Pergamon

Tetrahedron Letters 39 (1998) 3899–3902

TETRAHEDRON LETTERS

Synthesis of an Oligothymidylate Containing Boranophosphate Linkages

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Received 4 March 1998; revised 20 March 1998; accepted 23 March 1998

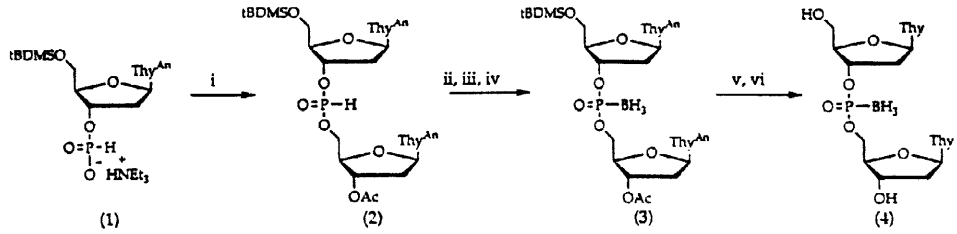
Abstract An oligothmidylate containing boranophosphates was synthesized *via* silylation of *H*-phosphonate linkages followed by boranation. These compounds formed duplexes having reduced stability with complementary RNA/DNA and stimulated RNase H activity.

The search for DNA analogues meeting the requirements necessary for antisense therapeutics has led to a wide range of backbone modifications.[1,2] Although phosphorothioate/dithioate analogues possess many of these requirements such as RNase H activation, there is still room for improvement. A new and promising member of this class, in which a non-bridging oxygen has been replaced by a borane group, has been reported by Shaw and co-workers,[3] and the RNA analogue by Chen *et al.*[4] This group is closely related to normal phosphodiesters and methylphosphonates as it is isoelectronic and isostructural with the methylphosphonates and carries a negative charge. Boranophosphate containing dimers have also been shown to be stable towards hydrolysis and resistant to phosphodiesterase degradation.[3] Due to the lack of a good route for the synthesis of boranophosphate oligonucleotide linkages, recent studies have been restricted to single replacement oligonucleotides.[5] Previous syntheses have been based on phosphoramidite chemistry where the borane group is introduced at the trialkyl phosphite stage *via* borane complex exchange. Given that the key synthetic step is borane exchange, we decided to base our work on reactive silyl phosphites. Prepared from either silyl phosphoramidites[6] or *via* silylation of the corresponding *H*-phosphonate,[7] dinucleotide silyl phosphites have proved to be versatile intermediates. We decided to prepare an oligothymidylate containing boranophosphate linkages from the corresponding *H*-phosphonate oligomer via a global silyl activation and borane treatment (Scheme 1). While this work was ongoing Wada and Sekine published an analogous procedure for the synthesis of hydroxymethylphosphonate internucleotide linkages.[8]

Model studies were performed on a 5'-O-dimethoxytritylthymidin-3'-yl 3'-O-acetylthymidin-5'-yl *H*-phosphonate diester. Conversion of the *H*-phosphonate diester to the silyl phosphite with *N,O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) (3 equiv.) in THF proceeded rapidly (10 minutes) and quantitatively. *In situ* treatment of the silyl phosphite with borane-THF complex resulted in rapid formation of the boranophosphate (<10 minutes, time required to acquire ^{31}P nmr spectrum), aqueous work up resulted in the loss of the trimethylsilyl group giving the boranophosphate diester with a purity of 98% as judge by ^{31}P nmr. UV analysis of the product showed a large decrease (60-75%) in absorbance indicating a borane mediated reduction of the base. Alteration of the borane concentration or the use of less reactive borane complexes, such as the amine or dimethylsulfide derivatives leads to a decrease in the amount of thymine reduction but we were unable to totally prevent it. Analysis of the side reaction showed a link between the silylation step and

