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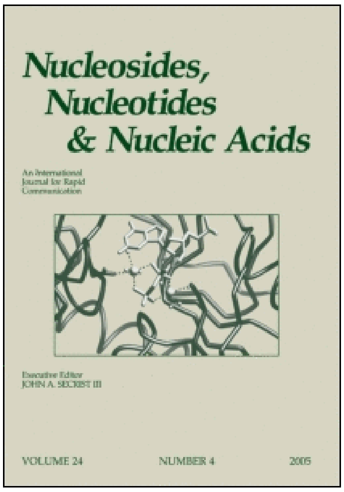

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Boranophosphate Oligonucleotides: New Synthetic Approaches

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NUCLEOSIDES & NUCLEOTIDES, 16(7-9), 1533-1538 (1997)

BORANOPHOSPHATE OLIGONUCLEOTIDES: NEW SYNTHETIC APPROACHES

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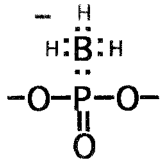
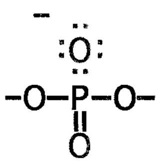
ABSTRACT. Recently our laboratory reported a new backbone-modified class of oligonucleotides, with a borane (BH_3-) group replacing one of the non-bridging oxygen atoms. Here we present two new approaches to synthesize the boranophosphate oligonucleotides. All-stereoregular boranophosphate oligonucleotides can be prepared by enzymatic template extension reactions using nucleoside α-boranotriphosphates, which are good substrates for a number of polymerases. Larger scale synthesis of boranophosphate oligonucleotides can be carried out by effective chemical synthesis using the H-phosphonate approach, instead of previously used phosphoramidite methodology. The main advantage of H-phosphonate methodology is the ability to carry out one boronation reaction, after oligonucleotide chain elongation has been completed, using mild conditions without base damage and producing the desired boranophosphate oligonucleotides in high yield.

INTRODUCTION. In recent years considerable attention has been devoted to the design of oligonucleotide analogs to improve their antisense and antigene characteristics and to create more powerful therapeutic remedies. This includes various backbone modifications to attain nuclease resistance, better permeability into the cell, and enhanced duplex stability. A great variety of modifications involve the phosphate group of an oligonucleotide chain. Phosphorothioate, methylphosphonate, phosphoramidate and other phosphate analogs now are under intensive investigation as possible therapeutic agents in medicine and as useful tools in molecular biology applications.

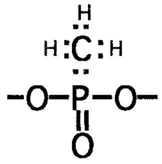
Recently, a new class of modified oligonucleotides with a boron-substituted phosphate group has been designed[1,2]. This analog contains a borane moiety in place of one of the non-bridging oxygen atoms. The boranophosphate group is isoelectronic to the phosphate group and sterically resembles the neutral methylphosphonate group, yet it bears a negative charge.



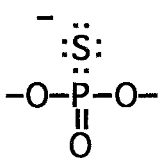
Boranophosphate



Phosphate



Methylphosphonate



Phosphorothioate

The negligible conformational changes of an oligonucleotide chain induced by the boranophosphate group and the presence of unmodified nucleoside units provide an opportunity to explore the use of boranophosphate oligonucleotides as antisense and antigene agents. Even though boranophosphates retain a negative charge that makes them water soluble, they are more hydrophobic than normal phosphates due to the presence of a borane group[1-3] that should increase their membrane permeability. Moreover, boranophosphate oligonucleotides are remarkably stable at physiological conditions in spite of the presence of boron-hydrogen bonds[1-3]. Additionally, substitution of the phosphate groups in oligonucleotides by boranophosphate leads to significant increase in resistance towards various endo- and exonucleases[3-5] that is important for successful antisense and antigene applications.

RESULTS AND DISCUSSION. In this study we describe two different synthetic approaches for boranophosphate oligonucleotides. The first is based on the capability of a number of polymerases to perform template extension reactions utilizing nucleoside α-boranotriphosphates ([α-P-BH_3]-dNTPs) as substrates[5-7]. This allows us to synthesize oligonucleotides containing boranophosphate internucleotide linkages by enzymatic methods. The α-phosphorus atoms in [α-P-BH_3]-dNTPs and in internucleotide boranophosphate linkages are chiral, as in methylphosphonate or phosphorothioate analogs. Enzymatic synthesis provides an opportunity to synthesize *all-stereoregular* boranophosphate oligonucleotides by using one of the α-boranotriphosphate stereoisomers. These oligos seem to be very interesting for studying protein-nucleic acids interactions, particularly endo- and exonuclease cleavage mechanisms.

