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Oxidative Substitution of Boranephosphonate Diesters as a Route to Post-synthetically Modified DNA

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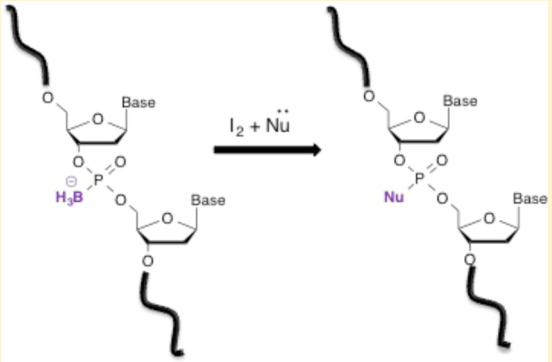
Supporting Information

ABSTRACT: The introduction of modifications into oligonucleotides is important for a large number of applications in the nucleic acids field. However, the method of solid-phase DNA synthesis presents significant challenges for incorporating many useful modifications that are unstable to the conditions for preparing synthetic DNA. Here we report that boranephosphonate diesters undergo facile nucleophilic substitution in a stereospecific manner upon activation by iodine. We have subsequently used this reactivity to post-synthetically introduce modifications including azides and fluorophores into DNA by first synthesizing boranephosphonate-linked 2′-deoxyoligonucleotides and then treating these oligomers with iodine and various nucleophiles. In addition, we show that this reaction is an attractive method for preparing stereodefined phosphorus-modified oligonucleotides. We have also examined the mechanism of this reaction and show that it proceeds via an iodophosphate intermediate. Beyond nucleic acids synthesis, due to the ubiquity of phosphate derivatives in natural compounds and therapeutics, this stereospecific reaction has many potential applications in organophosphorus chemistry.



INTRODUCTION

Chemically modified oligodeoxynucleotides (ODNs)[1−5] are used for applications in diagnostics,[6] biomedical research, therapeutics,[7] forensics,[8] and material sciences.[9−11] For therapeutic applications, the modifications are introduced to confer properties such as enhanced nuclease stability, improved pharmacokinetics, stronger and more selective binding to complementary DNA/RNA, and improved cellular uptake. On the other hand, the use of DNA-based sensors, nanomaterials, diagnostic tools, and catalysts requires the attachment of labels such as fluorophores,[12] quenchers, metal complexes,[13] and electron paramagnetic resonance (EPR) labels.[14]

The phosphoramidite approach[15] is the most commonly used procedure for chemical synthesis of DNA, RNA, and their derivatives. This method requires the stepwise treatment of the solid-support-linked, growing oligonucleotide as follows: (i) treatment with a strong acid, (ii) coupling with a phosphoramidite synthon to generate a phosphite triester, and (iii) oxidation of the phosphite to phosphate. Treatment with a strong base is required to remove the protecting groups from phosphate and the nucleoside bases. Thus, chemical modifications that are unstable toward acids and strong bases or react with P(III) compounds lead to side reactions with resultant diminishing yields of the desired product. Therefore, a general method for introducing modifications after completion of the synthesis would greatly expand the repertoire of nucleic acids analogues that can be synthesized.

Here we report the use of a boranephosphonate diester when incorporated into the DNA backbone (bpDNA)[16,17] as a highly versatile analogue that can be used to post-synthetically introduce modifications into ODNs. The method is based upon iodine activation of boranephosphonates toward substitution by nucleophiles (including alcohols, thiols, primary and secondary amines, and carbon nucleophiles). Furthermore, as this reaction occurs in a stereospecific manner, it is ideally suited for the purpose of synthesizing chiral phosphate derivatives. As a proof-of-principle, we show that this reaction allows the incorporation of stereospecific backbone modifications into 2′-deoxyoligonucleotides.

We also note that, because this chemistry is orthogonal to other methods of DNA modification, such as copper-activated click reactions[18] or palladium-catalyzed Stille couplings,[19] it opens avenues for labeling DNA with multiple functional groups and fluorophores. Finally, although the focus of the present report is toward modification of ODNs, this reaction is of broader interest in fields involving the synthesis of stereodefined phosphate-containing compounds, such as glycophosphate analogues[20−23] and nucleoside triphosphates.[24]

RESULTS

Substitution Reactions of Boranephosphonate Dimers Activated by Iodine. We have previously reported

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that boranephosphonate diesters reduce strongly oxidizing metal ions such as Au(III), Pt(II), and Ag(I) and produce metallic nanoparticles.[25] Concomitantly, the boranephosphonate diester undergoes nucleophilic substitution by the solvent (water or methanol) to generate the phosphate diester or triester, respectively. These observations hinted at a useful pathway to oxidatively activate the highly stable boranephosphonate internucleotide linkage toward nucleophilic substitution reactions. However, the use of metal ions for carrying out this process with a broad range of nucleophiles proved to be problematic due to the cross-reactivity of Au (III) with organic functional groups such as amines or thiols and also because the formation of nanoparticles in the reaction rendered workup and purification difficult. These challenges therefore led us to explore alternate oxidizing agents that would similarly activate a boranephosphonate toward substitution. In this regard, the instability of boranephosphonates toward aqueous iodine solutions has been observed in the past,[26] but there have been no investigations into the products formed from this reaction. In order to investigate whether iodine could activate boranephosphonates, we chose to study the reaction with diastereomerically pure 5′-(*tert*-butyldimethylsilyl)-2′-deoxythymidyl, 3′-(*tert*-butyldimethylsilyl)-2′-deoxythymidyl boranephosphonates (**9** and **10**) as model substrates. (Details of synthesis and purification are provided in the Supporting Information, including Scheme S1.) We refer to **9** and **10** as the fast and slow isomers, respectively, based upon their mobility on a silica column.

Initially, we reacted each diastereomer with a 30-fold excess of iodine dissolved in an anhydrous solution of 0.4 M methylamine and tetrahydrofuran (THF) (Figure 1A). The ^{31}P NMR spectra of the crude reaction mixtures (Figure 1B,C) showed formation of single peaks at 9.97 and 10.28 ppm when **9** and **10** were used as starting materials, respectively. The resonances observed in each case were distinct, which demonstrated the formation of only one diastereomer via a stereospecific reaction. The products were subsequently isolated and analyzed by proton NMR and mass spectrometry (Table 1, entry 2, also see Supporting Information). These results confirmed the formation of the phosphoramidates (**11** and **12**) in accordance with our hypothesis of iodine-activated, nucleophilic substitution of the BH_3 group. We also varied the amount of iodine added to these reaction mixtures, while keeping an excess of the methylamine (Supporting Information, Figure S1). The results from these experiments indicated that the complete conversion of boranephosphonate to the phosphoramidate required 1 equiv of iodine. This was an interesting observation as it points to the fact that it is not necessary to oxidize all three hydrides on borane in order to convert it into a good leaving group.

