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Chemical and enzymatic incorporation of N²-(p-n-butylphenyl)-2′-deoxyguanosine into an oligodeoxyribonucleotide

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ABSTRACT
An 18mer oligodeoxyribonucleotide containing a N²-(p-n-butylphenyl)-2′-deoxyguanosine (BuPdG) residue at the 3′ end has been synthesized by both chemical and enzymatic methods. Chemical synthesis involved attachment of 5′-DMT-BuPdG as the 3′-H-phosphonate to uridine-controlled pore glass (CPG), followed by extension via H-phosphonate chemistry. After oxidation of the backbone, deprotection of bases, and removal from CPG, the uridine residue was removed by periodate cleavage and β-elimination. The resulting oligomer 3′-phosphate was digested with alkaline phosphatase to give the free BuPdG-18mer. E.coli DNA polymerase I (Klenow) incorporated BuPdGTP at the 3′ end of the corresponding 17mer primer annealed to a complementary 29mer template, and the properties of this product were identical to those of chemically synthesized BuPdG-18mer. E.coli DNA polymerase I (Klenow) was unable to extend the BuPdG-18mer, and the 3′ to 5′ exonuclease activity of the enzyme was unable to remove the modified nucleotide.

INTRODUCTION
N²-(p-n-Butyphenyl)-2′-deoxyguanosine 5′-triphosphate (butylphenyl-dGTP, BuPdGTP) is a potent and selective inhibitor of eukaryotic DNA polymerase α and α-like DNA polymerases such as bacteriophage T4 DNA polymerase.1-4 In contrast, E.coli DNA polymerase I (pol I) has been found to be quite insensitive to this nucleotide.5 BuPdGTP is not a substrate for DNA polymerase α, but appears to be a substrate for the T4 enzyme.6 Thus, the basis for sensitivity to BuPdGTP among DNA polymerases may be complex.

Among a series of N²-substituted dGTP derivatives, we recently reported that N²-phenyl-dGTP (and BuPdGTP) could be incorporated by E.coli DNA polymerase I into an oligodeoxyribonucleotide primer in place of dGTP, but that significant chain termination occurred at the incorporation sites.5 In order to study the effects of butylphenyl-dG residues at the primer terminus on BuPdGTP-sensitive (pol α, T4 pol) and insensitive (pol I) enzymes, and to have authentic material for comparison of the properties of enzymatically incorporated BuPdGTP, we have synthesized chemically a 3′-BuPdG-oligomer. We show that the properties of this oligomer annealed to a complementary template are identical with those of the oligomer into which BuPdGTP has been incorporated by pol I, namely that the BuPdG-oligomer cannot be extended with the next substrate and cannot be degraded by the 3′ to 5′ exonuclease activity of pol I.

