N^7-Cyanoborane−2′-Deoxyguanosine 5′-Triphosphate Is a Good Substrate for DNA Polymerase†

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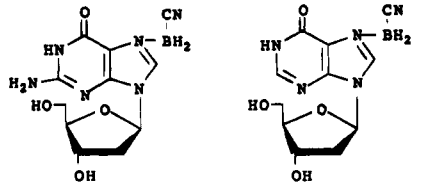
ABSTRACT: The 5′-triphosphate of the boronated nucleoside analog N^7-cyanoborane−2′-deoxyguanosine (^{7b}dGTP) was synthesized, and a series of experiments was initiated to assess the potential of the compound to serve as a substrate for DNA polymerases. We show here that ^{7b}dGTP can be incorporated into DNA by Sequenase. The resulting hemiboronated extension products are resistant to cleavage by treatment with either DMS and heat or a number of restriction enzymes. Further, in the polymerase chain reaction, ^{7b}dGTP can be utilized as a substrate for *Taq* polymerase. Finally, by kinetic analysis, we have found that ^{7b}dGTP is a more efficient substrate for exonuclease-free Klenow than normal dGTP. Thus, the introduction of a cyanoborane moiety to the N^7 position of dGTP results in a nucleotide that is accepted in lieu of normal dGTP by a number of DNA polymerases.

Our laboratory has synthesized a new class of modified 2′-deoxynucleosides that introduce a cyanoborane moiety to the purine bases of 2′-deoxyguanosine **1**, 2′-deoxyinosine **2**, and 2′-deoxyadenosine **3** and to the pyrimidine base of 2′-deoxycytidine **4** (Sood *et al.*, 1989). Preliminary *in vitro* and *in vivo* studies in rodents have shown that cyanoboronated nucleosides possess a number of potentially useful pharmacological properties, including antineoplastic (Sood *et al.*, 1992), hypolipidemic (Hall *et al.*, 1993), and anti-inflammatory activities (Rajendran *et al.*, 1994).



The pharmacological activity of a number of 2′-deoxynucleoside analogs depends on the ability of the compound to be phosphorylated by cellular kinases and subsequently incorporated into DNA by DNA polymerases (Reid *et al.*, 1988; Reardon & Spector, 1989; Reardon, 1992). Studies by Sood *et al.* (1992) suggest that phosphorylation and subsequent incorporation into DNA is a possible mechanism for the antineoplastic activity of the cyanoboronated nucleosides. Those results showed that N^3-cyanoborane−2′-deoxycytidine (**4**, ^{3b}dC[1]) was taken up rapidly by Tmolt$_3$ leukemia cells. After 24 h, a significant amount of the ^{3b}dC was found to be associated with DNA and/or RNA fractions of the Tmolt$_3$ leukemia cells.

Of the major cyanoboronated deoxynucleosides, the deoxyguanosine derivative (**1**) is the only compound in which the cyanoborane moiety does not interfere with the potential Watson−Crick hydrogen-bonding groups. Preliminary physical studies by the method of Williams *et al.* (1989) have shown that N^7-cyanoborane−2′-deoxyguanosine (^{7b}dG, (**1**)) is capable of base-pairing with 2′-deoxycytidine (B. Banks and B. R. Shaw, unpublished results). Therefore, it is possible that after cellular uptake and phosphorylation, the cyanoboronated deoxyguanosine triphosphate may be recognized by DNA polymerase and inserted into DNA opposite dC.

In this paper, we report the synthesis of the 5′-triphosphate of the boronated 2′-deoxyguanosine nucleoside (N^7-cyanoborane−2′-deoxyguanosine 5′-triphosphate, *i.e.*, ^{7b}dGTP (**5**)),

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[1] Abbreviations: ^{3b}dC, N^3-cyanoborane−2′-deoxycytidine; ^{7b}dG, N^7-cyanoborane−2′-deoxyguanosine; dG, 2′-deoxyguanosine; ^{7b}dGTP, N^7-cyanoborane−2′-deoxyguanosine 5′-triphosphate; dGTP, 2′-deoxyguanosine 5′-triphosphate; dA, 2′-deoxyadenosine; dT, thymidine; dC, 2′-deoxycytidine; dI, 2′-deoxyinosine; bp, base pairs; CD, circular dichroism; CM, carboxymethyl; DNA, 2′-deoxyribonucleic acid; DEAE, (diethylamino)ethyl; DMS, dimethyl sulfate; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; Klenow, the large fragment of *Escherichia coli* DNA polymerase I; MS, mass spectrometry; NMR, nuclear magnetic resonance; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; RT, room temperature; Tris, tris(hydroxymethyl)aminomethane; UV, ultraviolet.

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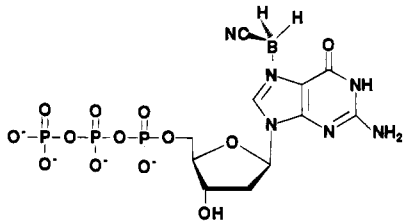
and an analysis of its incorporation into DNA by polymerases. Incorporation was performed using three prokaryotic DNA polymerases: Sequenase, *Taq* DNA polymerase, and an exonuclease-free mutant of the Klenow fragment from *Escherichia coli* DNA polymerase I. Sequenase and *Taq* polymerase were able to insert ^{7b}dGTP into DNA and extend the growing strand past the point of insertion. Exonuclease-free Klenow was used to determine the kinetic parameters for ^{7b}dGTP incorporation. Results show that ^{7b}dGTP was incorporated into DNA more efficiently than normal dGTP.