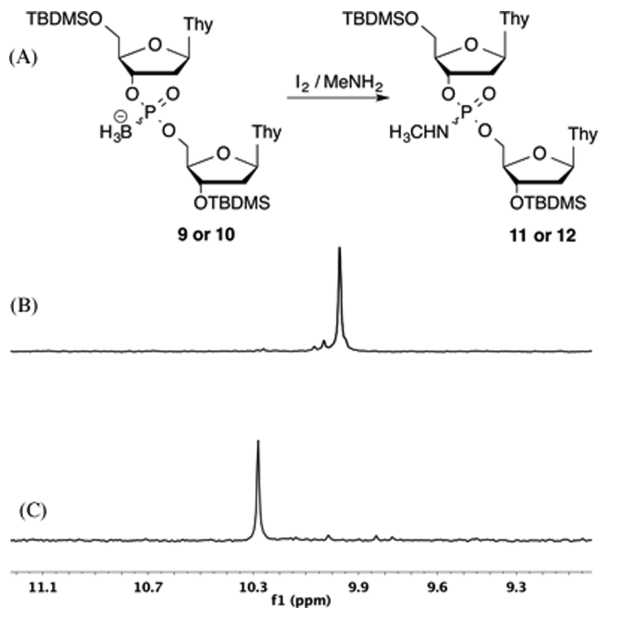

Figure 1. (A) Reaction of diastereomerically pure **9** or **10** with 30 equiv of iodine and a 0.4 M solution of methylamine in tetrahydrofuran, leading to formation of methylphosphoramidate **11** or **12** in a stereospecific manner. (B,C) ^{31}P NMR spectra of the crude reaction mixtures obtained from **9** and **10** as starting materials, respectively.

Subsequently we carried out the reactions of **9** and **10** with several different types of amines. The amines were added at a 10-fold excess over **9** and **10**. Although only an equimolar amount of iodine was needed with respect to the boranephosphonate, 3 equiv was used for these NMR-scale experiments in order to avoid variations in results due to random experimental error. Reactions with ammonia (Table 1, entry 1), a primary amine (Table 1, entry 2, methylamine), secondary amines (Table 1, entries 3 and 5, diethylamine and morpholine), and an aromatic amine (Table 1, entry 6, aniline) occurred quantitatively under these conditions (as estimated by ^{31}P NMR spectroscopy). Propargylamine and azido propanamine were also tested because they are known as "click" counterparts (Table 1, entries 4 and 7). Reactions with azides are particularly noteworthy as they are prone to undergo Staudinger reactions with P(III) compounds and cannot be incorporated using current methods of DNA synthesis. We were also able to successfully introduce arginine (Table 1, entry 9). This observation suggests that amino acids and peptides can be successfully linked to DNA as well. While pyrrole did not react under these conditions, 2-(2-aminoethyl)pyrrole readily formed the phosphoramidates (Table 1, entry 8). The efficacy of the reaction with amines was further demonstrated by quantitative conversion of the hindered camphenylamine (Table 1, entry 10; also see Supporting Information, Figures S2 and S3, for representative ^{31}P NMR spectra) to the corresponding amidates.

Similarly, when these dimers were treated with a solution of iodine in methanol, the expected methyl phosphotriesters were formed quantitatively based on ^{31}P NMR (Supporting Information, Figure S4). During the reaction with methanol, we found that the 5′-silyl protecting group (*tert*-butyldimethylsilyl, TBDMS) was removed. Presumably the production of hydroiodic acid from iodine oxidation of the BH_3 hydrides causes silyl ethers to be activated toward nucleophilic substitution (by methanol in this case). In contrast to the efficient reactivity with amines and methanol, we found that the reaction of **9** and **10** with *n*-propanol (either as the solvent or in 10-fold excess with THF as the solvent) did not occur. However, addition of a tertiary amine (diisopropylethylamine, DIPEA) to scavenge hydroiodic acid led to the desired *n*-propylphosphate triesters as determined by observing a sharp singlet in the ^{31}P NMR at −1.96 and −1.88 ppm when using **9** and **10**, respectively. The formation of the correct product was further ascertained by ESI-MS (Table 2, entry 1). In these experiments we found that the order of addition of the tertiary amine was important. The presence of DIPEA prior to addition

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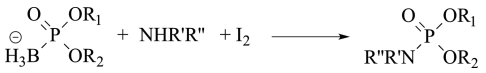
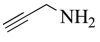
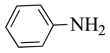
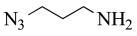
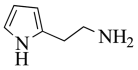
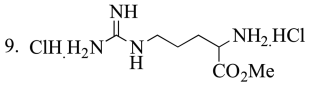
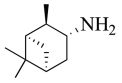
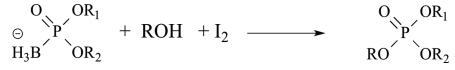
Table 1. Iodine-Activated Substitution of Boranephosphonate Dimers 9 and 10 by Amines[c]



Entry	NHR'R"	^{31}P NMR δ (ppm)		ESI+ (m/z)	
		Fast isomer	Slow isomer	Observed	Theoretical
1.	NH_3	10.37 (**13**)	10.43 (**14**)	774.31[a]	774.33
2.	CH_3NH_2	9.97 (**11**)	10.28 (**12**)	819.41[b]	819.39
3.	Et_2NH	9.49 (**15**)	10.15 (**16**)	903.64[b]	903.48
4.		8.03 (**17**)	8.56 (**18**)	867.40[b]	867.38
5.		6.92 (**19**)	7.39 (**20**)	931.50[b]	931.44
6.		2.14 (**21**)	2.62 (**22**)	850.40[a]	850.36
7.		8.89 (**23**)	9.32 (**24**)	957.62[b]	957.45
8.		8.76 (**25**)	8.59 (**26**)	868.54[a]	868.39
9.		6.57 (**27**)	6.75 (**28**)	945.60[a]	945.43
10.		8.06 (**29**)	8.49 (**30**)	1062.61[b]	1062.60

[c]Reactions were performed in acetonitrile using 0.01 mmol of the boranephosphonate dimer, 3.0 equiv of iodine, and 10.0 equiv of the amine. Diisopropylethylamine (20 equiv) was also added for entry 9. The observed ESI+ column gives the mass of the positively charged adduct of the phosphoramidates and the corresponding ammonium species ([a]) or the mass of the protonated phosphoramidate ([b]). R_1 = 5′-(*tert*-butyldimethylsilyl)-2′-deoxythymidyl; R_2 = 3′-(*tert*-butyldimethylsilyl)-2′-deoxythymidyl.

Table 2. Iodine-Activated Substitution of Boranephosphonate Dimers 9 and 10 by Alcohols, Thiols, and CH_3MgBr[c]



Entry	ROH	^{31}P NMR δ (ppm)		ESI+ (m/z)	
		Fast isomer	Slow isomer	Observed	Theoretical
1.	$CH_3CH_2CH_2OH$	-1.96 (**31**)	-1.88 (**32**)	839.3458	839.35[a]
2.	CF_3CH_2OH	-3.84 (**33**)	-3.66 (**34**)	985.4652	986.46[b]
3.	$CH_3CH(OH)CH_3$	-3.40 (**35**)	-3.19 (**36**)	945.5078	946.51[b]
4.		-2.41 (**37**)	-2.23 (**38**)	941.4765	942.48[b]
5.	$N_3(CH_2)_6OH$	-2.01 (**39**)	-1.87 (**40**)	1028.5564	1029.56[b]
6.	$CH_3CH_2CH_2SH$	30.03 (**41**)	29.40 (**42**)	855.3231	855.3254[a]
7.	CH_3MgBr	32.09 (**43**)	31.47 (**44**)	795.56	795.32[a]

[c]The theoretical ESI+ column gives data for $[M + Na]^+$ ([a]) and $[M+(i\text{-}Pr)_2EtNH]^+$ ([b]) ions. All the reactions were performed in acetonitrile with 0.01 mmol of **9** or **10** using acetonitrile as solvent, 3.0 equiv of iodine, 5.0 equiv of DIPEA, and 10.0 equiv of the nucleophile. For entries 3 and 4, the corresponding alcohol was the solvent. A total of 20.0 equiv of nucleophiles was used for entries 6 and 7. R_1 = 5′-(*tert*-butyldimethylsilyl)-2′-deoxythymidyl; R_2 = 3′-(*tert*-butyldimethylsilyl)-2′-deoxythymidyl.