MATERIALS AND METHODS
Syntheses
General. NMR spectra were determined with a Varian Unity 300 instrument. Chemical shifts in 1H spectra at 300 MHz are referenced to internal TMS, and in 31P spectra at 121.4 MHz to a capillary of 1% phosphoric acid. 5′-((Dimethoxytrityl)-N²-(p-n-butylphenyl)-9-(2-deoxy-β-D-ribofuranosyl)guanine (5′-DMT-BuPdG, 2). Triethylamine (200 µL) was added to a solution of N²-(p-n-Butylphenyl)-9-(2-deoxy-β-D-ribofuranosyl)guanine (BuPdG, 1)6 (145 mg, 0.363 mmol), dimethoxytrityl chloride (493 mg, 1.55 mmol) and 4-(dimethylamino)pyridine (1.3 mg) in dry pyridine (5 mL). After stirring for 2 hr at rt under Ar, the solution was poured into sat. NaHCO₃ solution. This mixture was extracted with ethyl acetate (3×5 mL), and the dried (Na₂SO₄) organic phase was concentrated and the residue applied to a silica gel column (51×2 cm). After washing with CHCl₃ the product was eluted with CHCl₃:MeOH (20:1). After evaporation of solvents the product was crystallized from MeOH giving 161 mg (61% of 2) as colorless crystals, mp 165–170°C. Anal. (C₄₇H₆₄N₂O₆): Calcd. C, 70.17; H, 6.18; N, 9.98. Found C, 69.98; H, 6.17; N, 9.90. 1H NMR (Me₂SO-d₆) δ 6.26 (t, 1H), 4.37 (m, 3H), 5.31 (d, exch, 3′-OH), 3.2 (d, 5′,5″H). 5′-((Dimethoxytrityl)-N²-(p-n-butylphenyl)-9-(2-deoxy-β-D-ribofuranosyl)guanine-3′-H-phosphonate, triethylammonium salt (3). 1,2,4-Triazole (21.7 mg, 0.18 mmol) was added to a stirred solution of PCl₃ (48.9 mg, 0.35 mmol) and N-
methylmorpholine (39.2 μL, 3.6 mmol) in dry CH₂Cl₂, and the mixture was stirred for 2 h at rt under argon. The mixture was cooled to −5°C, and a solution of 2 (50 mg, 0.71 mmol) in dry CH₂Cl₂ (3 mL) was added slowly. After 20 min at −5°C the reaction mixture was poured into 10 mL of 1 M triethylenammonium bichromate (TEAB, pH 8.5). The organic layer was separated, and the aqueous phase was extracted with CH₂Cl₂ (10 mL). The combined organic extracts were dried (Na₂SO₄) and the solvents removed to give a gum. This material was dissolved in CHCl₃ (0.5 mL), and the solution was slowly added to pentane (25 mL) cooled to −78°C. The colorless precipitate was collected by filtration to give 60 mg (97%) of 3. Analytically pure product was obtained by silica gel chromatography (10 × 2 cm; prepared in 1% NEt₃ in CHCl₃) and elution with 5% MeOH in CHCl₃ (81% recovery). Anal. (C₁₂H₁₄N₂O₈P): Calcd. P, 3.57. Found P, 3.25. NMR (CD₃CN:CD₃Ν₂, 1:1): δH = 7.17 (d, J = 608.5 Hz, PH), 6.46 (t, 1H), 5.29 (m, 3'H), 3.52 (d, J = 5.5, 5'-H). 31P δ = 3.05, Up = 60.85 Hz, UpH = 9.15 Hz. 3'-BuPdG-18mer (9). The oligodeoxyribonucleotide was synthesized via the H-phosphonate approach using an automated DNA synthesizer (8700 model, Millipore, MA). Synthesis was carried out on a 1 μmol scale using the H-phosphonate coupling cycle. The first step coupled the BuPdG-H-phosphonate (3) to uridine-controlled pore glass (CPG) support (Glen Research, Herndon, VA) with pivaloyl chloride catalyst. The required oligonucleotide sequence was assembled onto the CPG-bound dinucleoside H-phosphonate (4) with 5'-DMT-2'-deoxynucleobonucleoside-3'-H-phosphonates (4.0 mg/coupling). At the end of the assembly, the CPG-bound oligodeoxyribonucleotide H-phosphonate (5) was oxidized with 2% I₂ in pyridine:water (98:2, v:v) for 10 min to generate the phosphodiester backbone. Deprotection was carried out in conc NH₂OH for 10 hr at 55°C. Ammonia was removed by evaporation, and the solid mass of 6 was suspended in 1 mL of water. The crude oligonucleotide suspension was treated with sodium periodate in sodium acetate buffer, pH 5.5, for 30 min in the dark at ambient temperature. The progress of the reaction was followed by reverse phase HPLC (see below). The reaction mixture was desalted using a Sep-pak C-18 cartridge (Waters Associates, Milford, MA), and the eluate containing the dialdehyde form of the oligonucleotide (7) was treated with cyclohexylamine to remove uridine by β-elimination. The product, the 3'-phosphate of the oligodeoxyribonucleotide (8), was incubated with bacterial alkaline phosphatase to obtain the free BuPdG-18mer (9), which was purified by electrophoresis in a 8M urea-polyacrylamide gel.

HPLC. Analytical HPLC was carried out using a Waters 600E system controller, 745 data module and 481 UV-VIS detector, and a NovaPak C-18 column with a RCM 100 cartridge holder. Buffers were: A, 100 mM ammonium acetate; B, 20% buffer A in acetonitrile. The gradient used was 0–30% buffer B over 25 min and 30–100% buffer B over 15 min at a flow rate of 1.5 ml min⁻¹. Retention times were: oligonucleotide dialdehyde (7), 19.4 min; BuPdG-18mer-3'-phosphate (8), 20.2 min.