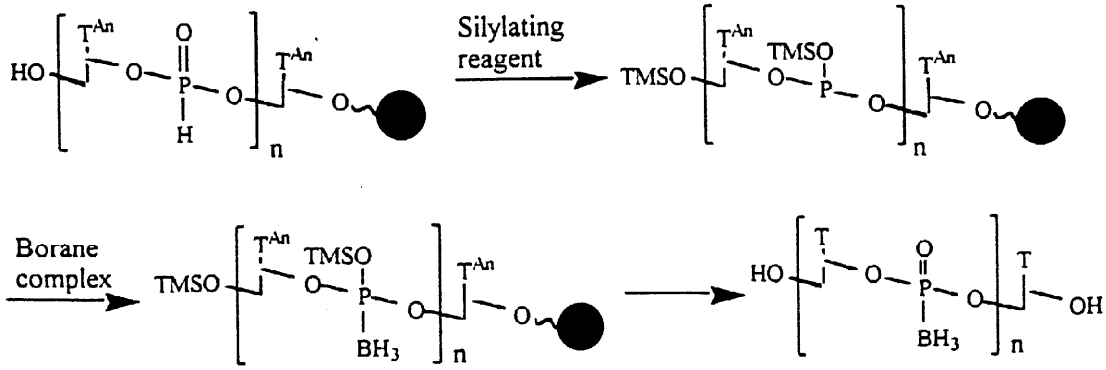
0040-4039/98/$19.00
PII: S0040-4039(98)00687-X

reduction of the thymine base. Presumably, BSTFA treatment causes silylation of the C4 oxygen introducing a N3-C4 double bond which appears susceptible to reduction by borane. In order to block the formation of this double bond, the N3 position was protected with the anisoyl group.[9] These model studies also showed the boranophosphate linkage to be incompatible with dimethoxytrityl deblocking procedures.[10] Therefore for the synthesis of a dithymidinyl boranophosphate, we protected the 5'-hydroxyl as the t-butyldimethysilyl ether and thymine N3 as the anisoyl amide (Scheme 1).



Scheme 1. Synthesis of Dithymidinyl Boranophosphate. Reagents i) 3-N-Anisoyl-3'-O-acetyl-thymidine, $(EtO)_2P(O)Cl$, ii) BSTFA, iii) BH_3-iPr_2NEt, iv) aq. work up, v) $MeOH/NH_3$, vi) TBAF.

The anisoyl derivative (1) was coupled to 3-N-anisoyl-3'-O-acetylthymidine using diethylchlorophosphate as the activator giving (2) in 74% yield. The *H*-phosphonate diester (2) was treated with BSTFA (3 equiv.) in THF for 10 minutes followed by *in situ* conversion to the boranophosphate using borane-diisopropylethylamine complex (6 equiv. 3 hours). The volume of THF was calculated to give a 0.1 M borane concentration. Aqueous work up resulted in loss of the trimethylsilyl group. The product was purified immediately by silica gel flash chromatography to remove excess borane complex and provide the boranophosphate (3) in 91% yield. Analysis by ^{31}P nmr spectroscopy showed a broad peak at 96ppm corresponding to the boranophosphate group and a small phosphate contamination at 1 ppm (<2%). The ^{11}B nmr spectrum of (3) contained a single broad peak at -40.5 ppm (relative to BF_3) corresponding to the boranophosphate. Removal of the acetyl and anisoyl groups with ammonia proceeded as expected without affecting the boranophosphate linkage. The 5' t-butyldimethylsilyl group was removed with TBAF (5 equiv.). The final product (4) had the UV absorbance expected of a dinucleotide.



Scheme 2. Solid Phase Synthesis of Oligothymidinyl Boranophosphate.