Previously, [α-P-BH_3]-dNTPs have been used in mixtures with normal dNTPs to create partially boronated oligonucleotides by enzymatic template extension[8,9]. These statistically boronated oligonucleotides have been successfully applied for sequencing purposes[9]. Yet oligonucleotide chains containing all-boranophosphate internucleotide bonds have not been reported until now. Here, the 57-meric and 5'-^{32}P-labeled 15-meric DNA oligonucleotides have been chosen as the template and primer, respectively (FIG. 1).

5'-AGTGTAACTTGGATCCGAATTCGATATCGGCGCCGGCAAGCTTCGATCGGGCTTAGC-3'
3'-CTAGCCCGAATCGAGp-5'

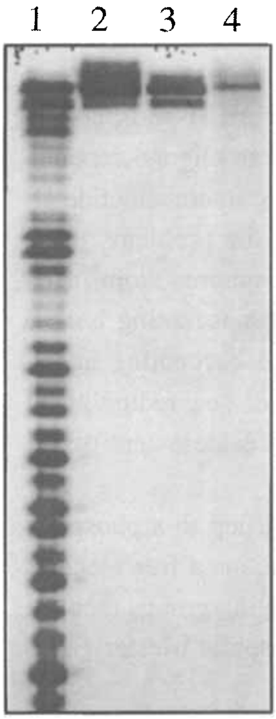


FIG. 1. 10% denaturing PAGE analysis of enzymatic template extension reaction. Reaction mixtures (0.3 mL) contained 5.4 pmol 57-meric DNA template, 4.6 pmol 5'-^{32}P-labeled 15-meric DNA primer, 600 pmol of the appropriate dNTP mixture and 60 units of 3'-5' exonuclease free Klenow polymerase in 47 mM Tris-HCl (pH 7.5), 6.8 mM $MgCl_2$, and 3.7 mM DTT. The mixture was incubated at 37 °C for 6 hours. Lane 1: sequencing lane; lane 2-4: [α-P-O]-, [α-P-S]-, or [α-P-BH_3]-dNTPs, respectively, in the reaction mixture.

Enzymatic template extension was carried out by 3'-5'-exonuclease-free Klenow polymerase and [α-P-BH_3]-dNTPs (tentatively the R_p stereoisomer) as substrates. As controls, the same reaction was performed with normal dNTPs and the conformationally equivalent S_p-isomer of [α-P-S]-dNTPs. It was found that concentrations as low as 2 μM [α-P-O]- or [α-P-S]- or [α-P-BH_3]-dNTPs were sufficient for the complete extension of 8 nM of the 15-meric primer. The formation of correct size product was inferred from its electrophoretic mobility (FIG. 1). The presence of more than one band in all three cases ([α-P-O]-, [α-P-S]-, [α-P-BH_3]-dNTPs, lanes 2-4, respectively) indicated incomplete or oversize extension reaction. The resulting all-boronated DNA stretch contains a number of well-known restriction enzyme recognition sites (EcoRI, EcoRV, HindIII, TagI, BamHI) distributed throughout the oligonucleotide. Studies of interactions of the restriction endonucleases with the boranophosphate oligonucleotide are currently in progress.

