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Chemical synthesis of an oligodeoxythymidylate containing boranephosphate and phosphate linkages

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Abstract—Oligodeoxythymidylate 14mers containing combinations of boranephosphate and phosphate linkages have been synthesized. These compounds show improved binding affinity and RNase H activation over fully modified boranephosphate DNA. © 2002 Elsevier Science Ltd. All rights reserved.

The search for DNA analogues useful for antisense applications has led to a wide range of backbone modifications. However, among these various derivatives, only phosphorothioate and phosphorodithioate modified DNA have been shown to be RNase H active, capable of forming duplexes with complementary oligonucleotides, and resistant to nuclease degradation.[1,2] Because these analogs, perhaps in part due to their sulfur content, appear to have certain undesirable biological properties,[3] there is room for the development of new derivatives. One of these is an analog whereby borane replaces a nonlinking oxygen in an internucleotide phosphate.[4] To date fully modified oligodeoxythymidylate 12 and 14mers have been reported[5,6] as well as a dimeric RNA derivative.[7] The fully modified 14mer,[6] dT_{14}-BH_3FM, hybridizes with complementary DNA and RNA but the binding affinity is less than unmodified controls as both duplexes have Tm depressions of 29°C. This analog also activates RNase H although there is a three-fold reduction in the cleavage rate compared to dT_{14}. When tested against nucleases, dT_{14}-BH_3FM showed resistance to snake venom phosphodiesterase and DNase I. Similar results[5] are obtained with the fully modified 12mer. While the potential antisense properties of this analog are encouraging, certain properties such as the significant loss of duplex stability require further exploration. One possibility is to reduce the boranephosphate content as work with phosphorodithioates has shown that oligomers having alternating phosphorodithioate/phosphate linkages have enhanced Tms but retain stability toward nucleases and RNase H activity.[2] This manuscript outlines the development of an approach for the synthesis of these analogs having boranephosphate and phosphate internucleotide linkages in the same oligodeoxynucleotide (ODN).

The synthesis of fully modified boranephosphate DNA relies on H-phosphonate chain elongation followed by global boranation.[5,6] This approach cannot be used to prepare ODNs having interspersed boranephosphate and phosphate internucleotide linkages. Our search for a more versatile procedure led us to re-examine the method whereby the borane group is introduced at the trialkylphosphite stage via borane complex exchange.[4] This method, however, is limited to the preparation of a dimer as removal of the dimethoxytrityl protecting group leads to loss of borane from the internucleotide linkage.[6,8] We reasoned that introduction of the recently developed bis-(trimethylsiloxy)cyclododecyloxysilyl ether (DODSi)[9] at the 5′-position of a deoxynucleoside phosphoramidite would eliminate this problem and lead to a satisfactory synthesis strategy. Initial model experiments demonstrated that the borane phosphate linkage is stable to 5′-silyl deprotection conditions with fluoride ion. Further studies also demonstrated that this silyl ether is stable to boranation conditions. These findings led us to develop the synthesis strategy outlined in Scheme 1.

For the solid-phase preparation of a boranephosphate/phosphate DNA, the synthetic cycle consists of several unique steps.[10] Preparation of compound **1** followed a published procedure[9] in 50–60% yield. Preceding the automated cycle, 5′-*O*-dimethoxytrityl-2′-deoxythymidine attached to the polystyrene support was detritylated with trichloroacetic acid (TCA). The synthesis cycle then begins by activation of **1** with tetrazole,