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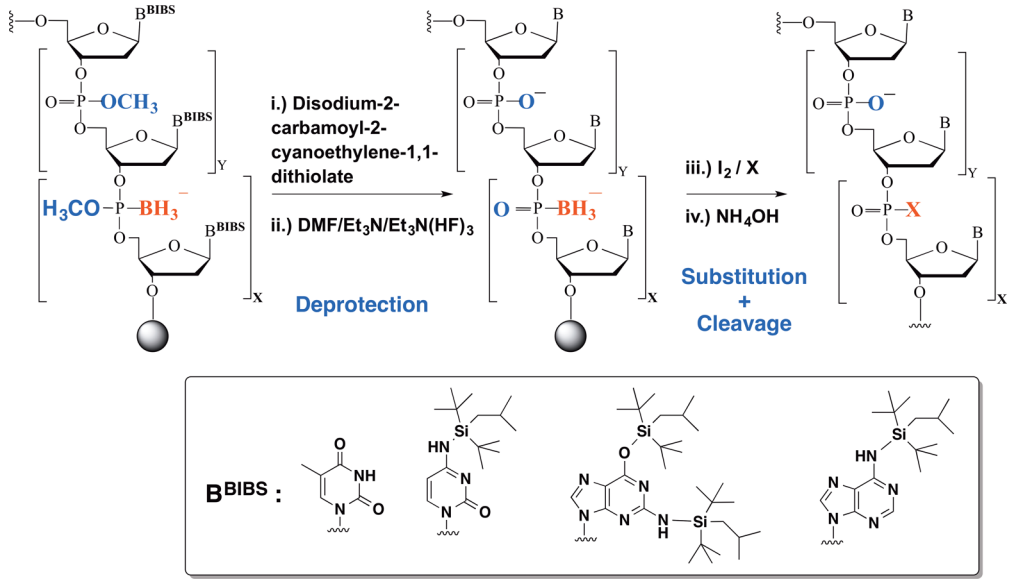
Scheme 1. Post-synthetic Modification of 2′-Deoxyoligonucleotides by Iodine-Activated Substitution of bpDNA Oligomers



of iodine prevented any reaction. This was likely due to coordination of iodine with the tertiary amine. Under optimized conditions, with DIPEA added following iodine oxidation, the formation of phosphotriesters could be accomplished with several alcohols including 2,2,2-trifluoroethanol and 2-propanol (Table 2, entries 2 and 3). As with amines, the reaction with alcohols was also useful for introducing groups such as alkynes and azides (Table 2, entries 4 and 5).

Finally, propanethiol (Table 2, entry 6) and methylmagnesium bromide (Table 2, entry 7) were also found to be effective nucleophiles for testing the synthesis of phophorothioate esters and alkylphosphonates, respectively. As these nucleophiles are reactive toward iodine, the reactions in these cases were carried out in two steps. Initially, 3 equiv of iodine was added to the boranephosphonate solution. After this mixture was stirred for 5 min, an excess (20 equiv) of the nucleophile and 5.0 equiv of the base (DIPEA) were added. The excess thiol or Grignard reagent neutralized any unreacted iodine present in the reaction mixture. The precipitates generated from these side reactions were removed by filtration or an aqueous workup. Subsequent analysis revealed formation of the desired products.

Phosphoramidate DNA Synthesis from Boranephosphonate Oligomers. The highly efficient reaction of boranephosphonate dinucleotides with amines encouraged us to explore the use of bpDNA as a substrate for generating modified ODNs having phosphoramidates at predefined positions within DNA. When coupled with the synthesis of bpDNA, we expected that these studies would lead to a methodology for introducing post-synthetically a large number of modifications. The approach (Scheme 1) was as follows: (1) Synthesize 2′-deoxyoligonucleotides having boranephosphonates at specific sites. (2) Remove all protecting groups from ODNs but retain attachment to the support. (3) Introduce phosphoramidates at sites that contain boranephosphonate linkages. (4) Use a mild, brief treatment to hydrolyze the succinate linkage that attaches the ODN to the solid support. Our current method for synthesizing boranephosphonate DNA[27] proved to be particularly attractive for developing this approach as it relies upon using the di-*tert*-butylisobutylsilyl group (BIBS) for protection of the nucleobase exocyclic amines. Following synthesis, the methyl protection on phosphorus is removed using disodium 2-carbamoyl-2-cyanoethylene-1,1-dithiolate, and BIBS protection on the nucleobases is eliminated with a fluoride ion solution buffered to neutral pH. At this point, ODNs free of protecting groups remain joined to the support. Following site-specific modification of boranephosphonate linkages using iodine and a nucleophile, the ODN can be released from the support by a 30 min treatment with aqueous ammonia (or other similarly mild conditions such as a 1 h treatment with ethylenediamine). In order to use even milder conditions for removal of ODNs from the supports, both the Q and oxalyl linkers will be tested. Thus, modifications are introduced by an orthogonal approach and are not exposed to reagents of DNA synthesis or prolonged ammonia treatment.

In order to carry out a systematic evaluation of this methodology, bpDNAs containing boranephosphonate diester internucleotide linkages at defined positions were synthesized (Table 3) and protecting groups removed. The support-linked 2′-deoxyoligonucleotides were then reacted with a solution of 0.05 M iodine and various amines at a concentration of 0.2 M in anhydrous acetonitrile for 48 h. The resins were repeatedly washed with acetonitrile and the ODNs cleaved using 30% NH_4OH (30 min at room temperature). After removal of ammonia under reduced pressure, the oligomers were dissolved in water and analyzed by LC-MS and ^{31}P NMR (Table 3 and Supporting Information, Figures S5−S25).

Initially we treated a resin-bound 2′-deoxyhexathymidine containing a single boranephosphonate diester linkage adjacent to the 5′ end with a solution of 0.05 M I_2 and 0.4 M ammonia in dioxane (ODN 1, Table 3). Upon cleavage and LC-MS analysis, we found that the desired oligomer having one phosphoramidate linkage was produced in quantitative yield. Similarly, the reaction proceeded smoothly with diethylamine and the highly hindered isopinocamphenylamine (Table 3, ODNs 2 and 5, respectively). In order to demonstrate the introduction of other functional groups (alkyne and azide) into DNA, the reaction was carried out with propargylamine (Table 3, ODN 3) and 3-azido-1-propanamine (Table 3, ODN 4).

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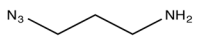
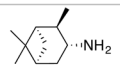
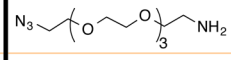
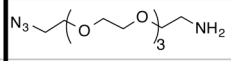
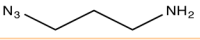
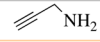
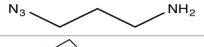
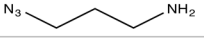
Table 3. Synthesis and Reaction of 2′-Deoxyoligonucleotides Containing Boranephosphonate Diester Linkages with Various Amines To Produce the Corresponding Phosphoramidate Oligodeoxynucleotides[a]