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MATERIALS AND METHODS

Materials. *N,N*-Dimethylformamide was distilled from CaH_2. Trimethyl phosphate (Aldrich, 99%) was distilled at 1 mmHg and stored over 4-Å molecular sieves. Phosphoryl chloride was distilled and stored in sealed ampules at 4 °C. Bis(tri-*n*-butylammonium) pyrophosphate (0.5 M) in *N,N*-dimethylformamide was prepared as described by Tomasz (1983). DEAE-cellulose (DE-32) and CM-cellulose (CM-32) were purchased from Whatman. Kieselgel 60 F_{254} 0.2-mm chromatoplates were purchased from Merck. Oligonucleotides were synthesized on an ABI 380B DNA synthesizer, purified by denaturing PAGE, and recovered by electroelution. Sequenase version 2.0 (modified T7 DNA polymerase) and exonuclease-free Klenow were purchased from USB. *Taq* polymerase was purchased from Cetus. M13mp2 DNA was provided kindly by Ted Gonzalez (Frederico *et al.*, 1990). [γ-^{33}P]ATP and [γ-^{35}S]ATP (>1000 Ci/mmol) were purchased from Amersham.

NMR. NMR spectra of an approximately 0.1 M solution were recorded at ambient temperature on a Varian Associates Model XL-300 spectrometer at 300 (^{1}H), 121.44 (^{31}P), or 75.57 MHz (^{13}C). ^{1}H and ^{13}C chemical shifts were referenced to tetramethylsilane, while ^{31}P shifts were referenced to 85% H_3PO_4.

Mass Spectrometry. MS spectra were taken on a VG 70S tandem hybrid MS/MS spectrometer run in the MS only mode. The accelerating voltage was 10 kV. A cesium ion gun at 35 kV was used for sample ionization using glycerol as a matrix.

Evaporations were carried out at RT using a rotary evaporator.

N^7-Cyanoborane−2′-Deoxyguanosine 5′-Triphosphate. N^7-Cyanoborane−2′-deoxyguanosine (^{7b}dG, **1**) was prepared according to the method of Sood *et al.* (1989, 1992). The method of Ludwig (1981, 1987), as modified by Kovács and Ötvös (1988), was adapted to the synthesis of N^7-cyanoborane−2′-deoxyguanosine 5′-triphosphate (**5**).

Proton sponge (214 mg, 1.0 mmol) was added to a solution of ^{7b}dG (153 mg, 0.5 mmol) in trimethyl phosphate (1.25 mL). Phosphoryl chloride (68.2 μL, 0.75 mmol) was pipeted into the reaction mixture under vigorous stirring at 0 °C. Stirring was continued with the exclusion of atmospheric moisture at 0 °C for 2 h. Bis(tri-*n*-butylammonium) pyrophosphate (0.5 M) in *N,N*-dimethylformamide (5.0 mL, 2.5 mmol) and tri-*n*-butylamine (0.5 mL, 2.1 mmol) were added quickly and synchronously to the reaction mixture. The reaction mixture was stirred intensively for 1 min; then aqueous triethylammonium hydrogen carbonate (20 mL, 0.5 M, pH 7.5) was added. Stirring was continued for an additional 1.5 h at RT. The solution was evaporated to dryness. The residue was freed from excess triethylammonium hydrogen carbonate by repeated evaporation with methanol. The residue at evaporation was dissolved in deionized water (5 mL). The solution was applied onto a DEAE-cellulose [HCO_3^-] column (2.1 × 52.0 cm). The column was eluted at 10 °C by using a linear gradient of aqueous triethylammonium hydrogen carbonate, pH 7.5 (2000 mL, 0−0.5 M; speed, 20 mL/fraction/20 min). Appropriate fractions were pooled and evaporated to dryness. Excess triethylammonium hydrogen carbonate was removed from the residue as described above. The evaporated residue was dissolved in deionized water (1 mL). The solution was percolated through a CM-cellulose [Na^+] column (1.4 × 12.0 cm). The column was washed with deionized water (speed, 4.0−4.5 mL/10 min). Fractions that showed UV absorbancy were combined and freeze-dried to give 139 mg (40%) of a white solid containing less than 5% contaminants (by thin-layer chromatography on a silica gel chromatoplate in *n*-PrOH/concentrated NH_4OH/H_2O (11/7/2), R_f = 0.26, and by ^{31}P NMR): ^{1}H NMR (D_2O) δ 8.369 (s, 1H, H8), 6.615−6.145 (unres. 1H, H1′), 4.607-4.600 (unres. 1H, H3′), 4.130−4.062 (2m, 3H, H4′ + H5′ + H5″), 2.608−2.568 and 2.461−2.380 (2m, 4H, H2′ + H2″ + BH_2); ^{31}P NMR (D_2O) δ −7.59 (d, J_{PP} = 19.4 Hz, 1P, P3), −10.32 (m, J_{PP} = 19.3 Hz, J_{PH} = 5.5 Hz, 1P, P1), −21.37 (t, J_{PP} = 19.0 Hz, 1P, P2); ^{13}C{^{1}H} NMR (D_2O) δ 155.80 (s, C6), 154.94 (s, C2), 150.72 (s, C4), 138.75 (s, C8), 110.89 (s, C5), 86.04 (d, J_{CP} = 8.6 Hz, C4′), 84.86 (s, C1′), 70.42 (s, C3′), 65.10 (d, J_{CP} = 5.5 Hz, C5′), 38.63 (s, C2′); MS *m/z* [$C_{11}H_{14}BN_6O_{13}P_3Na_4H$]$^+$ = 635.

