1788–1794 *Nucleic Acids Research, 1999, Vol. 27, No. 8*

Synthesis of 5-substituted 2′-deoxycytidine 5′-(α-P-borano)triphosphates, their incorporation into DNA and effects on exonuclease

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Received October 8, 1998; Revised and Accepted February 25, 1999

ABSTRACT

Direct PCR sequencing with boronated nucleotides provides an alternative to current PCR sequencing methods. The positions of boranophosphate-modified nucleotides incorporated randomly into DNA during PCR can be revealed directly by exonuclease digestion to give sequencing ladders. Cytosine nucleotides, however, are especially sensitive to exonuclease digestion and provide suboptimal sequencing ladders. Therefore, a series of 5-substituted analogs of 2′-deoxycytidine 5′-(α-P-borano)triphosphates (dCTPαB) were synthesized with the hope of increasing the nuclease resistance of deoxycytosine residues and thereby enhancing the deoxycytosine band intensities. These dCTP analogs contain a boranophosphate modification at the α-phosphate group in 2′-deoxycytidine 5′-triphosphate (dCTP) as well as a 5-methyl, 5-ethyl, 5-bromo or 5-iodo substitution for the 5-hydrogen of cytosine. The two diastereomers of each new dCTP derivative were separated by reverse phase HPLC. The first eluted diastereomer (putatively R_p) of each dCTP analog was a substrate for T7 DNA polymerase (Sequenase) and had an incorporation efficiency similar to normal dCTP and dCTPαB, with the 5-iodo-dCTPαB analog being the least efficient. Substitution at the C-5 position of cytosine by alkyl groups (ethyl and methyl) markedly enhanced the dCTPαB resistance towards exonuclease III (5-Et-dCTPαB > 5-Me-dCTPαB > dCTPαB ≈ 5-Br-dCTPαB > 5-I-dCTPαB), thereby generating DNA sequences that better define the deoxycytosine positions. The introduction of modified dCTPαB should increase the utility of direct DNA sequencing with boronated nucleoside 5′-triphosphates.

INTRODUCTION

Modified nucleoside 5′-triphosphates (dNTPs or NTPs) are widely used in biochemistry and molecular biology (1–4) as well as in various sequencing applications (5–8). Nucleoside boranophosphates (9–11) comprise a new class of modified nucleotides in which one non-bridging oxygen atom in the α-phosphate of nucleoside 5′-triphosphate is replaced by a borane group (BH_3) (12,13). Our studies have shown that 2′-deoxynucleoside 5′-(α-P-borano)triphosphates (dNTPαB, N = A, T, G, C) can be successfully incorporated into DNA by DNA polymerases (14). Once in DNA, the boranophosphate linkage is more resistant to degradation by endo- and exonucleases (15–18) relative to a normal phosphodiester linkage. Treatment of the primer-extended product with exonuclease III (which hydrolyzes phosphodiester bonds in the 3′→5′ direction along double-stranded DNA) generates fragments ending in a base-specific 3′-boranophosphate, thereby revealing the positions of the boranophosphates and producing the sequencing fragments. Direct PCR sequencing with boranophosphates has been shown to generate accurate and reproducible base calls over extended sequences (15). Yet, in order for boranophosphate sequencing to be more generally useful, this method requires improvement. For example, the preference of exonuclease III for cleaving cytidine nucleotides at a faster rate than adenosine, guanosine and thymidine nucleotides (19) is also observed for boronated cytidylate, which can result in faint base calls at some cytosine positions.

Alkylation or halogenation of the 5-position of deoxycytidine can influence the properties of modified DNA (20,21) and even the substrate specificity of an enzyme (22,23). It has been well-established that 5-methyldeoxycytidine (24,25), 5-ethyldeoxycytidine (26,27), 5-bromo- and 5-iododeoxycytidine (28) can induce a B-to-Z conformational transition in DNA (particularly for alternating pyrimidine–purine sequences). Thermodynamic stability of the DNA duplex is increased upon changing cytosine to 5-methylcytosine (29) or 5-halogenated cytosines (29,30). More than 320 restriction endonucleases are sensitive to base modifications that lie within the DNA recognition site and most of these enzymes will not cut DNA if their cleavage site contains a methylated base such as 5-methylcytosine (31). Incorporation of 5-bromodeoxycytidine or 5-iododeoxycytidine into double-stranded DNA completely stops cleavage by restriction enzymes *Alu*I and *Dde*I, and slows the rate of cleavage by *Hin*fI and *Rsa*I (32). It has also been demonstrated that incorporation of 5-methyl-2′-deoxycytidine into DNA can substantially protect DNA from exonuclease III cleavage (33).

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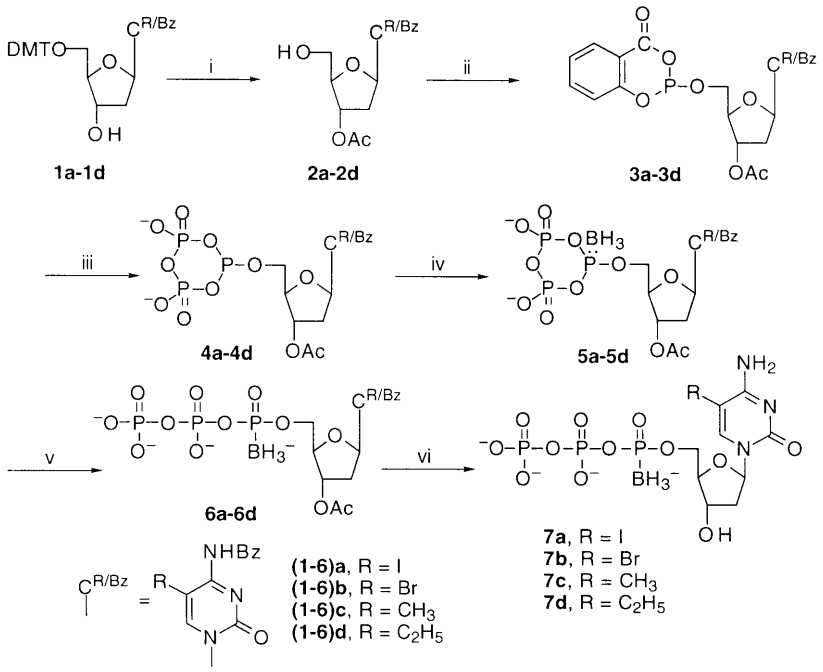