ODN No.	Sequence	Amine	Mol. Wt. (Obs)	Mol. Wt. (theor)
ODN 1	T*TTTTT	NH_3	1761.326	1761.336
ODN 2	T*TTTTT	$(CH_3CH_2)_2NH$	1817.381	1817.399
ODN 3	T*TTTTT		1799.336	1799.352
ODN 4	T*TTTTT		1844.372	1844.385
ODN 5	T*TTTTT		1897.451	1897.462
ODN 6	T*TTTTTTTTTTTTTTTTTT		6060.019	6061.02
ODN 7	T*TTTTTTTTTTTTTTTTTT		6058.902	6058.003
ODN 8	TTTTTTTTTTTTTTTTTT*T		6058.89	6058.003
ODN 9	T*TTTTTTTT*TTTTTTTTTT*T		7018.48	7018.64
ODN 10	T*TTTTTTTTTTTTTTTTTT		6354.073	6353.178
ODN 11	TTTTTTTTTTTTTTTTTT*T		6103.928	6103.036
ODN 12	T*TTTTTTTT*TTTTTTTTT*T	NH_3	6018.929	6018.019
ODN 13	T*T*T*TTTTTTTTTTTTT*T*T*T	NH_3	6016.001	6015.068
ODN 14	T*TTTT*TTT*TTT*TTT*TTT*TTT*T	NH_3	6319.038	6318.13
ODN 15	TGTAAACCATG*ATGTGCTGCTAT		7090.266	7091.7
ODN 16	TGTAAACCATG*ATGTGCTGCTAT		7135.16	7136.733
ODN 17	T*GTAAACCATGATGTGCTGCTAT		7091.175	7091.701
ODN 18	T*GTAAACCATGATGTGCTGCTAT		7136.169	7136.733
ODN 19	T*GTAAACCATG*ATGTGCTGCTA*T	NH_3	7051.108	7050.709
ODN 20	T*GTA*AAC*CAT*GAT*GTG*CTG*CTA*T	NH_3	7046.239	7045.789
ODN 21	T*T*T*T*T*T*T*T*T*T*T*T*T*T*T*T*T*T*T*T*T*T	NH_3	6002.56	6003.18

[a]Boranephosphonate diester linkages are denoted by red asterisks. For ODN 21, MALDI-TOF mass spectrometry was used, whereas LC-MS was used for the other ODNs.

These experiments also demonstrated that none of the amines led to measurable cleavage of the succinate linkage and loss of product. This conclusion was based upon the observation that the yields of the crude phosphoramidate-containing ODNs were close to the amount expected from synthesis on a 0.2 μmol scale.

In order to evaluate the effect of the position of the boranephosphonate linkage within the oligomer toward substitution by amines, we carried out similar reactions on 20-mers containing a single boranephosphonate diester linkage adjacent to either the 5′ or 3′ ends (Table 3, ODNs 6, 7, 8, 10, and 11). Substitution with propargylamine, 3-azido-1-propanamine, azido-PEG-amine, or 2-methylaziridine showed that conversion to the phosphoramidate occurred quantitatively at the 5′ or the 3′ end. Thus, the solid support matrix does not appear to interfere with this substitution reaction even when the boranephosphonate is located at the first internucleotide linkage adjacent to the support.

We next synthesized bpDNA containing three, six, or eight boranephosphonate diester linkages as well as a fully boronated 20-mer bpDNA sequence (Table 3, ODNs 12, 13, 14, and 21, respectively) in order to determine whether multiple modifications could be introduced within the same ODN. Substitution of borane with ammonia upon activation by iodine proceeded efficiently and analysis of the crude reaction mixtures by both LC-MS (Supporting Information, Figures S16−S18) and ^{31}P NMR spectroscopy (Supporting Information, Figure S25A−D) revealed that the desired phosphoramidates were formed in near quantitative yields. We also reacted azido-PEG-amine and iodine with a bpDNA 20mer that contained three boranephosphonate diester linkages (one each at the 5′ and 3′ ends and one internally) (Table 3, ODN 9). Three products were obtained. This was in contrast to the reaction of ODN 10 (one boranephosphonate at the 5′ linkage) where conversion to the phosphoramidate with azido-PEG-amine was quantitative. Based on LC-MS data, the product that was present in approximately 50% yield contained three azido-PEG-amine groups. In addition, two products having one or two azido-PEG-amines were also detected (Supporting Information, Figure S12). Using higher equivalents of the amine (up to 2.0 M) or iodine (1.0 M) and longer reaction times (96 h), and carrying out the reaction at elevated

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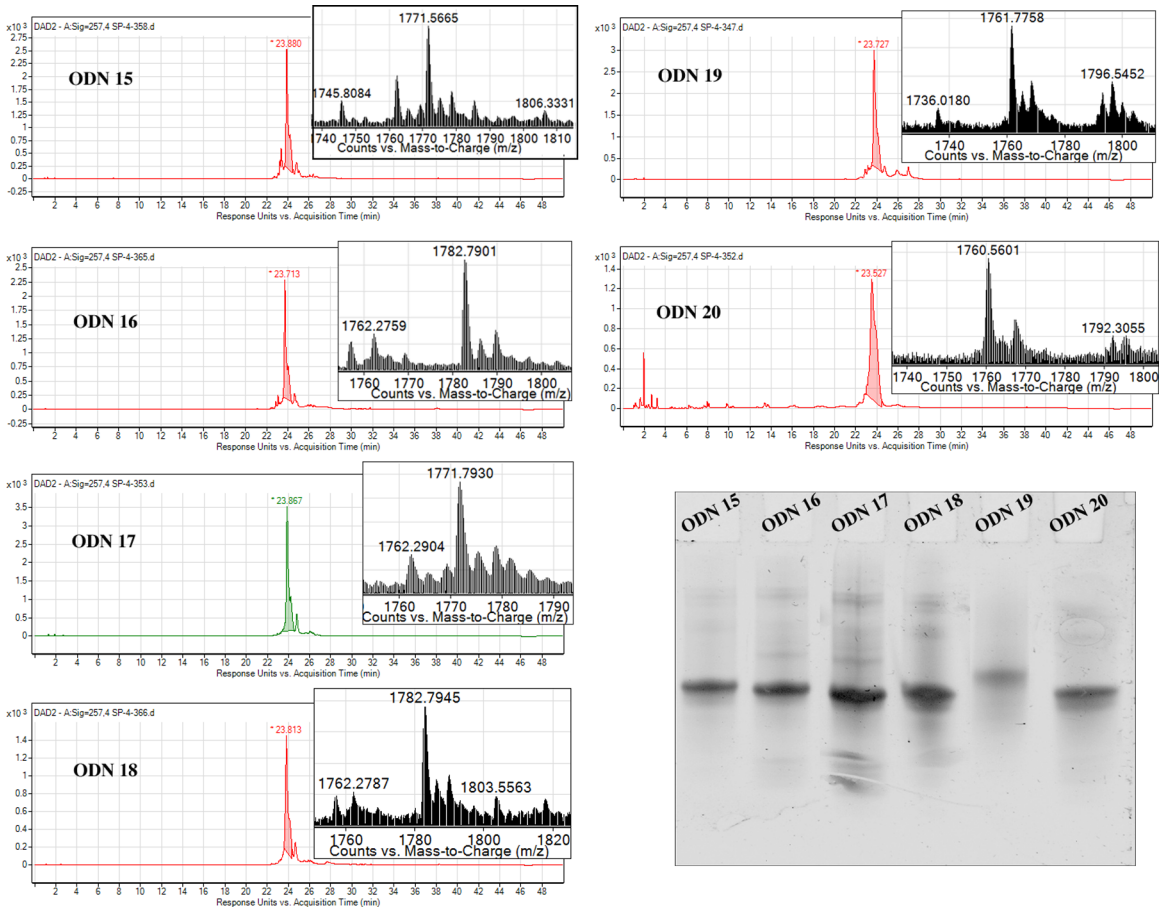

Figure 2. LC-MS and denaturing gel electrophoresis analysis of mixed-sequence oligonucleotides (ODNs 15−20). Each set of data shows the LC trace along with a zoomed-in view of the base peak in the mass spectra. The base peak in each case was a −4 charged species. The ODN numbers are indicated at the top of each lane in the gel image. Full mass spectra are provided in the Supporting Information.