HPLC. The compound was purified further by HPLC on a Delta-Pak C18 100-Å (3.9 × 300 mm, 15 μm spherical) column (Waters) using a linear gradient of methanol (0−10% in 10 min) in triethylammonium acetate (0.2 M, pH 7.5) with a flow rate of 4.0 mL/min (retention time, 16.32 min; retention time of 2′-deoxyguanosine 5′-triphosphate, 9.81 min). The HPLC-purified material, after the removal of triethylammonium acetate by lyophilization, was reconverted to the sodium salt as described before.

Incorporation of ^{7b}dGTP into M13mp2. Primer SS20 (5′-TATCGGCCTCAGGAAGATCG-3′, complementary to positions 6467−6448 of M13mp2; 10 pmol) was 5′-end-labeled with [γ-^{35}S]ATP (20 μCi) and polynucleotide kinase (10 units; New England Biolabs) in the manufacturer-supplied buffer (10 μL). The labeled primer was annealed to a single-stranded (+)M13mp2 DNA template (8 pmol) in NT buffer (50 mM Tris-HCl, pH 7.2, 10 mM $MgSO_4$, 0.1 mM DTT, and 50 μg/mL BSA; 30 μL) supplemented with dATP, dTTP, dCTP, and either dGTP or ^{7b}dGTP (83 mM each; ^{7b}dGTP ϵ_{260} = 12.5 × 10^3 L/mol·cm) by heating to 95 °C, incubating at 60 °C for 10 min, and then cooling on ice. Sequenase (6.5 units, 0.5 μL) was added, allowed to react for 15 min at room temperature, and stopped by heating at 70 °C for 10 min. The extended primer/template duplexes were used directly from the terminated Sequenase reactions.

DMS Cleavage. Extended M13 primer/template complexes (≈1 pmol, 3.5 μL) were exposed to 20 mM DMS for 10 min on ice. Reactions were stopped by adding DTT to 91 mM. The methylated DNA was cleaved by heating to 90 °C for 8 min and returned to ice. The cleaved DNA was recovered by ethanol precipitation.

Restriction Digestion. Extended M13 primer/template complexes (3.5 μL) were digested by *HaeIII* (16 units), *PvuI* (10 units), *PvuII* (12 units), or *Sau3AI* (4 units) in manufacturer-supplied buffer (20 μL) at 37 °C for 20 min. The DNA was recovered by ethanol precipitation.

Electrophoresis. Samples were resuspended in 0.5× denaturing loading buffer (95% formamide, 0.1% bromophenol blue, 0.1% xylene cyanol, and 20 mM Na_2EDTA), loaded onto an 8% polyacrylamide/7 M urea gel, and run for 2 h at 75 W in TBE buffer (89 mM Tris−borate and 2 mM Na_2-EDTA, pH 8.0). The gel was dried under vacuum, and the signal was detected by autoradiography. Quantitation was carried out on a Molecular Dynamics phosphorimager.

Polymerase Chain Reaction. Primers used for PCR were SS20 and RP (5′-TCACACAGGAAACAGCTATGAC-3′, M13mp2 positions 6200−6221). (+)M13mp2 DNA template (0.5 pmol) was mixed with primers (20 pmol each), dATP, dTTP, dCTP, and either dGTP or ^{7b}dGTP (100 mM each) in 100 μL of PCR buffer (50 mM KCl, 1.5 mM $MgCl_2$, and 10 mM Tris-HCl, pH 8.3, RT). The reaction mixture was heated to 95 °C for 1 min and returned to ice. *Taq* polymerase (0.5 μL, 2.5 units) was added, and the PCR was performed in an Ericomp thermal cycler for 25 cycles of 95 °C for 1 min, 56 °C for 1 min, and 72 °C for 1 min. Following PCR amplification, the DNA was ethanol precipitated and then resuspended in 20 μL of 10 mM Tris-HCl (pH 7.6). An aliquot (8 μL) was digested with *Sau*3AI (4 units) in the manufacturer-supplied buffer (10 μL) for 30 min at 37 °C. Unrestricted (8 μL) and restricted DNA (10 μL taken directly from the restriction digestion) were mixed with 1 μL of glycerol loading buffer (30% glycerol, 0.1% bromophenol blue, and 0.1% xylene cyanol) and separated on a 3% Nu-Sieve (FMC)/1% Sea-Kem (FMC) agarose gel.

Kinetic Analysis. The K_m and V_{max} values for the incorporation of normal dGTP and boronated ^{7b}dGTP at 37 °C were determined by a labeled primer/template polyacrylamide gel assay (Randall *et al.*, 1987; Boosalis *et al.*, 1987). The system consisted of a radioactively labeled synthetic primer annealed to a synthetic template, which is designed to code for a dG at the first position of primer extension. Primer extension was performed for a range of substrate concentrations. The extension products were separated by denaturing PAGE and quantitated. The initial reaction velocities (v_i) could be determined from the integrated band intensities. The intensity of the extended product (I_1) divided by the intensity of the unextended primer (I_0) plus one-half the intensity of the extended product ($0.5I_1$) gives a measure of v_i (Petruska *et al.*, 1988).