Enzyme assays

Materials. E.coli DNA polymerase I, Klenow fragment, was the sequencing grade obtained from Boehringer Mannheim. Terminal deoxynucleobonucleotidyl transferase (TdT) was obtained from New England Nuclear. Activated DNA was prepared as described. Operon Technologies Inc. supplied the 17mer primer (M13 universal primer SP010–1) and synthesized the 29mer template; sequences of these oligomers are shown in Fig 2. 5'-[³²P]-labelling of primers with polynucleotide kinase and annealing of primer:templates were done as described.

Standard pol I assay. Assays were done in a mixture of 30 mM Tris·HCl, pH 7.5, 20% glycerol, 4 mM DTT and 10 mM Mg(OAc)₂. Assay mixtures (25 μL each) contained 25 μM dCTP, dGTP and dATP and 10 μM [³²P]dGTP (1250 cpm/μmol), 400 μg/mL activated DNA and 0.5 unit of E.coli pol I, Klenow fragment. Truncated assays were identical except for the omission of dGTP. Processing and counting of acid-precipitable material were done as described.

Primer extension assay. Reactions were done in 25 μL of an assay mix containing 30 mM Tris·HCl, pH 7.5, 5% glycerol, 4 mM DTT, 10 mM Mg(OAc)₂ and 1.2 μM of labelled primer:template, with the addition of appropriate substrates. Assays for approximate Kₘ and kinetic determinations contained 0.35 μM primer:template. Reactions were initiated by addition of 0.1–0.5 units of pol I, and the mixtures were incubated at 30°C for 10 min. Procedures for termination and analysis of reaction products by 8M urea-polyacrylamide gel electrophoresis were as described. Assays with TdT and analysis of products were done as described.

RESULTS

Chemistry

We used H-phosphonate chemistry and a uridine-controlled pore glass (CPG) conjugate for synthesis of an oligomer containing the N²-(p-n-butylyphenyl)deGuo residue at the 3' position (Chart I). This strategy was dictated, in part, by our inability to prepare the 3'-hemisuccinate of 5'-DMT-BuPdG for direct attachment to aminomethyl-CPG (data not shown). 5'-DMT-BuPdG (2) and its 3'-H-phosphonate (3) were synthesized by standard methods. The H-phosphonate was obtained in 97% yield and was characterized by ¹H and ³¹P NMR and P analysis (see Materials and Methods).

Oligodeoxyribonucleotide synthesis was performed on a DNA synthesizer by reported procedures. Compound 3 was coupled to uridine-CPG with pivaloyl chloride (Chart I, step c). The chain was elongated with protected 5'-DMT-deoxynucleoside 3'-H-phosphonates, and the CPG-bound oligodeoxyribonucleoside-H-phosphonate was oxidized with iodine (step d) to generate the oligodeoxyribonucleotide phosphodiester. (The boldface sequence in structure 5, Chart I, implies protected bases.) After deprotection of the bases and release from CPG (step e), the free uridine-capped oligonucleotide (6) was treated with periodic acid at room temperature (step f) to generate the dialdehyde, 7. After desalting on a C-18 column the dialdehyde was treated with cyclohexylamine (step g) to effect β-elimination to yield the BuPdG-18mer as the 3'-phosphate, 8. After dephosphorylation with bacterial alkaline phosphatase, the BuPdG-18mer, 9, was purified by denaturing polyacrylamide gel electrophoresis. The preparation was done on a 1 μmole scale, and coupling efficiency was 98%.

The ability of the BuPdG-18mer to serve as a substrate for dNTP incorporation was shown by the results of extension reactions with TdT. Incubation of BuPdG-18mer with TdT in the presence of dGTP or BuPdGTP at 50 μM for 60 min at 35°C gave products as shown in Figure 1. The oligonucleotide gave

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a ladder of products with dGTP (lane A), but only supported extension by one molecule of BuPdGTP (lane B). These results indicate both that the terminal 3'-OH group of the BuPdG-18mer is free and that it can support dNTP incorporation.

BuPdGTP is a substrate for E.coli pol I

The primer-template illustrated in Figure 2 was used to monitor incorporation of nucleotides by E.coli pol I, Klenow fragment. 5'-32P-labelled 17mer primer, prepared with [γ-32P]ATP and polynucleotide kinase, was annealed to 29mer template as described previously. After incubation with pol I at 30°C for 10 minutes, the reaction mixtures were applied to a 8M urea-12% polyacrylamide gel and subjected to electrophoresis as described. As illustrated in Figure 2, incubation with all dNTPs at 10 μM produced fully copied 29mer product (lane I). Inclusion of 2 μM dGTP alone (lane C) or 2 μM each of dGTP and dTTP (lane E) resulted in nearly exclusive conversion of primer to the expected 18mer and 19mer, respectively. When 17mer:29mer was incubated with pol I and 100 μM BuPdGTP alone, a product migrating at nearly the same position as the 18mer was observed (lane D). This product appeared at the same position as 5'-32P-labelled BuPdG-18mer, synthesized as described above (lane B). Migration of the BuPdG-18mer in the gel was slightly slower than that of the conventional dG-18mer (Figure 2 and results not shown).