Scheme 1. Synthesis of 5-R-dCTPαB. (i) acetic anhydride and pyridine followed by 80% acetic acid; (ii) 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one; (iii) tributylammonium pyrophosphate; (iv) borane–*N*,*N*-diisopropylethylamine complex; (v) water; (vi) ammonium hydroxide/methanol (1:1 v/v).

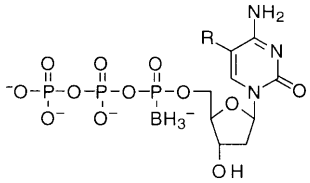

Figure 1. 5-Substituted analogs of dCTPαB. R = I, 5-iodo-dCTPαB; R = Br, 5-bromo-dCTPαB; R = CH_3, 5-methyl-dCTPαB; R = C_2H_5, 5-ethyl-dCTPαB.

Here, we synthesized, for the first time, a series of 5-substituted analogs of 2′-deoxycytidine 5′-(α-P-borano)triphosphates (dCTPαB), specifically 5-methyl-dCTPαB, 5-ethyl-dCTPαB, 5-bromo-dCTPαB and 5-iodo-dCTPαB (Fig. 1). We show that these four dCTP analogs can be successfully incorporated into DNA by T7 DNA polymerase (Sequenase). Once incorporated into DNA, the (α-P-BH_3)-5-methylcytidine and (α-P-BH_3)-5-ethylcytidine nucleotides are markedly more resistant to exonuclease III than the unsubstituted (α-P-BH_3)-cytidine nucleotide, thereby generating DNA products that better define the deoxycytosine positions in DNA sequence fragments.

RESULTS AND DISCUSSION

Synthesis

The starting base/sugar protected nucleosides for synthesis of 5-substituted dCTPαB (5-R-dCTPαB, R = methyl, ethyl, bromo, or iodo) are 3′-acetyl-N^4-benzoyl 5-substituted-2′-deoxycytidine (**2a**–**2d**). They were prepared by acetylation of 5′-*O*-(4,4′-dimethoxytrityl)-N^4-benzoyl 5-substituted-2′-deoxycytidine (**1a**–**1d**) followed by detritylation using acetic acid (Scheme 1). The 5′-*O*-(4,4′-dimethoxytrityl)-N^4-benzoyl 5-substituted-2′-deoxycytidine (**1a**–**1b**) were synthesized following the standard literature procedure (34).

Synthesis of the series of 5-R-dCTPαB was achieved (Scheme 1) via the salicyl phosphorochloridite approach we used to prepare deoxyribo- (10) and ribonucleoside (11) 5′-(α-P-borano)triphosphates (dNTPαB and NTPαB). The method involves a one-pot procedure and has the advantages of increasing yields and reducing time and cost for synthesis of nucleoside α-P-boronated 5′-triphosphates. The progress of the reactions in steps ii–v was monitored by ^{31}P NMR spectroscopy of the reaction mixtures (Table 1). The 3′-acetyl-N^4-benzoyl 5-substituted 2′-deoxycytidine **2** was phosphitylated by 2-chloro-4*H*-1,3,2-benzodioxa-phosphorin-4-one (salicyl phosphorochloridite) to yield the intermediate **3** (35,36). The ^{31}P NMR spectrum of the reaction mixture showed two signals at ~126 p.p.m. (representing two diastereomers of **3**). *In situ* treatment of **3** with pyrophosphate resulted in a rapid formation of **4**, P^2, P^3-dioxo-P^1-5-substituted-2′-deoxycytidyl-cyclotriphosphate. In the ^{31}P NMR spectrum, the two signals of compound **3** corresponding to α-phosphorus disappeared and a triplet centered at 105 p.p.m. was formed. This triplet, along with a doublet observed at –18 p.p.m., indicated the formation of **4** (35,36).

The intermediate **4**, without further purification, was immediately oxidized with borane–*N*,*N*-diisopropylethylamine complex to yield the boranocyclotriphosphate **5** (10,11). In this boronation step, the electron-deficient BH_3 coordinated with trivalent α-phosphorus. The boron–phosphorus complex **5** showed a characteristic broad peak centered at 85 p.p.m. in the ^{31}P NMR spectra. At the same time, the doublet for the two pentavalent phosphorus in compound **4** shifted from –18 to –21 p.p.m.. The product, **5**, was unstable in water and underwent hydrolytic ring-opening to the base/sugar protected 5-R-dCTPαB (**6**).