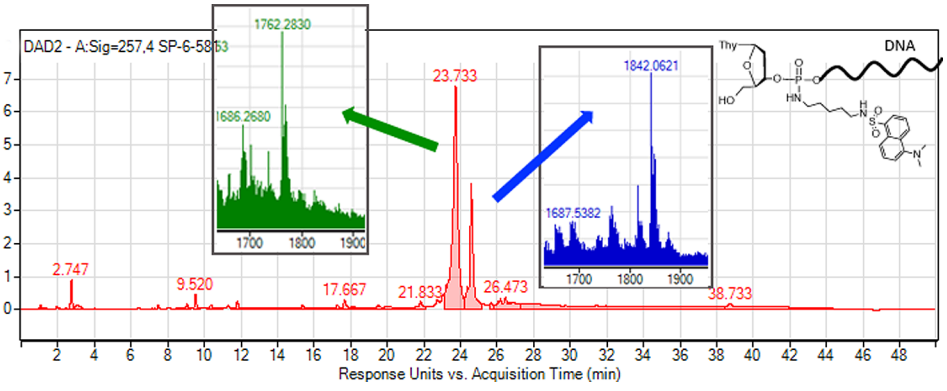

Figure 3. LC-MS analysis of a DNA oligomer (5′-T*GTAAACCATGATGTGCTGCTAT-3′, where the asterisk denotes a boranephosphonate diester linkage) that was reacted with iodine (0.05 and 0.2 M amino-dansyl, 48 h, room temperature) as described in the text and released from the solid support (30% NH_4OH, 30 min, room temperature). The peak at 24.5 min corresponds to the dansyl-conjugated product, whereas the peak at 23.73 min is due to conversion of the boranephosphonate to the phosphate. Insets: Expansion of the relevant regions of the mass spectra extracted from these peaks. The theoretical values for the −4 charged peaks for these oligomers are 1841.8 and 1762.4, respectively.

temperatures (up to 55 °C) and with alternate solvents (dichloromethane, dimethylformamide), did not lead to a significant improvement in yield of ODN 9, having three azido-PEG-amine phosphoramidates. It is possible that conjugation of one hydrophobic group causes the polystyrene-bound ODN to adhere to the solid matrix, thereby slowing down further reactions.

We also synthesized 2′-deoxyoligonucleotides containing all four nucleobases with variable locations and numbers of boranephosphonate diester linkages. Consistent with previous results, propargylamine and 3-azidopropanamine reacted with boranephosphonate diesters located internally as well as at the 5′ end of the oligodeoxynucleotide (ODNs 15−18). LC-MS analysis of the unpurified mixtures demonstrated formation of the desired phosphoramidates in high yields (Figure 2 and

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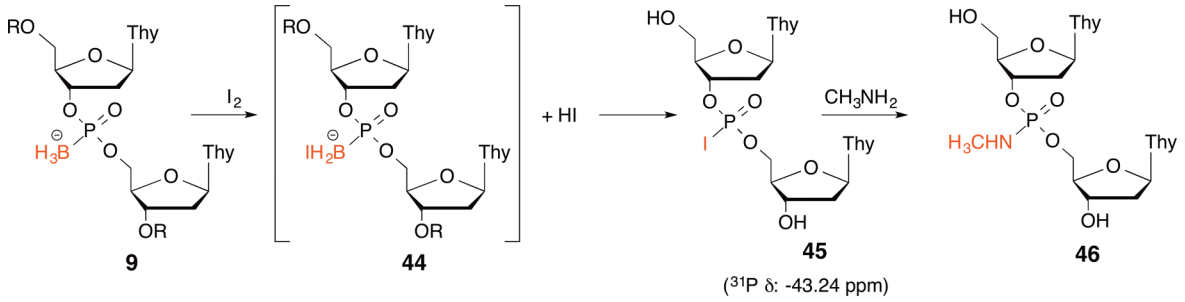
Scheme 2. Mechanism of Iodine-Activated Substitution of Boranephosphonate Diesters



Supporting Information, Figures S19–22). Mixed-base ODNs having ammonia substituted for borane at up to eight sites in bpDNA could be synthesized without any detectable "failure" products (Table 3, entries 19 and 20, Figure 2, and Supporting Information, Figures S23 and S24). This is evident from the ^{31}P NMR of the unpurified ODN 20 (Supporting Information, Figure S25C). The unpurified reaction mixtures for the mixed-base modified oligomers (ODNs 15–20) were also analyzed by denaturing gel electrophoresis. The only significant band in each case was the expected product (Figure 2).

Finally we tested whether this method could be used to attach common functionalities such as fluorophores to DNA oligomers. We carried out the reaction of an amino derivative of the dansyl dye with a 23-mer mixed-base ODN (see Figure 3) in which the last internucleotide linkage adjacent to the 5′ end was boranephosphonate. The desired product was obtained in 40% yield. Internucleotide phosphates are typically inaccessible and do not efficiently undergo reactions. Thus, fluorophores and other groups are usually conjugated to the 5′ hydroxyl or through a linker attached to the C5 position of pyrimidines. In this light, the reactivity observed here is significant as probes can be potentially introduced throughout the oligomer at any internucleotide linkage. Many useful fluorophores such as the cyanine family of dyes are sensitive to strong base and therefore must be incorporated via solution-phase conjugation reactions after cleavage of the oligomers from the solid support. This adds extra synthetic and purifications steps. Using the present chemistry, fluorophores would be introduced after removal of all protecting groups and need to survive only a very mild base for cleavage of modified oligomers from the support.

Mechanism of Iodine-Activated Substitution of Boranephosphonate Diesters. Iodine-activated substitution of the borane group, when carried out using diastereomerically pure **9** or **10**, yielded a single peak in the ^{31}P NMR (Figure 1) that indicates a stereospecific reaction pathway. These results are significant as synthesis of stereodefined phosphorus-containing compounds such as ODN derivatives, phosphotones and other phosphate-containing therapeutics are challenging. These factors therefore motivated us to further investigate the potential mechanism of this reaction. Similar to analogous reactions of amino boranes[28] we expected the present reaction to begin via oxidation of the borane hydrides by iodine. Moreover, based on our observation that 1 equiv of iodine is sufficient to activate the phosphorus center toward nucleophilic attack, an active intermediate of the type $(OR_1)(OR_2)(PO)(BH_2I)$ (**44**, Scheme 2) was hypothesized.

When **9** reacted with 3 equiv of iodine in acetonitrile, we observed the formation of a precipitate after approximately 10 min of stirring. Concomitantly a loss in ^{31}P NMR signal in the supernatant was observed, while addition of methylamine to the precipitate produced the methylamino phosphoramidate. This result indicated that the precipitate was likely the sought-after reactive intermediate. Initial attempts to characterize the precipitate were unsuccessful, as it was found to be insoluble in nonpolar solvents such as dichloromethane, toluene, or ethyl acetate, while in polar, non-nucleophilic solvents such as DMSO, DMF, and pyridine, various adducts (based on ^{31}P NMR spectra) that could not be characterized were produced (data not shown). During the course of investigating the reaction of boranephosphonate dimers with iodine and alcohols as nucleophiles (Table 2), we had found that the reaction with 2,2,2-trifluoroethanol, which is a polar solvent but a poor nucleophile, was quite slow. Letting this reaction mixture stand overnight did lead to the 2,2,2-trifluoroethylphosphate triester. This result indicated that the reactive intermediate was stable in this solvent for a long period of time. Consistent with these observations, we dissolved the precipitate in 2,2,2-trifluoroethanol and added a few drops (approximately 50 μL) of CD_2Cl_2 as a lock solvent for NMR. The ^{31}P NMR of this solution revealed a single resonance at −43 ppm (Figure 4). Although produced via a different pathway, a previous report