Primer (5′-CAGGAACAGCTATGGCCTCA-3′; 30 pmol) was end-labeled with 10 μCi of [γ-^{33}P]ATP, annealed to an equal amount of template (5′-GTGTAGCTGAGGCCAT-AGCTGTTCCTG-3′; 30 pmol), and mixed with 0.109 unit (0.0167 unit/reaction) of exonuclease-free Klenow in buffer A (50 mM Tris-HCl, pH 7.6, and 5 mM DTT; 32.5 μL). The primer was extended by mixing 5 μL of the primer/template duplexes with 5 μL of various concentrations of dGTP or ^{7b}dGTP in buffer B (50 mM Tris-HCl, pH 7.6, 5 mM DTT, and 20 mM $MgCl_2$). The reaction was carried out for 1.5 min at 37 °C and stopped by the addition of 10 μL of loading buffer (95% formamide, 20 mM Na_2EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol). The samples were separated on a 16% polyacrylamide/7 M urea sequencing gel, and the intensities of the bands were quantitated. The K_m and V_{max} values were calculated from the initial reaction velocities by nonlinear regression analysis. To verify that the initial velocities were obtained at an enzyme-limiting condition, the amount of enzyme was adjusted to produce about 20% extension at the highest substrate concentrations (Goodman *et al.*, 1993).

Time Course Reactions. We verified that the initial velocities were obtained during the period of linear accumulation of product by performing a time course experiment for the highest concentrations of substrate. Labeled primer/template (30 pmol) and exonuclease-free Klenow (0.109 unit) in buffer A (32.5 μL) were mixed with dGTP (0.2 μM) or ^{7b}dGTP (0.1 μM) in buffer B (32.5 μL) at 37 °C. Aliquots were withdrawn at 30-s intervals and mixed with loading buffer. Analysis showed that the product accumulated linearly from 30 s to 3 min (r^2 = 0.994 for dGTP; r^2 = 0.996 for ^{7b}dGTP).

RESULTS

Once pure N^7-cyanoborane−2′-deoxyguanosine 5′-triphosphate (**5**, ^{7b}dGTP) was prepared, two questions had to be answered to determine whether the ^{7b}dGTP was a substrate for DNA polymerase. (1) Could a primer be extended in the presence of ^{7b}dGTP? (2) Could the ^{7b}dG moiety be incorporated stably into the newly synthesized duplex? To answer the first question, an oligonucleotide primer was extended with DNA polymerase in the presence of dATP, dCTP, dTTP, and either dGTP or ^{7b}dGTP. Results, described here, show that the primer could be extended by Sequenase using ^{7b}dGTP for a substrate. To answer the second question, the presence of the ^{7b}dG residue in the newly synthesized duplex was verified by a methylation protection experiment and several restriction enzyme protection experiments. When ^{7b}dGTP was used for a substrate, results show that (a) the DNA product was protected from cleavage by treatment with dimethyl sulfate (DMS) and heat, and (b) the dG-containing recognition sites in the product were protected from cleavage by the restriction enzymes *Hae*III, *Pvu*I, *Pvu*II, and *Sau*3AI.

Incorporation of ^{7b}dGTP by Sequenase. The labeled primer, SS20, was annealed to the (+) strand of M13mp2 and extended by Sequenase in the presence of dATP, dCTP, dTTP, and either dGTP (lanes labeled G) or ^{7b}dGTP (lanes labeled b). Figure 1 shows that the SS20 primer was extended in the presence of dGTP (lane 1) as well as ^{7b}dGTP (lane 2). The dGTP products extended well into the upper ranges of the gel (500 to several thousand base pairs; lane 1). Likewise, the ^{7b}dGTP (lane 2) was incorporated into duplexes extending to hundreds of base pairs, indicating multiple incorporations and extensions of the boronated deoxynucleoside triphosphate. However, the pattern of extension products shown in lane 2 differed somewhat from the pattern produced by normal dGTP primer extension, in that there were pronounced dark bands at positions that required three or more consecutive ^{7b}dGTP incorporations. For example, there are three intense groups of bands at position 6356 (GGG), at position 6343 (GGG), and especially