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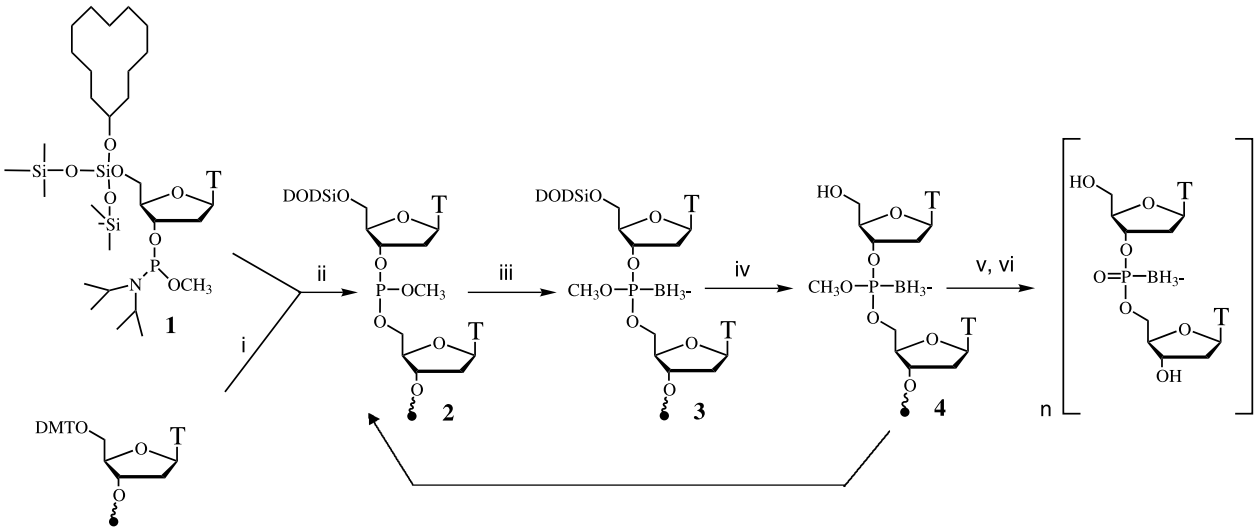

Scheme 1. Solid-phase synthesis of DNA having boranephosphate and phosphate internucleotide linkages. *Reagents*: (i) TCA/DCM; (ii) tetrazole/acetonitrile; (iii) BH_3·THF; (iv) TEA·HF/DMF; (v) disodium-2-carbamoyl-2-cyanoethylene-1,1-dithiolate/THF; (vi) NH_4OH. For introduction of a phosphate linkage, substitute *t*-butylperoxide/acetone at step iii.

coupling to form **2**, and boronation with BH_3·THF to yield **3**. Removal of DODSi with triethylammonium fluoride yields **4** which is now ready for repetition of the cycle. By oxidizing **2** with *t*-butylperoxide, an unmodified phosphate triester is generated instead of **4**. Following completion of the total synthesis, treatment with disodium-2-carbamoyl-2-cyanoethylene-1,1-dithiolate removes the methyl group from **4** yielding boranephosphate and from the methylphosphate triester internucleotide linkage to generate the natural phosphodiester. Following treatment of the support with aqueous ammonia at 55°C overnight, the product ODN is purified by RP-HPLC (Hypersil-BDS) in 20–25% overall yield. Analysis of the product by ^{31}P NMR (Fig. 1) showed a broad peak at 96 ppm and a sharp peak at −2 ppm. These peaks are consistent with boranephosphate and phosphodiester linkages, respectively.[4] Peak intensities vary according to the number of each linkage present. A small peak at 1 ppm appears to be boric acid coordinated to the unmodified phosphodiester linkage as it can be removed by anion-exchange HPLC. ^{11}B NMR showed a broad peak at −40 ppm corresponding to boranephosphate linkages and an impurity at 19 ppm which is assigned as the coordinated boric acid.

ODNs containing boranephosphate at every other position, $dT_{14}BH_3EO$, and every third position, $dT_{14}BH_3E3$, have undergone preliminary biochemical analysis. The hybridization properties of $dT_{14}BH_3EO$ and $dT_{14}BH_3E3$ with complementary rA_{14} and dA_{14} are summarized in Table 1. Lowering the borane content to 54% (7/13 linkages, $dT_{14}BH_3EO$) and 31% (4/13, $dT_{14}BH_3E3$) does indeed improve the binding affinity. The ΔTm/linkage of 2.2°C compares favorably with nonstereospecific dT_{14} phosphorothioate oligomers which exhibit ΔTm/linkage of 1.4–1.5°C.[11] When G:C base pairs are introduced into nonstereospecific phosphorothioate DNA, the ΔTm/linkage drops to 0.5–0.65°C.[11] Perhaps similar reductions will be observed with mixed sequence boranephosphate ODNs. The ability of these mixed backbone ODNs to direct *E. coli*