Table 1. ^{31}P NMR data of the intermediates **3a**–**3d** ($CDCl_3$), **4a**–**4d** (DMSO-d_6), **5a**–**5d** (DMSO-d_6) and **6a**–**6d** (D_2O)

Intermediate	^{31}P NMR Chemical Shifts (ppm)
3a	126.88, 126.22
3b	126.91, 126.72
3c	128.65, 125.99
3d	127.65, 125.16
4a	105.49, 105.23, 104.97 (t); -18.56, -18.82 (d)
4b	105.63, 105.36, 105.09 (t); -18.53, -18.80 (d)
4c	106.50, 106.23, 105.96 (t); -18.34, -18.39, -18.61, -18.65 (dd)
4d	105.65, 105.37, 105.10 (t); -18.45, -18.73 (d)
5a	87.05 (br); -21.60, -21.70, -21.88, -21.98 (dd)
5b	88.54 (br), -21.72, -22.00 (d)
5c	87.74 (br); -21.64, -21.93 (d)
5d	85.92 (br); -21.71, -21.80, -21.99, -21.08 (dd)
6a	81.62 (br); -10.22 (m); -22.52, -22.69, -22.89, -23.00 (dd)
6b	85-90 (br); -8.01 (m); -21.16 (m)
6c	84.95 (br); -9.02, -9.12 (d); -21.35 (m)
6d	84.32 (br); -8.93, -9.05 (d); -21.23, -21.33, -21.44, -21.55 (dd)

Spectra were recorded for individual reaction mixtures.

Removal of N^4-benzoyl and 3′-acetyl groups with methanol/concentrated ammonia (1:1 v/v) resulted in crude 5-R-dCTPαB (**7**), which was purified on anion-exchange chromatography. The two diastereomers of each 5-R-dCTPαB were separated by reverse phase HPLC (Table 2) and arbitrarily named as isomer I (putatively R_p) and isomer II (putatively S_p) (14).

Table 2. Reverse phase HPLC separation of diastereomers of **7a**–**7d**

Compound	Buffers[a]	Retention time (min)	
		Isomer I (%)	Isomer II (%)
7a: 5-I-dCTPαB	85% TEAA / 15% MeOH	8.83 (53%)	10.74 (47%)
7b: 5-Br-dCTPαB	0-30% MeOH in TEAA (25 min)	7.18 (47%)	9.93 (53%)
7c: 5-Me-dCTPαB	92% TEAA / 8% MeOH	10.78 (48%)	14.80 (52%)
7d: 5-Et-dCTPαB	90% TEAA / 10% MeOH	10.84 (53%)	14.92 (47%)

[a]Buffer A: 100 mM TEAA, pH 6.8, buffer B: MeOH.

Using the general procedure (Scheme 1), the preparation of 5-methyl dCTPαB (**7c**) and 5-ethyl dCTPαB (**7d**) was easily achieved with desirable yields. But in the case of 5-iodo and 5-bromo dCTPαB, the yields were very low, especially for the synthesis of 5-Br-dCTPαB. The major products (30–40%) in the synthesis of 5-I-dCTPαB (**7a**) and 5-Br-dCTPαB (**7b**) were shown to be normal dCTPαB (10). In order to examine the cause of debromination, a series of model experiments were carried out using authentic 5-bromo-2′-deoxycytidine and UV spectroscopy. Treatment of 5-bromo-2′-deoxycytidine with borane–*N*,*N*-diisopropylethyl complex (6 h), borane–dimethylsulfide complex (6 h) or concentrated ammonia (24 h) resulted in no change in the UV spectrum of the reaction mixture (37), which is the same as that of authentic 5-Br-dC. On the contrary, when 5-bromo-2′-deoxycytidine was treated with borane–*N*,*N*-diisopropylethyl complex followed by ammonia, the λ_{max} of the reaction mixture in the UV spectrum was significantly down-shifted from 288 nm for 5-Br-dC to 278 nm (data not shown). The major product of the reaction (40% yield) was identified by NMR and MS as normal 2′-deoxycytidine. The observations suggest

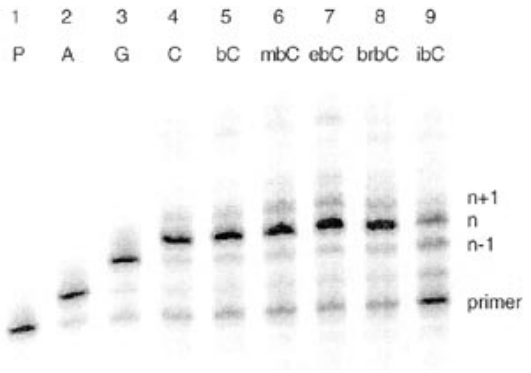

Figure 2. Primer extension analysis with dCTPαB. Primers were extended by dATP (lane 2), dATP + dGTP (lane 3), dATP + dGTP + dCTP (lane 4), dATP + dGTP + dCTPαB (lane 5), dATP + dGTP + 5-Me-dCTPαB (lane 6), dATP + dGTP + 5-Et-dCTPαB (lane 7), dATP + dGTP + 5-Br-dCTPαB (lane 8) and dATP + dGTP + 5-I-dCTPαB (lane 9).

that the combination of borane–amine complex and ammonia used in the one-pot synthesis can cause debromination of 5-bromocytosine and results in the low yield of 5-Br-dCTPαB, although the mechanism remains unclear. One possible reason could be that a more active borane–amine complex, which might reduce and debrominate 5-bromo-dC to give normal dC (38,39), is formed in the mixture of borane–*N*,*N*-diisopropylethyl complex and ammonia. We believe that a similar side-reaction (deiodination) may have occurred in the synthesis of 5-I-dCTPαB, resulting in a low yield of the desired 5-I-dCTPαB.