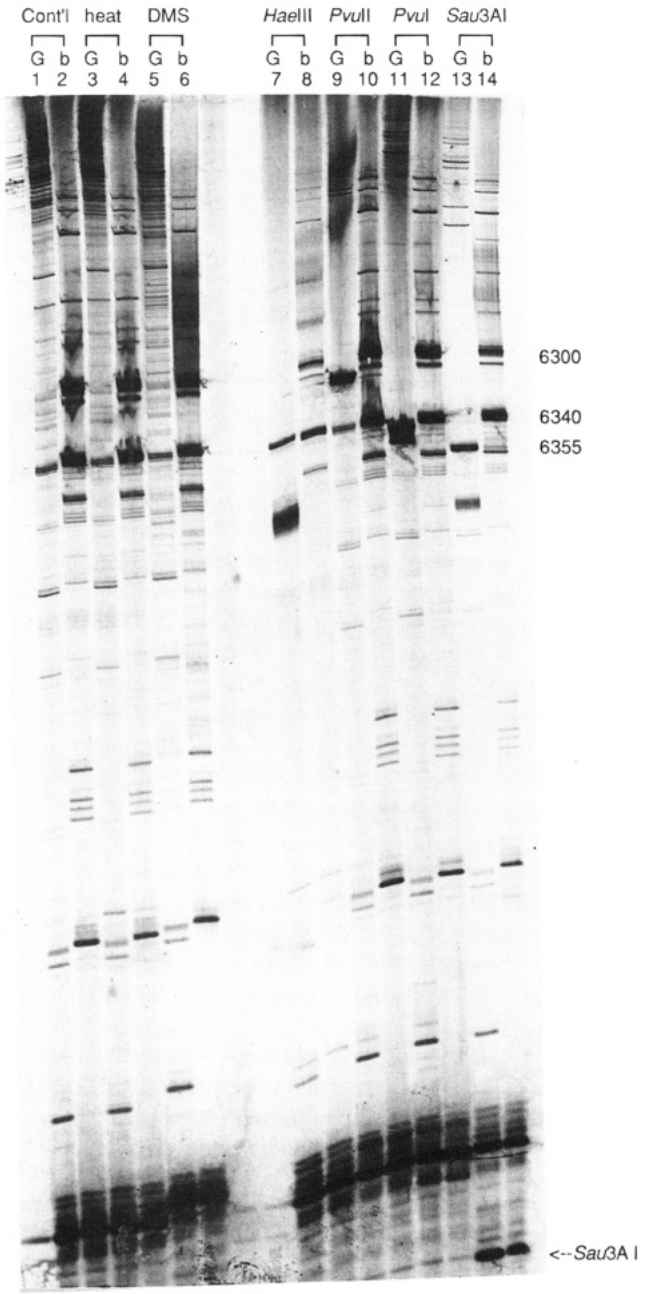

FIGURE 1: Incorporation of ^{7b}dGTP by Sequenase into an M13mp2 duplex and inhibition of restriction endonucleases by ^{7b}dG. Labeled primer SS20 was annealed to a single-stranded (+)M13mp2 DNA template, mixed with dATP, dTTP, dCTP, either dGTP (lanes labeled G) or ^{7b}dGTP (lanes labeled b), and Sequenase, and allowed to react for 15 min at room temperature, and the reactions were stopped by heating at 70 °C for 10 min. Extended primers are shown in lanes 1 and 2. The extension products were either heated to 90 °C for 8 min (lanes 3 and 4) or exposed to 20 mM DMS for 10 min on ice. The reactions were stopped with 91 mM DTT and then heated to 90 °C for 8 min (lanes 5 and 6). In lanes 7−14, extended M13 primer/template complexes were digested with *Hae*III (lanes 7 and 8), *Pvu*II (lanes 9 and 10), *Pvu*I (lanes 11 and 12), or *Sau*3AI (lanes 13 and 14) in manufacturer-supplied buffer at 37 °C for 20 min. All samples were precipitated with ethanol, resuspended in 0.5× denaturing loading buffer, loaded onto an 8% polyacrylamide/7 M urea gel, and run for 2 h at 75 W in TBE buffer.

at position 6309 (GGGGG). The intense bands indicate a stall of the polymerase at these points. It was not clear whether the polymerase extended through these points by incorporating a ^{7b}dGTP into each position, albeit slowly with respect to dGTP, or whether the polymerase waited for and incorporated a normal dGTP that could be present at a low concentration due to contamination in the starting material or deboronation of ^{7b}dGTP. To confirm that the ^{7b}dGTP had been incorporated stably into the extension products, we performed methylation protection and restriction enzyme protection assays, which are described in the following sections.

DMS Protection. DMS methylates DNA preferentially at the N^7 position of 2′-deoxyguanosine; consequently, upon heating, the glycosidic bond of the methylated 2′-deoxyguanosine can be broken and the DNA strand is cleaved (Maxam & Gilbert, 1977). Unlike dG, however, ^{7b}dG cannot be methylated because the BH_2CN moiety occupies the N^7 methylation site. Further, the purine ring of ^{7b}dG is uncharged, and consequently it is more stable to depurination than the positively charged N^7-methylated dG. Therefore, it was anticipated that DMS/heat treatment should not cleave DNA at ^{7b}dG sites.

As shown by Figure 1, primers extended with either dGTP or ^{7b}dGTP were unaffected by heat treatment (compare lanes 3 and 4 to lanes 1 and 2). However, when each sample was treated with DMS prior to heat treatment, the dG-containing extension products (DMS, lane G) were cleaved (compare the control lane 1 with the DMS lane 5, especially in the region from 6350 to about 6200). In contrast, the extension products polymerized in the presence of ^{7b}dGTP were resistant to cleavage by DMS/heat treatment (compare the control lane 2 with the DMS lane 6). Even though lane 6 exhibits a high degree of radioactive background in the 6350-6200 region, it appears that the higher molecular weight extension products were not cleaved significantly by the DMS/heat treatment. Therefore, the data suggest that the N^7-cyanoborane group blocks the 2′-deoxyguanosine residues of the extended primers from methylation by DMS.

Restriction Enzyme Protection. Restriction enzymes can be inhibited sterically by bulky adducts in the major groove of DNA (Gromova & Shabarova, 1990). If a cyanoborane moiety at the N^7 position of guanosine were to inhibit restriction digestion, and if ^{7b}dG were incorporated stably into duplex DNA during primer extension, then the duplexes produced by primer extension in the presence of ^{7b}dGTP should be resistant to cleavage by restriction endonucleases.

