77. Found: C, 60.48; H, 6.45; N, 14.82.

N^2-(*p*-n-Octylphenyl)-2'-deoxyguanosine. 2-(*p*-n-Octylanilino)-6-chloro-9-(2-deoxy-β-D-ribofuranosyl)purine (0.239 g, 0.5 mmol) was dissolved in abs. ethanol (11.4 mL), and 2-mercaptoethanol (0.2 mL) and 1N sodium methoxide in methanol (0.85 mL) were added. The reaction mixture was heated at reflux for 48 h; 1N sodium methoxide in methanol (0.85 mL) was added and reflux was continued for 24 h. The solution was cooled, and water (12 mL) was added. The resulting suspension was neutralized with AcOH, and the product was filtered and washed with water to give 0.202 g (88%) of N^2-(*p*-n-octylphenyl)-2'-deoxyguanosine, mp 200-203 °C. *Anal.* Calcd. for $C_{24}H_{33}N_4O_3$: C, 63.28; H, 7.30; N, 15.37. Found: C, 63.22; H, 7.57; N, 15.68.

N^2-(*p*-n-Octylphenyl)-2'-deoxyguanosine 5'-phosphate. N^2-(*p*-n-Octylphenyl)-2'-deoxyguanosine (70 mg, 0.15 mmol) was suspended in trimethyl phosphate (0.87 mL), and the mixture was cooled to -4 °C. Phosphoryl chloride (22.9 μL, 0.24 mmol) was added, and the resulting solution was kept at -4 °C for 15 h. Water (4 mL) was added, and the mixture was neutralized with triethylamine. The solution was filtered and purified by HPLC in 6 portions on a Synchropak column (8.9x250 mm). After elution for 1 min with

water:acetonitrile (65:35), the product was eluted with a linear gradient of the starting mixture to 0.4 M aqueous ammonium bicarbonate:acetonitrile (65:35) during 45 min at a flow rate of 3 mL/min. All fractions containing the 5'-phosphate (retention time 18 min) were evaporated to dryness, and the residue was dissolved in deionized water and lyophilized giving 66 mg (75%) of product. For NMR spectra, small samples of each fraction, after lyophilization, were dissolved in water, passed through a Chelex 100 column (Na form, 2 mL) and lyophilized. ^{31}P NMR (D_2O): δ 4.5 (t, J=4.6 Hz).

N^2-(*p*-n-Octylphenyl)-2'-deoxyguanosine 5'-triphosphate. N^2-(*p*-n-Octylphenyl)-2'-deoxyguanosine 5'-phosphate (43 mg, 0.047 mmol) was suspended in hexamethylphosphoric triamide (HMPA) (1.5 mL), and 1,1'-carbonyldiimidazole (35.6 mg, 0.22 mmol) was added. The reaction mixture was stirred at rt for 5 h and quenched by the addition of methanol (31 μL). A solution of tri(n-butyl)ammonium pyrophosphate (103.2 mg, 0.26 mmol) in HMPA (1.75 mL) was added, and the mixture was stirred at rt for 24 h. The resulting mixture was treated with ice (5 g), and the product was purified by HPLC in 6 portions on a Synchropak column (8.9x250mm). After elution for 1 min in water:acetonitrile (65:35), the product was eluted in a linear gradient of the starting mixture to 0.7 M aqueous ammonium bicarbonate:acetonitrile:water (45:35:20) during 45 min at a flow rate of 3 mL/min. Fractions containing the product were collected and evaporated to dryness, and the residue was dissolved in deionized water (15 mL) and lyophilized to give 29.2 mg (81%) of product. UV: λ_{max} (H_2O) 278 nm (ϵ 20480). ^{31}P NMR (D_2O): δ -10.6 (d, J=20 Hz, γP), -11.3 (dt, αP), -23.2 (t, J=20 Hz, βP).

Other inhibitors. N^2-(*p*-n-Butylphenyl)guanine (BuPG) and N^2-(*p*-n-butylphenyl)-2'-deoxyguanosine 5'-triphosphate (BuPdGTP) were prepared as described in refs. 17 and 4, respectively. 6-(*p*-n-Octylanilino)uracil (OctAU) was prepared as described in ref. 1.

Enzymes. DNA polymerases α, δ and ϵ were purified from calf thymus by published methods.[18,19] Full length bacteriophage T4 DNA polymerase[20] was a gift from Dr. Linda Reha-Krantz, University of Alberta. The enzyme was stored in aliquots of 4.1 mg protein/mL (2.4 units/μg) at -80 °C.

Enzyme Assays. Assays of DNA polymerases with activated DNA as primer:template were done in 25 μL volumes containing 30 mM Tris-HCl (pH 7.5), 20% glycerol, 4 mM dithiothreitol (DTT), 10 mM $MgCl_2$, 0.4 mg/mL activated calf thymus DNA (Worthington), 25 μM each dATP, dCTP and dGTP,

and 10 μM [^{3}H]dTTP (1250 cpm/pmol). Reactions were initiated by the addition of 0.1 unit of pol α or 3.2 ng T4 pol and incubated for 10 min. at 37 °C (pol α) or 30 °C (T4 pol). Reactions were quenched by the addition of 10% trichloroacetic acid (TCA) in 100 mM aqueous sodium pyrophosphate (0.5 mL). Acid-insoluble product was collected on GF/A filters, which were washed three times with 0.1 M HCl/100 mM sodium pyrophosphate and counted in Optifluor (1 mL). Assays of DNA polymerases with poly dA:oligo dT as template:primer were done in 25 μL volumes containing 30 mM Tris-HCl (pH 7.5), 20% glycerol, 4 mM dithiothreitol (DTT), 10 mM $MgCl_2$, 1 μg polydA:oligodT (base ratio 10:1), and 10 μM [^{3}H]dTTP (1250 cpm/pmol). Reactions were initiated by addition of 0.1 unit of pol α, δ or ϵ, or 3.2 ng of T4 pol, carried out as described above, and quenched by the addition of 0.5 mL of heat-denatured activated DNA (40 μg/mL). After standing for 10 min, DNA was precipitated by the addition of ice cold 10% trichloroacetic acid in 200 mM aqueous sodium pyrophosphate (0.5 mL). Collection and counting of acid-insoluble product were done as described above. Primer extension assays employed a 17mer primer, 5′ end-labelled with ^{32}P, and a complementary 29mer template (see legend to Figure 2 for sequences); reactions with T4 DNA polymerase and denaturing polyacrylamide gel electrophoresis of the products were done as described.[13]

Inhibitor assays. Compounds were tested by the addition of two-fold or three-fold serial dilutions of stock solutions of inhibitors in DMSO (BuPG, OctAU, OctPG) or 50 mM Tris-HCl (pH 7.5) (BuPdGTP, OctPdGTP). For determinations of apparent K_i or IC_{50} values, 6 concentrations of inhibitor were used, and the results of at least two independent experiments were plotted as % inhibition vs. log inhibitor concentration. For kinetic determinations, K_m and V_{max} values for variable substrates were measured in the absence and presence of inhibitors. Results were plotted by the method of Lineweaver and Burk (*SigmaPlot*) as 1/pmol [^{3}H]dTMP incorporated vs. 1/[^{3}H]dTTP or 1/dGTP concentration. Regression lines were obtained using the HyperCard program *Enzyme Kinetics* (D.G. Gilbert, Indiana University). Competitive K_i values were obtained from $K_i = I/[(K_m'/K_m)-1]$, where K_m' is the apparent K_m of variable substrate in the presence of inhibitor at concentration I.

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