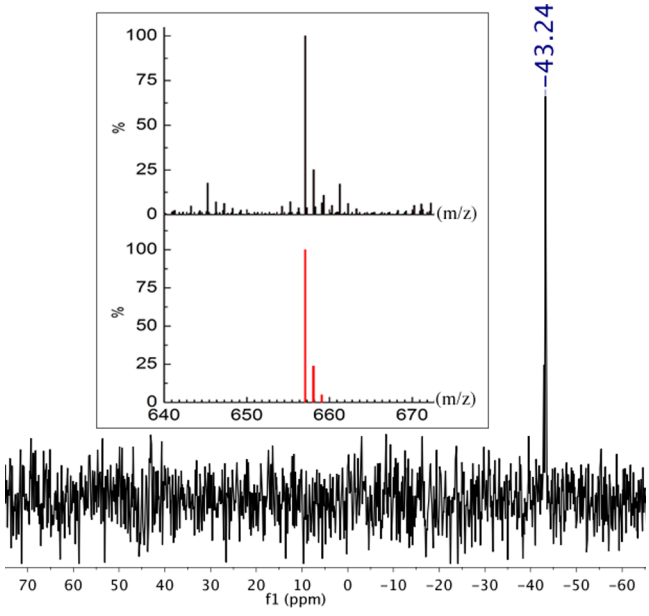


Figure 4. Characterization of the iodophosphate intermediate **45**. ^{31}P NMR spectrum in 2,2,2-trifluoroethanol, showing a peak at −43.24 ppm, with **9** as the starting material. Inset: ESI-MS (+ mode) shows that the recorded isotope distribution (top) matches the theoretical isotope distribution (bottom) of **45**. Theoretical mass, 656.0380; observed, 656.0371.

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from our laboratory[29] has described the formation of a reactive species having a similar ^{31}P NMR resonance that was postulated to be the iodophosphate intermediate. We also analyzed a solution of the precipitate dissolved in 2,2,2-trifluoroethanol by diluting it with anhydrous acetonitrile and directly infusing this solution into an ESI-MS (Figure 4). The resulting mass spectrum showed a high degree of correspondence between the theoretical and observed isotopic distribution for an iodophosphate (**45**) (Figure 4 inset). We noted that as per the mass spectrum, the precipitated product no longer contained the TBDMS protecting groups. Loss of TBDMS is likely caused by acid activation by the hydroiodic acid produced during oxidation of the hydrides of the borane group by iodine. The loss of the hydrophobic TBDMS groups is consistent with this species precipitating from the solution.

Thus, we conjecture that the initial reaction proceeds by oxidation of a hydride on the borane group to produce an iodoboranephosphonate intermediate (**44**, Scheme 2). Subsequently nucleophilic attack by iodide generates the iodophosphate (**45**). A second substitution reaction occurs at phosphorus through displacement of iodide with a nucleophile. Nucleophilic substitutions at phosphorus proceed via inversion of configuration.[30] Therefore, this reaction pathway, with two successive inversions of configuration, implies that formation of the final product occurs with an overall retention of configuration at phosphorus. These observations are supported by comparing the NOESY spectra (Supporting Information, Figure S26) of the starting boranephosphonate diastereomers (**9** and **10**) with the methyphosphotriesters that are produced from each isomer (**47** and **48**, respectively). **9** showed much stronger NOE peaks between the BH_3 hydrogens and the hydrogens on the ribose moiety when compared to **10**. Similarly **46** (the product from **9**) also showed stronger NOE signals between the methyl hydrogens and the sugar hydrogens than **47**. These results support the conclusion that the BH_3 group of **9** and the OCH_3 group of **46** are in the same relative orientation at phosphorus.

Stereospecific Synthesis of 2′-Deoxyoligonucleotide Analogues. Finally, the stereospecific nature of this reaction encouraged us to investigate whether the reactivity of boranephosphonates could be used to introduce stereospecific internucleotide phosphate linkages within 2′-deoxyoligonucleotides. We carried out the synthesis of diastereomerically pure 5′-O-dimethoxytrityl dideoxynucleotide boranephosphonate 3′-phosphoramidites d(TT), d(CT), d(AT), and d(GT) (**63**–**70**, Supporting Information, Scheme S2). Detailed synthetic procedure and methods for separation are provided in the Supporting Information. Briefly, after the tetrazole mediate coupling of 5′-*O*-dimethoxytrityl-*N*-BIBS-protected 2′-deoxynucleoside 3′-*O*-cyanoethyl-*N*,*N*-diisopropylphosphoramidites with 3′-*O*-acetyl-2′-deoxythymidine, the resulting triesters were boronated, the cyanoethyl groups removed, and the two diasteromers were separated via silica gel column chromatography. Subsequently, the 3′-acetyl was removed and the resulting free hydroxyl phosphitylated to yield the desired dimer phosphoramidites. Comparison of the ^{31}P NMR of the diastereomeric pairs revealed distinct chemical shifts in each case (Table 4). These studies demonstrate the feasibility of synthesizing and more importantly separating the diastereomeric pairs containing any of the four nucleobases. In fact, in our experience we have found that the bulky BIBS group allows more facile separation of the diastereomers by silica gel chromatography when compared to nucleobases containing standard amide protecting groups such as benzoyl and acetyl.

Table 4. ^{31}P NMR Chemical Shifts Corresponding to the Boranephosphonate Moiety in the Dimer Phosphoramidites

	^{31}P NMR (ppm)[a]	
	fast	slow
TT	94.97 (**63**)	93.74 (**64**)
CT	93.38 (**65**)	94.54 (**66**)
AT	93.75 (**67**)	94.71 (**68**)
GT	93.90 (**69**)	95.11 (**70**)

[a]The compound numbers are indicated in parentheses.

Subsequently we carried out solid-phase synthesis of 21-mer 2′-deoxyoligonucleotides where each ODN had one of the diastereomers (Table 4) incorporated at four positions. These ODNs were then reacted with 0.05 M iodine and 0.4 M ammonia in dioxane to yield oligomers containing stereospecific phosphoramidate internucleotide linkages (Table 5).

Table 5. 2′-Deoxyoligonucleotides Containing Stereospecific Phosphoramidate Linkages Synthesized Starting from Stereopure Boranephosphonate Dimer Phosphoramidites[a]

ODN No.	Sequence	Dimer Used	Tm
ODN 22	TTTTTTTT*TT*TT*TT*TTTTTTT	**63**	44.1 ± 0.6
ODN 23	TTTTTTTT*TT*TT*TT*TTTTTTT	**64**	45.8 ± 0.5
ODN 24	TTTTTTTC*TC*TC*TC*TTTTTTT	**65**	57.4 ± 0.01
ODN 25	TTTTTTTC*TC*TC*TC*TTTTTTT	**66**	55.4 ± 1.0
ODN 26	TTTTTTTTTTTTTTTTTTTTT	-	52.87 ± 0.8
ODN 27	TTTTTTTCTCTCTTTTTTT	-	64.5 ± 0.4

[a]Phosphoramidate linkages are denoted by red asterisks. Melting temperatures were measured for binding to a complementary RNA sequence. ODN 26 and ODN 27 correspond to the unmodified DNA sequences.

The incorporation of the d(TT) and d(CT) dimers occurred in high yields, although longer coupling time of 15 min were required. Unfortunately, the d(AT) and d(GT) dimers did not couple efficiently under these conditions and in amounts sufficient for further studies. Future research will be carried out to optimize conditions that will allow incorporation of these compounds as well.