In Figure 1, the extended primer/template duplexes were digested with *Hae*III, *Pvu*II, or *Pvu*I. In each case, the dG-containing sample (lanes labeled G) was cleaved at the appropriate site: *e.g.*, *Hae*III at position 6342 (GG↑CC, lane 7), *Pvu*II at position 6324 (CAG↑CTG, lane 9), and *Pvu*I at position 6353 (CGAT↑CG, lane 11). In contrast, the duplexes polymerized in the presence of ^{7b}dGTP were resistant to restriction digestion (compare lanes 8, 10, and 12, labeled b, with the control lanes labeled G). Resistance to restriction digestion suggests that a ^{7b}dG residue is present at each restriction site; however, it is also possible that resistance to cleavage could have been the result of nonspecific enzyme inhibition due to the presence of the boronated nucleotides in the DNA or reaction mix.

To rule out the possibility of nonspecific enzyme inhibition, the duplexes were digested with *Sau3AI*. Figure 1 shows that the duplexes polymerized in the presence of dGTP were cleaved at the appropriate site (↑GATC, position 6354, lane 13); in contrast, the duplexes polymerized in the presence of ^{7b}dGTP were not cleaved at position 6354 (lane 14). In addition, position 15 of the SS20 primer is a *Sau3AI*

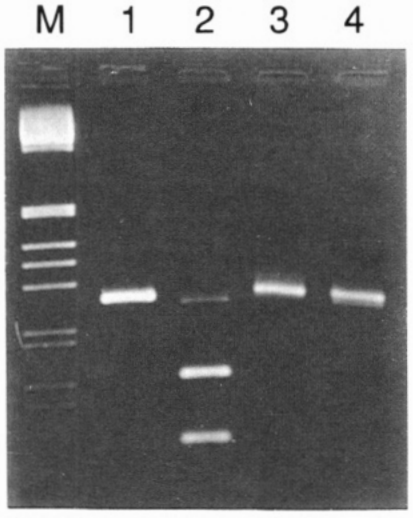

FIGURE 2: Polymerase chain reaction using ^{7b}dGTP. (+)M13mp2 DNA template (0.5 pmol) was mixed with RP and SS20 primers (20 pmol each) and 100 mM each dATP, dTTP, dCTP, and either dGTP (lanes 1 and 2) or ^{7b}dGTP (lanes 3 and 4) in PCR buffer. The reaction mixture was heated to 95 °C for 1 min and returned to ice. *Taq* polymerase (2.5 units) was added, and the PCR was performed for 25 cycles of 95 °C for 1 min, 56 °C for 1 min, and 72 °C for 1 min. Following PCR amplification, the DNA was ethanol precipitated and then resuspended in 20 μL of 10 mM Tris-HCl (pH 7.6). An aliquot (8 μL) was digested with *Sau*3AI (4 units) in manufacturer-supplied buffer (10 μL) for 30 min at 37 °C. Unrestricted (8 μL; lanes 1 and 3) and restricted DNA (10 μL, taken directly from the restriction digestion; lanes 2 and 4) were mixed with 1 μL of glycerol loading buffer and separated on a 4% agarose gel. Lane M: 1-kb ladder (BRL).

restriction site that can serve as an internal control. At the bottom of the autoradiograph, cleavage at primer position 15 can be seen plainly in both lanes 13 and 14, yet cleavage was inhibited at position 6354 in lane 14. Therefore, we conclude that the inhibition at site 6354 of lane 14 was due to the inability of the restriction enzyme to cope with the boronated base at the restriction site and not to nonspecific inhibition of the enzyme by free or polymerized boronated 2′-deoxyguanosine in the reaction mix. Thus, the specific inhibition of *Sau*3AI at position 6354 shows that ^{7b}dG was introduced stably into the duplex by polymerization with Sequenase.

^{7b}dGTP Can Substitute for dGTP in the Polymerase Chain Reaction. The polymerase chain reaction (Saiki *et al.*, 1985; Mullis & Faloona, 1987) was performed using normal dATP, dTTP, dCTP, and either dGTP or ^{7b}dGTP. The PCR products were separated on a 4% agarose gel and visualized by ethidium bromide fluorescence. The results presented in Figure 2 show that the correct 267-bp PCR product was produced using either dGTP (lane 1) or ^{7b}dGTP (lane 3), indicating that ^{7b}dGTP can substitute for dGTP in the polymerase chain reaction. The amount of DNA produced by both PCR reactions was similar; upon comparing the intensities of the product bands, the amount of DNA produced in the presence of ^{7b}dGTP (lane 3) was reduced only slightly relative to that produced in the presence of dGTP (lane 1). The presence of ^{7b}dG in the PCR products was verified by *Sau3AI* digestion. In the control lane 2, the PCR product produced in the presence of dGTP was cleaved completely at site 6354 into the two expected bands of 98 and 154 bp (the 15-mer band due to cleavage within the primer was beyond the resolution of the gel). In contrast,