Apparent Km values for pol I-catalyzed incorporation of dGTP and BuPdGTP, defined as the concentrations of the substrates required to convert 50% of starting primer to product in 10 minutes at 30°C, were determined in single site extension reactions. Quantitation of starting and product oligomers derived from [32P]-labelled 17mer:29mer, separated on denaturing gels and measured with a Betascope analyzer, revealed apparent Km values of 0.8 and 2.0 μM for dGTP and BuPdGTP, respectively. However, the rate of incorporation of BuPdGTP was considerably slower than that of dGTP when substrates were present at '3×Km' concentrations. At 30°C the half time for conversion of 17mer to BuPdG-18mer in the presence of 6 μM BuPdGTP in primer extension reactions was 5 minutes. The half time for conversion of 17mer to 18mer with 2.5 μM dGTP was less than

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**Figure 1.** Extension of BuPdG-18mer by TdT. Reactions (10 μL) containing 3.6 μM [5'-32P]BuPdG-18mer and 0.25 units of terminal deoxyribonucleotidyl transferase were incubated for 1 h at 35°C, and products were analyzed by 8M urea-PAGE as described. Lane A, +50 μM dGTP. Lane B, +50 μM BuPdGTP. Lane C, BuPdG-18mer alone.

**Figure 2.** Primer extension reactions with E.coli pol I. Reactions were done as described in Materials and Methods, and utilized as primer:templates either 5'-[32P]-labelled-17mer:29mer or 5'-[32P]-labelled-BuPdG-18mer:29mer and pol I, and with the additions indicated. Products were analyzed by 8M urea-PAGE. Lanes A and I, 17mer, no dNTPs. Lane B, BuPdG-18mer, no dNTPs. Lane C, 17mer plus 2 μM dGTP. Lane D, 17mer plus 100 μM BuPdGTP. Lane E, 17mer plus 2 μM dGTP and dTTP. Lane F, 17mer plus 100 μM BuPdGTP and 2 μM dTTP. Lane G, BuPdG-18mer plus 2 μM dTTP. Lane H, 17mer plus BuPdG-18mer, no dNTPs. Lane J, 17mer plus 25 μM dGTP, dATP, dCTP, and dTTP.
1 minute at this temperature. These results indicate similar affinity of the nucleotides for the pol I-primer:template complex, but a significantly reduced rate of incorporation of the synthetic nucleotide compared to the natural substrate.

**BuPdGTP is a terminating substrate for pol I**

The results of two experiments suggested that the BuPdG-18mer was resistant to extension by pol I. Incubation of the 17mer:29mer primer:template with 100 μM BuPdGTP and 2 μM dTTP (Figure 2, lane F) did not result in a product migrating near the 19mer of lane E, but only the BuPdG-18mer. Similarly, incubation of synthetic BuPdG-18mer:29mer with 2 μM dTTP did not result in any product (lane G). To determine the extent of this resistance, the BuPdG-18mer:29mer was incubated with dTTP and pol I under various conditions. Incubations at concentrations as high as 100 μM dTTP and for up to 60 minutes at 30°C did not lead to extension of the BuPdG-primer by pol I (data not shown).

**3'-BuPdG primer is resistant to pol I 3' to 5' exonuclease**

In control extension reactions of the primer:templates lacking dNTP substrates, the 17mer primer was partially degraded by the 3' to 5' exonuclease activity of pol I, but no such degradation was observed with the BuPdG-18mer (results not shown). Because exonucleolytic cleavage of single stranded DNA is more efficient than that of double stranded DNA,12 we incubated labelled 17mer and BuPdG-18mer separately with pol I at 30°C and removed aliquots of the reactions at various times. The aliquots were subjected to denaturing PAGE with the results shown in Figure 3. It is clear that the 17mer is degraded to smaller oligomers with a half-life of about 15 minutes and that essentially none remains after 60 minutes. In contrast, no demonstrable degradation of the BuPdG-18mer was observed after incubation for 60 minutes (Figure 3).