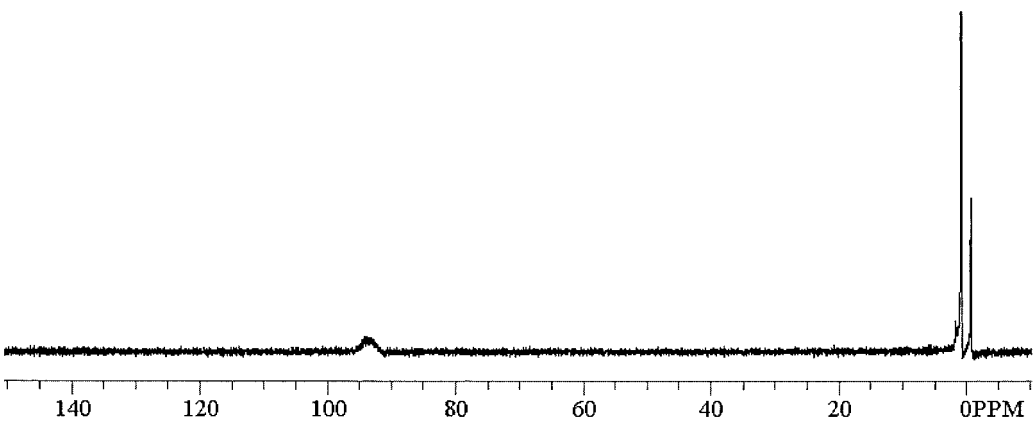

Figure 1. ^{31}P NMR of $dT_{14}BH_3EO$. The peak at 0 ppm is a phosphoric acid reference.

Table 1. A summary of melting temperatures for boranephosphate containing ODNs

Duplex[a]	Tm (°C)[b]	ΔTm (°C)	ΔTm (°C)/linkage
dA_{14}:$dT_{14}BH_3FM$	16.8	29.1	2.2
dA_{14}:$dT_{14}BH_3EO$	31.1	14.8	2.1
dA_{14}:$dT_{14}BH_3E3$	38.0	7.9	2.0
dA_{14}:dT_{14}	45.9	–	–
rA_{14}:$dT_{14}BH_3FM$	6.8	29.9	2.3
rA_{14}:$dT_{14}BH_3EO$	21.6	15.2	2.2
rA_{14}:$dT_{14}BH_3E3$	28.5	8.3	2.1
rA_{14}:dT_{14}	36.8	–	–

[a] $dT_{14}BH_3FM$, fully modified boranephosphate; $dT_{14}BH_3EO$, every other linkage boranephosphate; $dT_{14}BH_3E3$, every third linkage boranephosphate.

[b] Buffer: 0.1 M potassium phosphate and 0.1 M KCl at pH 7.0.

RNase H showed significant improvement as both $dT_{14}BH_3EO$ and $dT_{14}BH_3E3$ activated this activity to the same cleavage rate as dT_{14} and similarly modified phosphorothioate dT_{14}. Lowering the number of modified linkages did not negatively affect the nuclease resistance of boranephosphate/phosphate DNA. All variants of borane modified ODNs were 100% resistant to DNase I. Snake venom phosphodiesterase was able to degrade at the same rate both $dT_{14}BH_3E3$ and an analog with phosphorothioate linkages at every third position. The $dT_{14}BH_3FM$ and $dT_{14}BH_3EO$ ODNs were completely resistant to snake venom phosphodiesterase.

These results suggest that boranephosphate containing ODNs have biochemical properties useful for further research in the antisense area. Research directed toward the incorporation of guanine, cytosine and adenine nucleotide bases is currently underway.

Acknowledgements

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10. Syntheses were performed on an ABI 392 synthesizer. The HF content of the TEA·HF necessitates a few adaptations. The glass flow restrictor was replaced with a Teflon flow restrictor and the TEA·HF was stored on the machine in a nalgene bottle. Attack of the fluoride ion reagent on the silyl backbone of CPG entails the use of a polystyrene support. The automated cycle contains the following steps:
    i. Coupling: (a) 2×2.5 s simultaneous delivery of deoxynucleoside amidite 1 at 0.1 M in acetonitrile and 0.45 M tetrazole in acetonitrile followed by 60 s wait. (b) 1×20 s rinse in acetonitrile.
    ii. Boranation: (a) 1×15 s delivery 0.1 M BH_3·THF in THF followed by a 30 s wait. (b) 30 s rinse with acetonitrile. Due to the instability of the BH_3·THF complex on the synthesizer, a fresh solution was prepared for each boranation event.
    iii. 5′-Deprotection: (a) 30 s rinse with DMF. (b) 1×25 s delivery of 1 M HF, 3.3 M TEA in DMF followed by a 45 s wait. (c) 1×30 s rinse with DMF. (d)1×60 s rinse with acetonitrile.
    iv. Oxidation to phosphate: (a) 1×45 s 1 M *t*-butylperoxide in acetone. (b) 1×30 s rinse with acetonitrile.
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