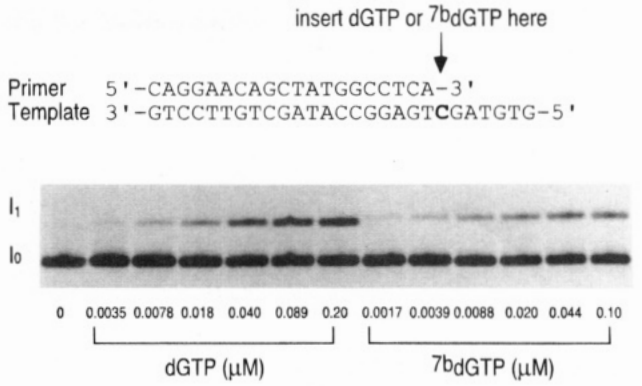

FIGURE 3: Primer extension analysis to the kinetics of dGTP and ^{7b}dGTP incorporation. Primer was end-labeled, annealed to an equal amount of template, and mixed with exonuclease-free Klenow in buffer A. The primer was extended by mixing the primer/template duplexes with various concentrations of either dGTP or ^{7b}dGTP in buffer B. The samples were separated on a 16% polyacrylamide/7 M urea sequencing gel, dried, and exposed to X-ray film. I_0, unextended primer; I_1, extension product.

the PCR product produced in the presence of ^{7b}dGTP was not cleaved at site 6354 (lane 4). Inhibition of *Sau*3AI confirms that ^{7b}dG was introduced stably into the PCR products. Therefore, ^{7b}dGTP is a substrate for *Taq* polymerase, and long duplexes of ^{7b}dG-containing DNA can be produced by PCR.

During PCR, the high-temperature conditions caused some deboronation of the ^{7b}dGTP to produce dGTP. HPLC analysis of a ^{7b}dGTP solution showed that, after 25 cycles of PCR, the amount of ^{7b}dGTP was reduced from 82% (the majority of the impurities was presumably boronated dGDP) to 55% and the amount of dGTP increased from undetectable levels to 20% (data not shown). Nevertheless, we found that the PCR product produced in the presence of ^{7b}dGTP was almost 100% resistant to *Sau3*AI digestion, indicating that ^{7b}dGTP was incorporated preferentially into the PCR product even in the presence of normal dGTP. The incorporation of ^{7b}dGTP in the presence of a significant amount of dGTP suggested that the efficiency of incorporation was higher for ^{7b}dGTP than for dGTP, which was confirmed by kinetic analysis.

Kinetic Analysis. The kinetic parameters for incorporation of dGTP and ^{7b}dGTP by exonuclease-free Klenow were determined by a primer extension/polyacrylamide gel assay (Randall *et al.*, 1987; Boosalis *et al.*, 1987). Radioactively labeled primer was annealed to a template and extended from a standing start with various concentrations of dGTP or ^{7b}dGTP, as shown in Figure 3. The calculated initial velocity vs substrate concentration plots for the illustrated experiment are shown in Figure 4. The results of nine experiments of the type shown in Figures 3 and 4 were averaged and are presented in Table 1.

While the V_{max} for ^{7b}dGTP was 40% lower than the V_{max} for dGTP (10.6% vs 18.5% extension/min), the K_m for ^{7b}dGTP was over 5 times lower than the K_m for dGTP (*i.e.*, 0.007 vs 0.39 μM). The ratio of V_{max} to K_m is the apparent second-order rate constant, or efficiency, of the reaction. Therefore, the large decrease in K_m relative to the small decrease in V_{max} leads to an increased efficiency for the incorporation of ^{7b}dGTP (17.1) as compared to dGTP (5.2). The incorporation efficiency of ^{7b}dGTP relative to dGTP, or f, is equal to 3.5. Thus, the efficiency of incorporation

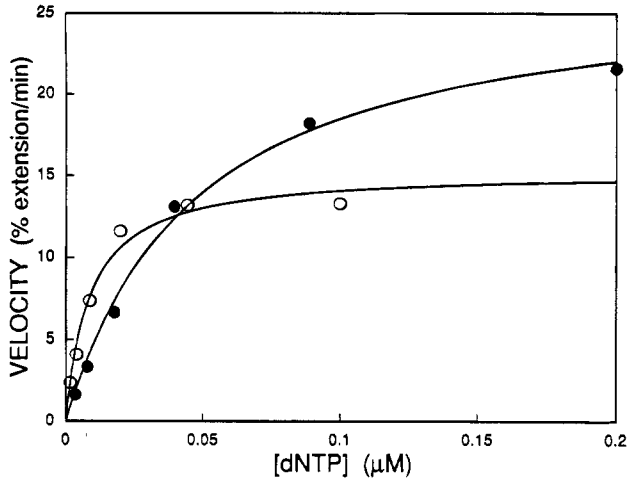

FIGURE 4: Representative Michaelis–Menten plot for the extension kinetics of dGTP (●) and ^{7b}dGTP (○). Extension products were imaged on a Molecular Dynamics phosphorimager, integrated, and used to calculate initial reaction velocities. Initial velocities were plotted vs substrate concentration.

Table 1: Kinetic Parameters for the Incorporation of dGTP and ^{7b}dGTP[a]

	V_{max} (% ext/min)	K_m (μM)	V_{max}/K_m	f[b]
dGTP	18.5 ± 5.9	0.039 ± 0.016	5.2 ± 1.6	1
^{7b}dGTP	10.6 ± 2.3	0.007 ± 0.002	17.1 ± 5.3	3.5

[a] Results are the averages of nine experiments. [b] f is the ratio of the incorporation efficiency of ^{7b}dGTP compared to that of normal dGTP.

by exonuclease-free Klenow is, indeed, higher for the base-boronated ^{7b}dGTP than for normal dGTP.