The oligomer pairs incorporating the d(TT) or d(CT) dimers (ODN 22 from **63** and ODN 23 from **64**, or ODN 24 from **65** and ODN 25 from **66**, respectively) were purified by reverse-phase HPLC and analyzed by LC-MS (Supporting Information, Figures S27–S30). The ^{31}P NMR spectra (Figure 5) of ODN 22 and ODN 23 show efficient conversion of the boranephosphonates to amidates. Interestingly, we found that multiple batches of ODN 22 showed the formation of a broader phosphoramidate peak (at 12 ppm) when compared to the corresponding peak for ODN 23. Moreover, this oligomer also proved to be challenging to purify by HPLC as evidenced by a higher than expected integration value of the phosphate peak (Figure 5). In contrast, ODN 23 yielded the correct 1:4 ratio of the areas under the phosphoramidate and phosphate peaks. These observed differences may be explained by different conformations adopted by oligomers containing distinct diastereomeric phosphoramidates.

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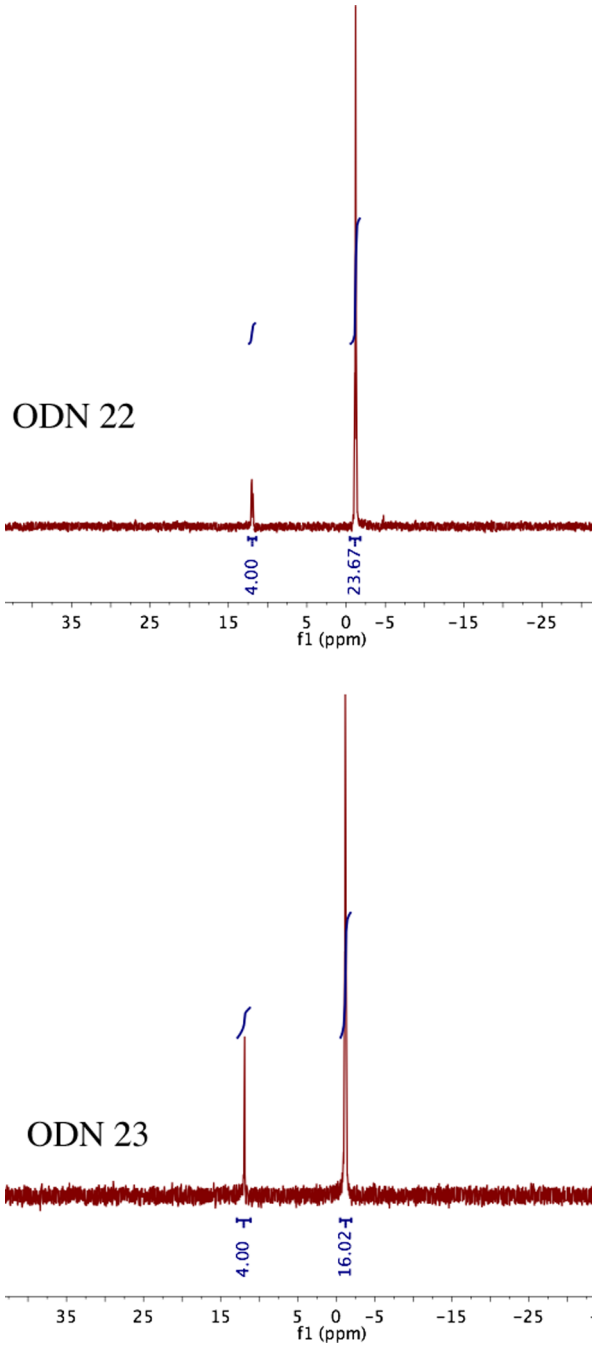

Figure 5. ^{31}P NMR of purified ODN 22 and ODN 23.

Further confirmation that the reaction for converting the boranephosphonate to phosphoramidate occurs stereospecifically was obtained by measuring the melting temperature of these 2′-deoxyoligonucleotides with complementary RNA sequences. As expected, in each case one of the oligomers was found to bind more strongly to the complementary sequence than its diastereomeric partner (Table 5).

DISCUSSION

Our discovery that the stable boranephosphonate internucleotide linkage is activated by iodine oxidation toward substitution reactions with various nucleophiles has created several possible avenues for using this new synthetic pathway not only in the nucleic acids field but other areas as well. This manuscript outlines a plausible mechanism for this reaction and also presents initial results illustrating how we can utilize the boranephosphonate analogue for various applications.

The high stability of boranephosphonates is based upon the fact that the borane moiety is a poor leaving group due to its low apicophilicity. It is well established that, during substitutions at phosphorus centers, the incoming and leaving groups need to be in the apical positions of the trigonal bipyramidal intermediate.[31] Apicophilicity is positively correlated with electronegativity.[32,33] Thus, theoretical calculations have shown that reaction intermediates with borane at the apical position are high energy and unfavorable. Additionally, comparisons of boranephosphonates with analogous aminoboranes, which are relatively unstable and readily hydrolyze in protic solvents such as methanol or water, suggest that the P-BH_3 bond is significantly stronger than N-BH_3. The added stability of this interaction is probably due to back-donation of electrons from the σ orbitals of the borane to empty (perhaps σ^*)[34] orbitals on phosphorus. In the present reaction the initial oxidation of one or more of the hydrides on borane leads to the formation of an iodoborane derivative (such as **44**) that likely causes disruption of such bonding interactions. Concomitantly the electronegative iodine atom makes the borane moiety more apicophilic and thus a better leaving group. The overall effect is to cause substitution at phosphorus to proceed very efficiently.

Wada and co-workers have reported a separate reaction pathway[35,36] whereby boranephosphonates react with trityl cations to produce an H-phosphonate, i.e., a P(III) compound. The present reaction, however, clearly proceeds through a P(V) intermediate. Taken together, these complementary reactivities illustrate that, in spite of their "P(V)-like" stability, boranephosphonates represent an intermediate oxidation state allowing access to both P(III) and P(V) compounds, depending on the reaction conditions used. We hope that such unparalleled versatility of these synthons will find broad usage in organophosphorus reaction schemes as well as further studies toward gaining a better understanding of the structure and bonding in these compounds.

The synthetic methodology for preparing bpDNA oligomers we have developed previously[27] has a two-fold benefit in enabling the versatile method of DNA modification described here. First, the ability to prepare 25−30-mer bpDNA, having all four nucleoside bases, in essentially quantitative yield far exceeds any previously reported procedure.[16,37−41] Thus, we are able to produce modified oligomers derived from boranephosphonates of lengths that are necessary for applications such as DNA-based materials, antisense agents and sensors. Second, the di-*tert*-butylisobutylsilyl group used to protect the exocyclic amines is removed by treatment with mild, buffered fluoride solutions. Thus, by introducing the modifications through substitution of boranephosphonates at the end of the synthetic cycle and after removal of nucleobase protecting groups, there is no need to expose the final ODN product to either strong acids or bases as is the case in all previously outlined chemistries.[42−44]

Here we illustrate the strength of this approach through the synthesis of ODNs having phosphoramidate internucleotide linkages. However, based on experiments with boranephosphonate dimers we anticipate that this approach will also apply to the synthesis of ODNs having alkyl/aryl phosphonate and phosphate triester linkages as well. The approach would be the same-complete synthesis and removal of protecting groups prior to conversion of the boranephosphonate linkages to

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phosphonates or triesters. Thus, the synthesis of ODNs having these linkages, which are highly susceptible to base and fluoride, should now be possible in very high yields and with excellent purity. For example previous research has shown that alkynyl phosphonates[45] (as clickable reagents when reacted with azide to yield triazoylphosphonates), phosphonoacetates[46,47] and phosphonoformates[48] all show biological and biochemical activity. However, with current chemistries such as the phosphoramidite approach, stepwise yields of 95% severely limits their value. The approach as outlined here will generate very high yields of boranephosphonate DNA that can then be converted via Grignard, Reformatski, or alkyl/aryl lithium reagents to the corresponding phosphonates.