Primer extension and exonuclease digestion

Direct PCR sequencing with boronated nucleotides involves two major steps (15). In the first step, boronated nucleotides, i.e. dNTPαB, are randomly incorporated into the product DNA by the polymerase chain reaction (PCR). In the second step, base-specific 3′-termini are obtained by exonuclease digestion, since the 3′→5′ exonuclease III-mediated degradation of DNA is efficiently blocked at the boranophosphate positions. Using the sequencing technique with the α-P-borano-deoxynucleoside triphosphate substitution we can define DNA sequences that are comparable with current PCR sequencing (15). In order to improve the boranophosphate sequencing method and make it more generally useful, some problems had to be solved. For example, the preference of exonuclease III for cytosine caused weak band intensities at some cytosine positions. By introducing a 5-alkyl or 5-halogeno modification into the cytidine nucleotide in DNA, we expected to retain the excellent incorporation properties of cytidine nucleotides by DNA polymerase yet increase the exonuclease-resistance of the resulting modified DNA.

To determine whether the modified boranophosphate dCTPs could substitute for the normal dCTP, extension of a 5′-fluorescently labeled primer was carried out in the presence of various normal and boronated dNTPs, and modified T7 DNA polymerase (Sequenase) (Fig. 2). The primer was extended to the first position by normal dATP (lane 2), to the second position by normal dGTP along with dATP (lane 3), and to the third position by either normal dCTP (lane 4) or by a modified dCTP [dCTPαB (lane 5), 5-Me-dCTPαB (lane 6), 5-Et-dCTPαB (lane 7), 5-Br-dCTPαB (lane 8) or 5-I-dCTPαB (lane 9)] along with dATP and dGTP. The incorporation patterns of dCTPαB and 5-substituted dCTPαB were almost indistinguishable from that of normal dCTP (Fig. 2).

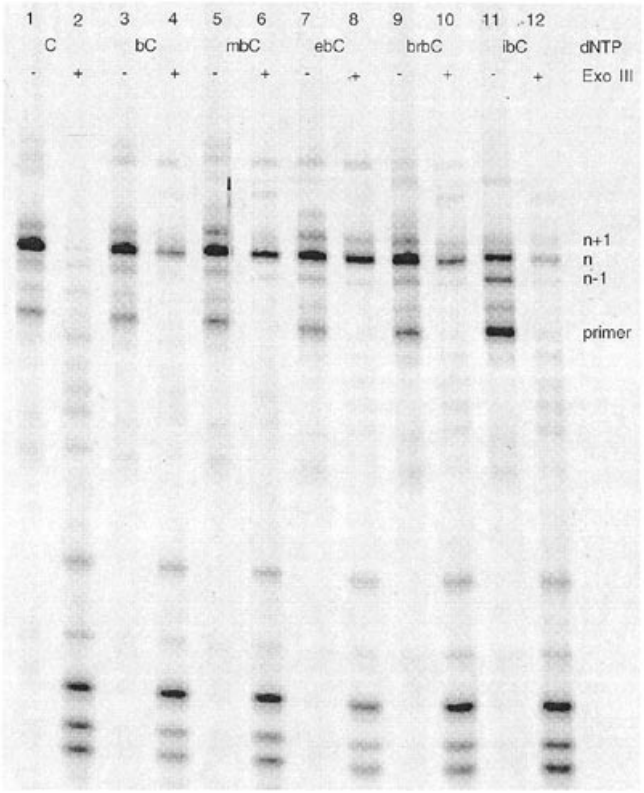

Figure 3. Exonuclease digestion of DNA containing 5-substituted 2′-deoxycytidine boranophosphate nucleotides. Following amplification, the dC terminated extended primers were treated with exonuclease III: dCTP (lanes 1 and 2), dCTPαB (lanes 3 and 4), 5-Me-dCTPαB (lanes 5 and 6), 5-Et-dCTPαB (lanes 7 and 8), 5-Br-dCTPαB (lanes 9 and 10) and 5-I-dCTPαB (lanes 11 and 12).

The 5-substituted boranophosphate dCTPs (5-Me-, 5-Et- and 5-Br-dCTPαB) were incorporated similarly to each other although, relative to the normal dCTP, they exhibited a slight degree of over-extension (at n + 1). The 5-I-dCTPαB was incorporated less efficiently than the other nucleotides, as evidenced by the high degree of unextended primer, as well as by a pause in extension immediately prior to 5-I-dCTPαB incorporation (at n – 1).

Our previous study has shown that dCTPαB (like dCTP) serves as a good substrate for DNA polymerases (15). Others have reported that 5-substituted dCTP analogs are generally well tolerated by DNA polymerases. With Klenow DNA polymerase, unboronated 5-ethyl-dCTP was able to substitute for dCTP in copying poly(dI-dC) but with a lower rate and to a lesser extent (27) relative to dCTP. Using a different DNA polymerase (Sequenase) and DNA primer-template, we found here that boronated 5-ethyl-dCTPαB is incorporated into DNA similarly to normal dCTP. With respect to other analogs, 5-methyl-dCTP and 5-bromo-dCTP are also incorporated into DNA by Klenow DNA polymerase with no difference in the efficiency of the primer extension compared to normal dCTP (30). With *Escherichia coli* polymerase I, the incorporation rate of 5-bromo-dCTP was more than twice that of 5-iodo-dCTP (32). As expected, we found that the boronated 5-methyl- and 5-bromo- analogs were incorporated into DNA with similar efficiency to dCTP. By comparison, the 5-iodo-dCTPαB analog was a worse substrate for Sequenase and appeared to cause premature chain termination. The poor incorporation of the 5-iodo-dCTPαB analog could result from conformational changes and/or improper folding at the polymerase active site (40) caused by the large size and hydrophobicity of 5-iodo group. It is also possible that the more acidic dissociation constant for the N-3 hydrogen of 5-iodo-dC would affect the nature of hydrogen bonding between 5-iodo-dC and dG (41).