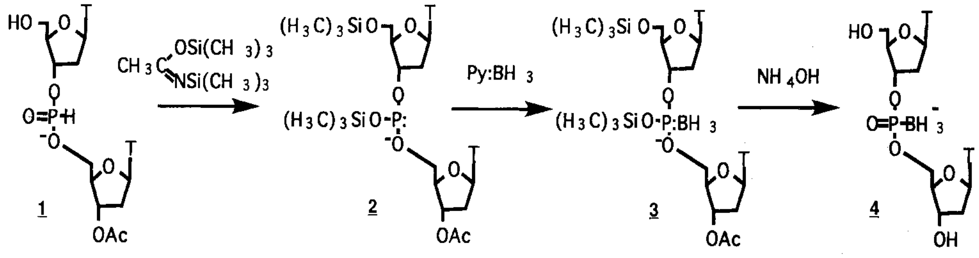
A main drawback of the enzymatic approach is the relatively small amounts of oligonucleotides that can be synthesized. Chemical methods of synthesis, however, can provide greater amounts of material. Previously, the phosphoramidite approach has been used for synthesis of boranophosphate oligonucleotides[1,2]. The triester phosphite group formed during the coupling reaction easily reacted with various borane complexes giving the desired boranophosphate. However, that methodology required boronation in each elongation cycle and therefore a high yield of this reaction was very critical for successful synthesis of long oligonucleotide chains. By contrast, the H-phosphonate methodology of oligonucleotide synthesis presents wide opportunities for modification of a H-phosphonate group and for obtaining analogs of phosphate oligonucleotides. Phosphorothioate, phosphoramidate and other analogs can be achieved from H-phosphonates by appropriate chemical treatment. It seems attractive to utilize the H-phosphonate approach for synthesis of boranophosphate oligonucleotides. This approach allows us to avoid multiple boronation steps and perform one boronation reaction for all H-phosphonate groups simultaneously after the complete chain elongation of an oligonucleotide.

We have developed an effective method for converting internucleotide H-phosphonate diester groups to boranophosphates. One of the problems to be overcome is the lack of a free pair of electrons on the phosphorus atom in H-phosphonates that complicates the reaction with the electron-accepting borane group. This problem may be solved by using a powerful boronating agent. However, such a boronating agent may cause base damage, i.e., reduction of carbonyl groups or hydroboration of double bonds to a noticeable extent that is undesirable.

A better way is to convert a H-phosphonate diester group to a phosphite triester, presented below. The phosphite triester group contains a free electron pair and is significantly more reactive towards electron-accepting groups then the H-phosphonate diester. The boronation reaction of the phosphite triester group can be done in mild conditions excluding any base damage.

2'-Deoxythymidylyl[3'-5'](3'-acetylthymidylyl) H-phosphonate (1) has been used to study the conversion of the H-phosphonate to the boranophosphate. The course of the reactions has been followed by ^{31}P-NMR spectroscopy. The first conversion of H-phosphonate diester 1 to the phosphite triester (2) has been achieved by silylation with bis-N,O-(trimethylsilyl)acetamide (see SCHEME).

During the reaction, which requires anhydrous conditions and inert atmosphere, the signals of H-phosphonate dinucleoside stereoisomers at δ=11.5 and 12.6 ppm disappear and new signals at δ=130.7 and 130.8 ppm corresponding to the phosphite triester stereoisomers appear (spectra were referenced externally to 85% H_3PO_4 in D_2O). After complete disappearance of the H-phosphonate signals and without separation of the intermediate product, a 20-fold excess of pyridine-borane complex is added to the reaction mixture. Our data indicate that pyridine-borane complex does not react with any of the



SCHEME

four DNA bases under the conditions used for phosphite boronation (not shown). The boronation reaction leads to disappearance of the phosphite triester signals and appearance of a broad signal at δ=96.1 ppm corresponding to the boranophosphate triester (3). Deprotection by ammoniacal treatment of the reaction mixture gives the desired dithymidine boranophosphate diester (4) (δ=93.2 ppm). Overall yield of the conversion 1 to 4 based on ^{31}P-NMR spectroscopy data is 70%.

The pure product has been isolated by reverse-phase HPLC. The ^{1}H, ^{31}P, and ^{11}B NMR spectroscopy data and FAB MS data of dithymidine boranophosphate completely coincide with that obtained for the same compound synthesized by the phosphoramidite method[1,2].

CONCLUSIONS. It has been demonstrated that all-boronated oligonucleotides can be prepared by enzymatic synthesis using normal DNA template and primer. This method allows us to produce stereoregular oligonucleotides that are important in studying enzyme-nucleic acid interactions. For scale up synthesis, we have developed a new procedure for chemical synthesis of boranophosphate oligonucleotides that surpasses the current phosphoramidite synthetic approach. This new method utilizes well-known H-phosphonate technology routinely used in automated oligonucleotide synthesizers and consequently gives us an opportunity to carry out synthesis in the automated regime. The opportunity to perform only one boronation reaction after the complete oligonucleotide chain elongation makes it possible to use mild conditions for this step, thereby excluding any base damage.

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