DISCUSSION

The base-boronated 2′-deoxynucleoside 5′-triphosphate, ^{7b}dGTP, proved to be a good substrate for Sequenase in a primer extension experiment. A labeled primer was extended successfully for several hundred bases, with only occasional pauses at sites that required three or more consecutive ^{7b}dGTP incorporations. The ^{7b}dG moiety was shown by DMS and restriction enzyme protection assays to have been introduced stably into the newly synthesized hemiboronated duplex. Sites occupied by ^{7b}dG are resistant to methylation and subsequent cleavage by heat, thereby providing a method for their detection. Unlike normal dG, the boronated dG is blocked at the N^7 position and thus would be expected to block recognition of the major groove by proteins or by a third potential triple-helical strand *via* Hoogsteen recognition.

^{7b}dGTP was incorporated successfully into DNA by PCR. Since PCR should be capable of producing microgram quantities of fully boronated duplexes, PCR amplifications can be designed to make longer duplexes of defined length than would be possible by primer extension.

^{7b}dGTP proved to be a more efficient substrate for incorporation by exonuclease-free Klenow than normal dGTP. In a series of kinetic experiments, the V_{max} for incorporation of ^{7b}dGTP was within a factor of 2 of the V_{max} for normal dGTP; however, a 5-fold lower K_m for ^{7b}dGTP relative to dGTP resulted in an efficiency of incorporation for ^{7b}dGTP that was 3.5 times greater than that of normal dGTP. The decrease in K_m possibly is due to the increased strength of the ^{7b}dG:dC base pair (B. Banks and B. R. Shaw, unpublished results). Caution must be exercised in drawing general conclusions from the observed increased efficiency, because the kinetic parameters were determined for only one sequence and the efficiency of incorporation depends on the sequence surrounding the incorporation site (Reeves & Beattie, 1985; Mendelman *et al.*, 1989; Singer *et al.*, 1989; Yu & Goodman, 1992; Joyce *et al.*, 1992). Nevertheless, we know of no other instance of a 2′-deoxyguanosine 5′-triphosphate analog that is incorporated into DNA more efficiently than the normal dGTP substrate.

The efficiency of enzymatic incorporation of 2′-deoxynucleoside 5′-triphosphate analogs into DNA by polymerase depends on the ability of the compound to form a stable Watson–Crick base pair (Echols & Goodman, 1991). For example, among 5′-triphosphate analogs of 2′-deoxyguanosine, modifications of the purine ring that disrupt hydrogen bonding, such as O^6-methyl-dGTP, dramatically decrease the efficiency of incorporation by Klenow (Snow *et al.*, 1984). More subtle modifications, such as 6-thio-dGTP, decrease the efficiency of incorporation to a lesser degree, *i.e.*, 5-fold less efficient than normal dGTP, presumably because the base-pairing characteristics of the analog are similar to those of normal dGTP (Rappaport, 1993).

In contrast, modification of the imidazole region of dGTP, which does not disrupt hydrogen bonding, does not dramatically decrease the efficiency of incorporation of various dGTP analogs. For example, N^7-methyl-dGTP has been shown to be incorporated by *E. coli* DNA polymerase I at about 20% the efficiency of normal dGTP (Hendler *et al.*, 1970). Further, an analog with a less disruptive modification, 7-deaza-dGTP, is incorporated with an efficiency that is approximately equal to that of normal dGTP in DNA sequencing reactions with Klenow (Mizuawa *et al.*, 1986; Barr *et al.*, 1986). Finally, the analog described here, N^7-cyanoborane–dGTP, which in NMR model studies *faciliated* base-pairing, was found to be incorporated by exonuclease-free Klenow 3–4 times *more* efficiently than normal dGTP. We predict that ^{7b}dG should form a strong Watson–Crick base pair with dC in duplex DNA.

It remains to be determined whether or not ^{7b}dG residues affect the stability or conformation of a short duplex. The primer extension experiments showed that three or more consecutive ^{7b}dG residues inhibited the rate of polymerization, suggesting a possible disturbance of the normal duplex. Physical reasons for the disturbance can be elucidated by melting temperature, calorimetric, and CD experiments, together with molecular dynamics calculations, but a more precise method would be to determine a three-dimensional solution structure by NMR methods. Primer extension can be used to produce micromolar solutions of selectively boronated duplexes for physical characterization.

In summary, we have synthesized the 5′-triphosphate of a novel boronated 2′-deoxynucleoside. The N^7-cyanoborane–2′-deoxyguanosine 5′-triphosphate is an excellent substrate for primer extension using Sequenase and for PCR using *Taq* polymerase. Kinetic experiments showed that ^{7b}dGTP is actually a better substrate for exonuclease-free Klenow than normal dGTP. Experiments are in progress to perform similar, but larger scale, primer extension and PCR experiments to enzymatically incorporate ^{7b}dGTP into DNA for characterizing the physical properties of the boronated duplexes. The key properties of ^{7b}dGTP, *i.e.*, it is readily and stably incorporated into DNA by polymerases and it is resistant to several chemical and endonuclease activities, may

be useful in a number of applications to produce DNA duplexes that are stable under a variety of conditions.

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