Following extension, an aliquot of each dC-terminated sample was digested with exonuclease III (Fig. 3). The normal dC-terminated sample was digested extensively (lane 2). In contrast, for each boranophosphate-terminated sample, exonuclease digestion was halted significantly at the position of the boronated substitution (lanes 4, 6, 8, 10 and 12). To give a measure for the degree of protection provided by the boronated compounds, the intensity of each full-length extension product digested with exonuclease III was compared with the intensity of the respective undigested extension product (Table 3). Among the boronated nucleotides, the degree of protection decreased, such that: 5-Et-dCTPαB > 5-Me-dCTPαB > dCTPαB ≈ 5-Br-dCTPαB > 5-I-dCTPαB. The significantly enhanced nuclease-resistance of the 5-alkyl (5-ethyl and 5-methyl) cytidine nucleotides, along with their excellent incorporation properties, make them ideal candidates to improve our PCR sequencing method.

Table 3. Exonuclease digestion of DNA containing 5-substituted 2′-deoxycytidine boranophosphate nucleotides

Nucleotide	Exo III	volume[a]	% protection
dCTP	-	626	2
	+	11	
dCTPαB	-	480	25
	+	120	
5-Me-dCTPαB	-	472	54
	+	254	
5-Et-dCTPαB	-	451	65
	+	292	
5-Br-dCTPαB	-	565	29
	+	166	
5-I-dCTPαB	-	290	17
	+	49	

[a]volume = area of band n × integrated fluorescent signal intensity.

The digestion of DNA by exonuclease III, a 3′→5′ double-stranded DNA specific exonuclease, is influenced by the nature of the phosphodiester linkage as well as the base. In natural DNA, cytosine residues with normal phosphodiester linkages are cleaved more rapidly than G-residues, while A and T residues are cleaved at an intermediate rate (19). Substitution of a phosphodiester bond with a phosphorothioate (42) or boranophosphate (15) greatly retards the cleavage of the phosphate linkage by exonuclease III. Modification of the base also affects the extent of cleavage. In our studies here, addition of an ethyl group at the 5-position of cytosine in the base pair immediately adjacent to the boranophosphate diester bond to be cleaved resulted in the most pronounced inhibitory effects, whereas an iodo group gave less protection. The reason for the higher resistance of 5-alkylated deoxycytidine but relatively poorer exonuclease III protection by 5-halogenated deoxycytidine is not clear.

Linxweiller and Horz (19) suggested that the local DNA conformation of a dinucleotide may govern the susceptibility of DNA to exonuclease III. Hence, perturbations in the local DNA conformation of substrate DNA induced by backbone (43,44) or base (45,46) modification may alter its recognition by exonuclease III. An alkyl or halogen atom at the C-5 position of pyrimidines is known to potentiate the B-to-Z transition in modified poly(dG-dC) (27,28), in the order iodo > bromo > methyl > ethyl, which follows the order of exonuclease III susceptibility observed here. Yet a single replacement of deoxycytidine by the 5-alkyl (47) or 5-halogeno (48) analog in a nonalternating CG sequence, as in our study here, would not be expected to lead to a major distortion of a nominal B-form double helix; nevertheless, the possible formation of a localized Z-type (24–28) or other DNA structure cannot be excluded. Other possibilities include effects on base pairing and stacking (49), hydration, ion binding sites and thermodynamic stability of the helix (29,30). Dual modification of a cytidylate residue by a 5-substitution and a boranophosphate linkage might also interfere with substrate binding and/or induce conformational changes (steric distortions) of the enzyme–substrate complex, which may alter its digestion by exonuclease III.

CONCLUSION

In order to overcome the preference of exonuclease III for cytidine residues, which produce occasional faint bands in the C lane in direct PCR sequencing (15), we synthesized a new series of 2′-deoxycytidine 5′-triphosphate analogs containing an α-borano-phosphate group and an alkyl or halogeno substitution at the 5-position of cytosine. With the exception of the 5-iodo derivative, our results show that the 5-bromo, 5-methyl and 5-ethyl derivatives of dCTPαB are good substrates for DNA polymerase and are incorporated into DNA similarly to unmodified 2′-deoxycytidine 5′-triphosphate. More importantly, after incorporation into DNA, the boronated 5-methyl and 5-ethyl cytidine nucleotides exhibit increased resistance to exonuclease digestion as compared to the unsubstituted (α-P-borano)-cytidine. The significantly enhanced nuclease-resistance of the 5-alkyl (e.g., 5-ethyl and 5-methyl) cytidine nucleotides, along with their excellent incorporation properties, makes them ideal candidates to improve our PCR sequencing method (15).

MATERIALS AND METHODS

Synthesis

All solvents, chemicals and reagents were used without further purification unless otherwise indicated. 5′-*O*-(4,4′-dimethoxytrityl)-N^4-benzoyl-5-iodo-2′-deoxycytidine **1a** was synthesized by N^4-benzoylation of 5-iodo-2′-deoxycytidine (R.I. Chemicals) followed by 5′-detritylation of the resulting N^4-benzoyl-5-iodo-2′-deoxycytidine. 5′-*O*-(4,4′-dimethoxytrityl)-N^4-benzoyl-5-bromo-2′-deoxycytidine **1b** was synthesized by 5′-tritylation followed by N^4-benzoylation of 5-bromo-2′-deoxycytidine (Sigma). 5′-*O*-(4,4′-dimethoxytrityl)-N^4-benzoyl-5-methyl-2′-deoxycytidine was purchased from ChemGenes Corporation. 5′-*O*-(4,4′-dimethoxytrityl)-N^4-benzoyl-5-ethyl-2′-deoxycytidine was purchased from R.I. Chemicals.