This reaction pathway also opens opportunities to generate new analogues that are limited by current chemistries. Here we show that azide functionalities (which previously could not be introduced according to the phosphoramidite approach) are accessible in high yield simply by iodine oxidation of boranephosphonates following ODN synthesis. Thus, this pathway will be useful, via azides, for introducing fluorophores, EPR probes, and other diagnostic reagents throughout an ODN at any predetermined internucleotide linkage.

Oligonucleotides containing phosphorus modifications provide stability against nucleases and therefore have been extensively used in oligonucleotide-based therapeutics. In these DNA analogues, the presence of a chiral phosphorus has significant implications for their properties such as binding to complementary sequences. Although the field of stereo defined oligonucleotide synthesis has advanced significantly, perhaps the most relevant advances are the synthesis of stereo defined phosphorothioate DNAs and boranephosphonate DNAs using monomer synthons.[49−52] For all these methods there are several limitations such as cycle yields during synthesis of oligomers, difficult purifications of isomers, and stereo excess. Here we use iodine-activated substitution of boranephosphonates with ammonia to produce stereodefined phosphoramidate internucleotide linkages in 2′-deoxythymidine and 2′-deoxycytidine ODNs. The approach involves first the synthesis in high yields of 5′-*O*-dimethoxytrityl-2′-nucleosidyl-3′ 3′-*O*-acetylthymidyl-5′ boranephosphonates with sequences TT and TC. These diastereomers were separated on silica gel, the acetyl group removed, the resulting dimers converted to the 3′- phosphoramidites, and these synthons used in the preparation of 2′-deoxyoligonucleotides having four stereocontrolled internucleotide linkages (21-mers). As is the case with other dimer synthesis strategies, yields are relatively low due to the slow reaction rates of these dimers. This is currently a limitation of our research with stereo defined dimers of AT and GT. Although the yields of AT and GT dimers as boranephosphonates are very good (see experimental details in the Supporting Information) and separation of the isomers is straightforward via silica gel chromatography, the incorporation of these synthons into ODNs proceeds slowly at best, and further research with activators, more reactive 3′-phosphoramidites, longer reaction times, and double coupling must be carried out. However, given the efficient reaction of these boranephosphonates dimers with iodine and a variety of nucleophiles, current work is underway in our laboratory to use this reaction pathway to prepare various stereodefined, P-modified oligomers such as phosphotriesters, phosphorthioates, and alkylphosphonates.

Finally, the discovery of this new reaction pathway has stimulated us to investigate several novel applications. For example we are currently attempting to use this reaction sequence to prepare imidoamidate DNA (both nonlinking valencies have nitrogen substituted for oxygen) and as a method for templated chemical ligation of two ODNs to produce longer ODNs linked via a natural phosphodiester bond. Another research direction we are exploring is to use this chemistry to insert organometallic reagents (most are impossible at this time because of the chemistries used to synthesize DNA) at phosphorus and specifically complementary duplexes. We have also recently demonstrated that natural internucleotide linkages and morpholino DNAs can be prepared via iodine oxidation of boranephosphonate synthons.

■ CONCLUSIONS

Boranephosphonate diesters are shown to undergo efficient substitution reactions upon oxidation of the BH_3 hydrides by iodine. Reactions with amines, alcohols, propanethiol, and methylmagnesium bromide led to the formation, in near-quantitative yields, of phosphoramidates, phosphotriesters, phosphorothioates, and methylphosphonates, respectively. This reaction proceeds through an iodophosphate intermediate in a stereospecific manner with an overall retention of configuration. We have used this reaction to develop a method for the incorporation of modifications into DNA in a post-synthetic manner. Given the presence of phosphate groups in a large number of biologically relevant molecules, the present reaction pathway has the potential to open up many new avenues in synthetic organophosphorus chemistry.

■ EXPERIMENTAL SECTION

Synthesis of Phosphoramidate, Phosphotriester, Phosphorothioate, and Alkylphosphonate Dinucleotides from Boranephosphonate Dinucleotides. The boranephosphonate dinucleotide (**9** or **10**, 0.01 mmol) was added to a 10 mL pear-shaped flask stoppered with a rubber septum under an atmosphere of argon. Anhydrous acetonitrile (1.0 mL) was added to the flask via a syringe. In a separate flask, iodine (0.03 mmol) was dissolved in acetonitrile (0.5 mL). For reactions listed in Table 1, the nucleophile (0.1 mmol) was added to the flask containing the boranephosphonate diester followed by addition of the iodine solution. For reactions in Table 2, the iodine solution was first added and allowed to stir for approximately 1 min, followed by addition of diisopropylethylamine. Immediately afterward, the nucleophile was added. Reaction mixtures were then allowed to stir overnight. Except for entries 6 and 7 in Table 2, an aliquot of each reaction mixture was transferred to an NMR tube, and 50 μL of CD_2Cl_2 was added as the lock solvent for recording the ^{31}P NMR spectrum. For reactions with propanethiol or methylmagnesium bromide (Table 2, entries 6 and 7), a white precipitate formed upon addition of the nucleophile. After stirring overnight, these reaction mixtures were diluted with dichloromethane (5 mL) and extracted with water (twice, 5 mL). The organic layers were evaporated to dryness. The residues were dissolved in CD_2Cl_2 for recording the ^{31}P NMR spectra or in methanol for mass spectrometric analyses.

Post-synthetic Modification of ODNs. ODNs containing boranephosphonate linkages were synthesized as described previously[27] at a 0.2 μmol scale on highly cross-linked polystyrene resin (Glen Research). Subsequently each resin-linked ODN was treated with disodium 2-carbamoyl-2-cyanoethylene-1,1-dithiolate in *N*,*N*-dimethylformamide (1.0 M) for 45 min in order to remove the methyl protecting group on phosphorus, washed repeatedly with anhydrous DMF and acetonitrile and dried under a stream of argon. The resins were then treated with a fluoride deprotection mixture (940 μL of DMF + 470 μL of Et_3N + 630 μL of $Et_3N{\cdot}(HF)_3$) overnight in order to remove the silyl protecting groups on the 2′-deoxynucleoside bases. Subsequently the resins were washed repeatedly first with DMF and

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then with anhydrous acetonitrile, dried under a flow of argon, and transferred to glass vials. A solution of iodine (0.05M) and amine (0.2 M) in anhydrous acetonitrile was added to each glass vial. In the case of reactions with ammonia, appropriate amounts of iodine were dissolved in solutions of ammonia (0.4 M) in dioxane, and these solutions were added to the resins. The glass vials were then placed on a mechanical shaker for 48 h.

Vials containing the resins were centrifuged at 4000 rpm and the supernatants removed with a pipet. Subsequently the resins were washed 4−5 times with 2 mL aliquots of acetonitrile, shaken vigorously, placed in a centrifuge at 4000 rpm for 5 min and supernatants removed. The resins were then treated with 1 mL of 30% ammonium hydroxide in water for 30 min and the ammonia was removed by evaporation in a SpeedVac instrument. The ODNs were dissolved by adding 800 μL of water. The polystyrene resin beads were then removed using a 0.2 μm centrifugal filters. The filtrates were used for subsequent analyses.

■ ASSOCIATED CONTENT

Supporting Information

Detailed synthetic procedures, characterization information, LC-MS spectra of the oligomers synthesized, and NOESY spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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