**BuPdGTP is a weak inhibitor of pol I and protects activated DNA from exonucleolytic degradation**

BuPdGTP was added to assays of pol I with activated DNA in the presence 25 μM each of dATP, dCTP and dGTP, and the incorporation of 10 μM [3H]dTTP into acid-precipitable material was measured. Only 24% inhibition of label incorporation was observed at 100 μM BuPdGTP under these conditions. In contrast, when pol I was assayed in the absence of dGTP (the truncated assay), BuPdGTP stimulated labelled dTTP incorporation at low concentrations (Table 1). Because the results above showed that BuPdGTP is a terminating substrate for pol I, one would expect either no effect of the compound or inhibition rather than stimulation of enzyme activity in the truncated assay. The results are, however, consistent with two possibilities. First, BuPdGTP may inhibit the 3' to 5' exonuclease activity directly. This is probably not the case, because BuPdGTP at 100 μM did not inhibit exonucleolytic release of [3H]dTMP after incubation of [3H]-labelled single stranded DNA with pol I (data not shown). Second, the low level of BuPdGTP incorporation at G-requiring sites in the absence of dGTP may protect the primers from 3' to 5' exonuclease degradation; thus the control counts measured in the absence of dGTP would represent the net difference between incorporation and removal of nucleotides when the enzyme is unable to bind the next substrate, i.e. dGTP.

### DISCUSSION

BuPdGTP inhibits eukaryotic DNA polymerase α with high potency (K<sub>i</sub>, ca. 1–5 nM), but is not a substrate for the enzyme.2 Thus its mechanism of inhibition of pol α likely involves sequestration of the enzyme in a tight, but reversible, complex consisting of the nucleotide, the primer:template and the enzyme. Indeed, sequestration experiments have shown that the latter mechanism pertains not only for BuPdGTP, but for other non-incorporable DNA polymerase inhibitors.2,4 We recently found that the lack of ability to incorporate BuPdGTP was not shared by another sensitive DNA polymerase, that from the bacteriophage T4. The T4 enzyme is inhibited with nanomolar potency by BuPdGTP, but is able to incorporate the analog.4 The need for an authentic 3'-BuPdG oligomer to test hypotheses about the mechanism(s) of DNA polymerase inhibition by BuPdGTP prompted the chemical synthesis reported in this paper.

*E. coli* DNA polymerase I has been considered resistant to BuPdGTP, based on the weak inhibition (see above) or lack of inhibition observed in standard assays of the enzyme. In this paper we have shown that, in the absence of competing dGTP, BuPdGTP stimulates residual synthesis catalyzed by pol I in a truncated assay lacking dGTP, and is a substrate with surprisingly good affinity for the enzyme. The apparent K<sub>i</sub> values of 0.1 μM and 2.0 μM for dGTP and BuPdGTP in single site extension reactions (see above) indicate similar affinities of the nucleotides for the enzyme-primer:template complex. However, the profoundly reduced incorporation rate of the modified substrate (see above) explains the apparent resistance of pol I activity toward the compound in standard assays containing dGTP. These

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**Table 1. Effect of BuPdGTP on *E. coli* pol I.**

<table>
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<th>BuPdGTP, μM</th>
<th>pmol [3H]dTTP incorporated&lt;sup&gt;(4)&lt;/sup&gt;</th>
<th>+ dGTP</th>
<th>− dGTP</th>
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<tr>
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<td>15.8</td>
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<sup>(4)</sup> Assays were done with activated DNA as described in Materials and Methods. Standard deviations of duplicate experiments averaged ± 0.1 pmol, and background incorporation in assays lacking enzyme was 0.04 pmol. + dGTP corresponds to 25 μM of dGTP, and − dGTP corresponds to the truncated assay.

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**Figure 3.** Exonucleolytic cleavage of single stranded primers by pol I. 5'-[32P]-labelled 17mer and BuPdG-18mer (1.2 μM) were separately incubated with pol I in extension buffer (see Materials and Methods) for various periods of time, and the products were analyzed by 8M urea-PAGE.
results suggest that the BuPdG-primers, when formed, do not have substantial affinity for the enzyme. In contrast, the high potency of BuPdGTP for T4 DNA polymerase may result directly from incorporation of the analog. Our application of the primer extension reactions and synthetic BuPdG-primer:templates will help to clarify these issues.

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