^{1}H and ^{31}P NMR spectra were acquired at 400.0 and 161.9 MHz, respectively, using a Varian Inova-400 spectrometer. The signals are expressed as s(singlet), d(doublet), t(triplet), q(quartet), m(multiplet) and br(broad). Ultraviolet (UV) spectra were recorded on a Milton Roy Spectronic 3000 Array spectrometer. Mass spectra were recorded on a JEOL-JMS-SX-102 using FAB-MS at 3000 resolution. 3-Nitrobenzyl alcohol or 2-(2-aminoethylamino)ethanol in glycerol was used as matrix.

Thin layer chromatography (TLC) was performed on silica gel 60 F-254, 25 DC-Alufolien (EM Industries, Inc.). TLC plates were visualized under short wave UV light or heating the chromatogram after spraying with 10% sulfuric acid in methanol. Flash column chromatography was performed with 100–200 mesh silica gel (Fisher Scientific). Ion-exchange chromatography was performed with QA-52 quaternary ammonium cellulose (Whatman International Ltd.) packed into a 1.5 × 30 cm LC column. Reverse phase HPLC was performed using a Delta Pak C18 reverse phase column with a Waters system consisting of a 600E system controller, a 991 photodiode array UV detector and a NEC Powermate 386 computer.

3′-Acetyl-N^4-benzoyl-5-substituted 2′-deoxycytidine (2a–2d): general procedure

5′-*O*-(4,4′-dimethoxytrityl)-N^4-benzoyl-5-substituted 2′-deoxycytidine **1a**–**1d** was dissolved in anhydrous pyridine. Excess acetic anhydride was added. The reaction was continued at room temperature for 24 h. The TLC (solvent: 6% MeOH in CH_2Cl_2) showed complete disappearance of starting material and formation of a less polar compound. The solvent and excess reagents were removed *in vacuo*. The residue was dissolved in dichloromethane (50 ml) and washed with a saturated solution of sodium bicarbonate (2 × 25 ml) followed by water (25 ml). The aqueous layers were combined and re-extracted with dichloromethane (25 ml). The organic portion was dried by using Na_2SO_4 and filtered. The solvent was removed under reduced pressure to afford crude product, which was treated with 80% acetic acid without purification. After 6 h reaction at room temperature, the TLC (solvent: 4% MeOH in CH_2Cl_2) showed completed conversion of starting material. The solvent was removed under reduced pressure; the residue was dissolved in dichloromethane (50 ml), and washed with saturated sodium bicarbonate solution (2 × 25 ml) followed by water (25 ml). The aqueous layers were combined and re-extracted with dichloromethane (25 ml). The organic phase was dried (Na_2SO_4), filtered, and solvent was removed in vacuum. The residue was purified by column chromatography on silica gel (0–2% methanol in dichloromethane) to yield the desired product.

3′-Acetyl-N^4-benzoyl-5-iodo-2′-deoxycytidine (2a)

Compound **2a** was prepared in 45% yield (640 mg) following the general procedure using 5′-*O*-(4,4′-dimethoxytrityl)-N^4-benzoyl-5-iodo-2′-deoxycytidine **1a** (2.18 g, 2.87 mmol) and acetic anhydride (4 ml) in anhydrous pyridine (40 ml) followed by treatment with acetic acid (80 ml). FAB-MS: 500.00 [M+H] (calcd 499.16 for $C_{18}H_{18}IN_3O_6$). ^{1}H NMR (DMSO-d_6): δ 8.62 (s, 1H, H-6), 8.23 (d, J. = 6.4 Hz, 2H, Ar-H), 7.62 (d, J. = 7.2 Hz, 1H, Ar-H), 7.53 (t, J. = 7.2 Hz, 2H, Ar-H), 6.14 (t, J. = 6.4 Hz, 1H, H-1′), 5.37 (t, J. = 4.8 Hz, 1H, H-4′), 5.23 (s, 1H, 5′-OH), 4.10 (s, 1H, H-3′), 3.67 (m, 2H, H-5′), 2.38 (m, 2H, H-2′), 2.06 (s, 3H, CH_3).

3′-Acetyl-*N*^{4}-benzoyl-5-bromo-2′-deoxycytidine (2b)

Compound **2b** was prepared in 54% yield (244.5 mg) following the general procedure using 5′-*O*-(4,4′-dimethoxytrityl)-*N*^{4}-benzoyl-5-bromo-2′-deoxycytidine **1b** (716.0 mg, 1.0 mmol) and acetic anhydride (0.8 ml) in anhydrous pyridine (5 ml) followed by treatment with acetic acid (10 ml). FAB-MS [M+H] 452.03 (calcd 452.26 for $BrC_{18}H_{18}N_3O_6$). ^{1}H NMR (DMSO-d_6): δ 8.54 (s, 1H, H-6), 8.10 (m, 2H, Ar-H), 7.57 (t, J. = 7.2 Hz, 1H, Ar-H), 7.47 (t, J. = 7.6 Hz, 2H, Ar-H), 6.10 (t, J. = 6.8 Hz, 1H, H-1′), 5.34 (t, J. = 4.8 Hz, 1H, H-3′), 5.19 (m, 1H, H-4′), 4.06 (s, 1H, 5′-OH), 3.68–3.52 (m, 2H, H-5′), 2.34 (m, 2H, H-2′), 2.02 (s, 3H, $COCH_3$).

3′-Acetyl-*N*^{4}-benzoyl-5-methyl-2′-deoxycytidine (2c)

Compound **2c** was prepared in 41% yield (201.1 mg) following the general procedure using 5′-*O*-(4,4′-dimethoxytrityl)-*N*^{4}-benzoyl-5-methyl-2′-deoxycytidine **1c** (647.7 mg, 1.0 mmol) and acetic anhydride (400 µl) in anhydrous pyridine (5 ml) followed by treatment with acetic acid (10 ml). FAB-MS: [M+H] 388.14 (calcd 387.39 for $C_{19}H_{21}N_3O_6$). ^{1}H NMR ($CDCl_3$): δ 8.30 (d, J. = 7.2 Hz, 2H, Ar-H), 7.72 (s, 1H, H-6), 7.49 (d, J. = 7.2 Hz, 1H, Ar-H), 7.32 (q, J. = 7.2 Hz, 2H, Ar-H), 6.28 (t, J. = 7.0 Hz, 1H, H-1′), 6.09 (s, 1H, 5′-OH), 5.35 (t, J. = 3.2 Hz, 1H, H-3′), 4.12 (d, J. = 2.4 Hz, 1H, H-4′), 3.98–3.89 (2m, 2H, H-5′), 2.44–2.39 (m, 2H, H-2′), 2.09 (s, 3H, $COCH_3$), 1.24 (s, 3H, 5-CH_3).

3′-Acetyl-*N*^{4}-benzoyl-5-ethyl-2′-deoxycytidine (2d)

Compound **2d** was prepared in 66% yield (266.4 mg) following the general procedure using 5′-*O*-(4,4′-dimethoxytrityl)-*N*^{4}-benzoyl-5-ethyl-2′-deoxycytidine **1d** (662.4 mg, 1.0 mmol) and acetic anhydride (800 µl) in anhydrous pyridine (5 ml) followed by treatment with acetic acid (10 ml). FAB-MS [M+H] 402.2 (calcd 401.4 for $C_{20}H_{23}N_3O_6$). ^{1}H NMR ($CDCl_3$): δ 8.28 (d, J. = 6.8 Hz, 2H, Ar-H), 7.65 (s, 1H, H-6), 7.50 (t, J. = 7.2 Hz, 1H, Ar-H), 7.41 (t, J. = 7.2 Hz, 1H, Ar-H), 6.26 (t, J. = 7.2 Hz, 1H, H-1′), 5.34 (m, 1H, H-3′), 4.11 (q, J. = 2.4 Hz, 1H, H-4′), 3.97–3.89 (m, 2H, H-5′), 2.57 (q, J. = 7.6 Hz, 2H, 5-CH_2CH_3), 2.08 (s, 3H, $COCH_3$), 1.22 (t, J. = 7.6 Hz, 3H, 5-CH_2CH_3).

5-R-dCTPαB (7a–7d): general procedure

One of the 3′-acetyl-*N*^{4}-benzoyl-5-substituted 2′-deoxycytidine **2a**–**2d** was dissolved in anhydrous DMF under an argon atmosphere. Anhydrous pyridine was added and a freshly prepared solution of 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one (1.5 eqv) in anhydrous DMF was injected. After 10 min reaction, tributylamine was added followed by a 0.5 M solution of tributylammonium pyrophosphate (1.2 eqv) in anhydrous DMF and the mixture was stirred for 10 min. Excess borane–*N*,*N*-diisopropylethylamine complex (10 eqv) was added to the reaction mixture and stirring was continued for 6 h. Deionized water was added and the mixture was stirred for 1 h. After removal of the solvent under vacuum, a mixture of ammonium hydroxide and methanol (1:1 v/v) was added. The mixture was stirred for 24 h and diluted with deionized water (50 ml). The resulting solution was extracted with ether (50 ml). The solvent was removed from the aqueous layer under reduced pressure and the residue was applied to a QA-52 cellulose (HCO_3^-) column and eluted with a linear gradient of 800 ml each of 0.005 and 0.2 M ammonium bicarbonate buffer, pH 9.6. Fractions containing the desired product were collected and evaporated to dryness. Excess ammonium bicarbonate was removed by lyophilization from deionized water to give the ammonium salt of 5-R-dCTPαB **7a**–**7d**.

5-Iodo-2′-deoxycytidine 5′-(α-P-borano)triphosphate (7a)

Compound **7a** (5-iodo-dCTPαB) was prepared in 11% yield following the general procedure using 3′-acetyl-*N*^{4}-benzoyl-5-iodo-2′-deoxycytidine **2a** (100 mg, 0.2 mmol). FAB-MS: 589.91 $[M+3H]^-$ (calcd 586.85 for $BC_9H_{14}IN_3O_{12}P_3$). ^{1}H NMR (D_2O): δ 8.04 (s, 1H, H-6), 6.06 (t, J. = 6.4 Hz, 1H, H-1′), 4.47 (m, 1H, H-3′), 4.00 (m, 1H, H-4′), 3.00, 2.98 (2m, 2H, H-5′), 2.26, 2.11 (2m, 2H, H-2′), 0.55–0.05 (2br, 3H, BH_3). ^{31}P NMR (D_2O): δ 85.76 (br, 1P, α–P), –8.89 (m, 1P, γ–P), -21.12 (m, 1P, β–P). UV (H_2O) λ_{max} 295 nm.

5-Bromo-2-deoxycytidine 5′-(α-P-borano)triphosphate (7b)

Compound **7b** (5-bromo-dCTPαB) was prepared in 7% yield following the general procedure using 3′-acetyl-*N*^{4}-benzoyl-5-bromo-2′-deoxycytidine **2b** (127 mg, 0.3 mmol). FAB-MS: $[M+3H]^-$ 541.9 and 543.9 (calcd 538.9 and 540.9 for $BBrC_9H_{14}N_3O_{12}P_3$). ^{1}H NMR (D_2O): δ 7.99, 7.97 (2s, 1H, H-6, 2 isomers), 6.08 (q, J. = 6.8 Hz, 1H, H-1′), 4.50 (m, 1H, H-3′), 4.07, 4.02 (2m, 3H, H-4′, H-5′), 2.25, 2.12 (2m, 2H, H-2′), +0.80 to –0.20 (br, 3H, BH_3). ^{31}P NMR (D_2O) δ 84.97 (br, 1P, α–P), –8.47 (d, J. = 16.84 Hz, 1P, γ–P), –21.22 (dd, J. = 20.24 Hz, 24.29 Hz, 1P, β–P). UV (H_2O) λ_{max} 286 nm.

5-Methyl-2′-deoxycytidine 5′-(α-P-borano)triphosphate (7c)

Compound **7c** (5-methyl-dCTPαB) was prepared in 33% yield following the general procedure using 3′-acetyl-*N*^{4}-benzoyl-5-methyl-2′-deoxycytidine **2c** (113 mg, 0.3 mmol). FAB-MS: $[M+3H]^-$ 478.1 (calcd 475.0 for $BC_{10}H_{17}N_3O_{12}P_3$). ^{1}H NMR ($D_2O$): δ 7.65, 7.62 (2s, 1H, H-6, 2 isomers), 6.18 (t, J. = 6.0 Hz, 1H, H-1′), 4.50, 4.43 (2m, 1H, H-3′), 4.05, 3.86 (2m, 3H, H-4′, H-5′), 2.21, 2.15 (2m, 2H, H-2′), 1.87 (s, 3H, 5-CH_3), 0.43, 0.11 (2br, 3H, BH_3). ^{31}P NMR (D_2O): δ 84.39 (br, 1P, α–P), –8.91 (m, 1P, γ–P), –21.21 (m, 1P, β–P). UV (H_2O) λ_{max} 278 nm.

5-Ethyl-2-deoxycytidine 5′-(α-P-borano)triphosphate (7d)

Compound **7d** (5-ethyl-dCTPαB) was prepared in 52% yield following the general procedure using 3′-acetyl-*N*^{4}-benzoyl-5-ethyl-2′-deoxycytidine **2d** (155 mg, 0.4 mmol). FAB-MS: $[M+3H]^-$ 492.0 (calcd 489.02 for $BC_{11}H_{19}N_3O_{12}P_3$). ^{1}H NMR ($D_2O$): δ 7.57, 7.50 (2s, 1H, H-6, 2 isomers), 6.16 (t, J. = 8.0 Hz, 1H, H-1′), 5.87 (m, 1H, H-3′), 4.02 (m, 3H, H-4′, H-5′), 3.02 (q, J. = 7.2 Hz, 2H, 5-CH_2CH_3), 2.27, 2.15 (2m, 2H, H-2′), 1.10 (t, J. = 7.2 Hz, 3H, 5-CH_2CH_3), +0.60 to –0.30 (br, 3H, BH_3). ^{31}P NMR (D_2O): δ 85.58 (br, 1P, α–P), –7.68 (d, J. = 19.10 Hz, 1P, γ–P), –21.01 (dd, J. = 31.25 Hz, 19.83 Hz, 1P, β–P). UV (H_2O) λ_{max} 277 nm.

Reverse phase HPLC separation of two diastereomers of 5-R-dCTPαB

The separation of diastereomers of 5-R-dCTPαB **7a**–**7d** was achieved by ion-pairing chromatography on reverse phase column (Delta Pak C18, 7.8 × 300 mm, 15 µ, 300 Å) using isocratic or linear elution [buffer A: 100 mM triethylammonium

acetate (TEAA), pH 6.8, buffer B: methanol]. After HPLC purification, the solvents were removed under reduced pressure. The buffer component, TEAA and methanol were removed by lyophilization. The triethylammonium cation (TEA^+) was then converted to sodium cation (Na^+) by passing through sodium form cation exchange resin Dowex-50WX8-200 (Sigma).

Primer extension and exonuclease digestion

Oligonucleotides used as primer and template were purchased from Operon. 2′-Deoxycytidine 5′-triphosphate (dCTP) was purchased from USB. dCTPαB was synthesized as described (10). The diastereomer (isomer I, putatively R_p) corresponding to the first-eluted peak in HPLC profiles was used here in the extension reaction.

Fluorescently labeled primer {[5′-HEX-d(CAGGAACAGC-TATGGCCTC)-3′]; 10 pmol} was annealed to unlabeled template {[5′-d(GTGTAGCTGAGGCCATAGCTGTTCCTG)-3′]; 30 pmol}. Extension was accomplished with modified T7 DNA polymerase (Sequenase; 0.5 U) in the presence of various dNTPs (10 µM each; see Fig. 2 legend) in buffer (50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT; 10 µl) for 10 min at 37°C. The samples were diluted with 40 µl of Tris–HCl (10 mM; pH 7.5) and heated to 95°C for 1 min to inactivate the polymerase. Equivalent aliquots of each extended primer–template duplex (as determined by fluorescence imaging) were digested with exonuclease III (8 U) for 20 min at 37°C. Samples were separated on 20% polyacrylamide, 8 M urea gels and analyzed on a Hitachi FMBIO-100 fluorescence imager. For comparison of the digested/undigested samples, a volume integration of the full-length product was calculated [length × width × (intensity–background intensity)].

ACKNOWLEDGEMENTS

We thank Dr Dmitri Sergueev for assistance with performing reverse phase HPLC. This work was supported by DOE grants DE-FG05-94ER61882 and 97ER62376 to B.R.S.

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