We were interested to see if this chemistry could be applied to solid phase synthesis of oligonucleotide boranophosphates (Scheme 2). Due to incompatibility of the dimethoxytrityl group with boranophosphates, a modified strategy was followed. This involved presynthesis of oligothymidine as the H-phosphonate via standard procedures, removal of the 5'-dimethoxytrityl group, silylation, and boronation. It was considered unlikely that the presence of an oligonucleotide 5'-hydroxyl group would lead to side products as it was probably protected as the silyl ether following BSTFA treatment. 5'-Dimethoxytrityl-3-N-anisoylthymidine as the 3'-H-phosphonate was used as the synthon. Loading involved converting 5'-dimethoxytrityl-3-N-anisoylthymidine to the succinate ester and attaching this derivative to the LCA-CPG support by 1,3-dicyclohexylcarbodiimide (DCC) mediated coupling. The concentration of support linked nucleoside was controlled to give 20-30 μmol g^{-1}.Using the strategy outlined in Scheme 2, a tetradecathymidinyl boranophosphate was synthesized. The *H*-phosphonate oligonucleotide was prepared on a 1 μmol scale using an automated synthesis cycle with stepwise coupling being >98% as judged by the trityl cation assay. Silylation and boranation were completed manually using the double syringe technique. The *H*-phosphonate groups were first converted to the silyl phosphite using 0.4 M BSTFA in THF (4 hrs, 1 ml), the support was washed with 0.1 M borane-diisopropylethylamine in THF (1 ml) and allowed to react (7 hrs) with a fresh 2.0 ml of 0.1 M borane-diisopropylethylamine in THF. After washing the support with THF (2 x 1 ml) and dichloromethane (10 ml) followed by drying with argon, the oligonucleotide was cleaved from the support and anisoyl groups removed using concentrated aqueous ammonia (2 ml, 48 hours) at r. t. Analysis of the crude product by ^{31}P nmr spectroscopy showed a broad peak at 94 ppm consistent with the boranophosphate linkage; this peak accounted for 85-90% of the phosphorus content with the remaining 10-15% located as several peaks between -2 and 7 ppm. The phosphate (-1 ppm, single peak in proton coupled ^{31}P nmr spectrum) impurity is related to removal of the trimethylsilyl group from the phosphorus center. Desilylation with water prior to ammonia treatment leads to an increase in impurity and fluoride mediated desilylation gave an impurity content of 10-15%. Purification of the oligonucleotide was achieved by reverse phase HPLC (ODS Hypersil) giving 64 A_{266} units (58%). The ^{11}B nmr spectrum showed a broad peak at -40.5 ppm consistent with the boranophosphate.

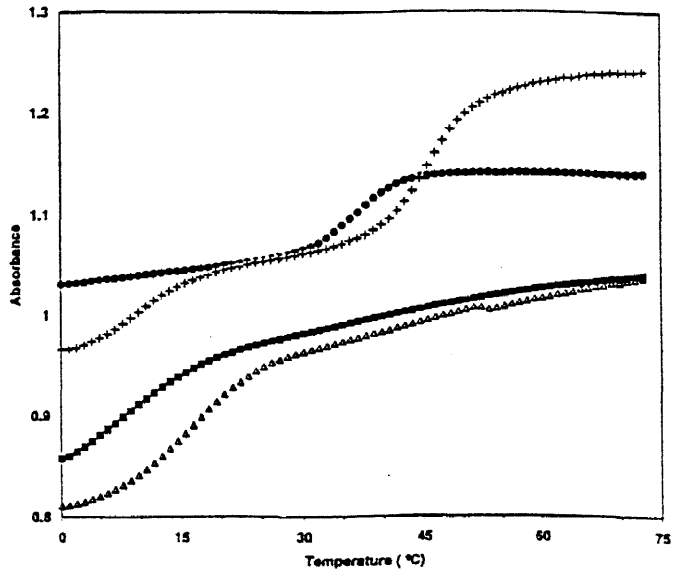


Figure 1. Melting curves of dT_{14} phosphodiester and dT_{14} boranophosphate with rA_{14} and dA_{14} in 0.1 M KCl, 0.1 M phosphate buffer, pH 7. (●) rA_{14}:dT_{14}; (+) dA_{14}:dT_{14}; (■) rA14:BH_3dT_{14}; (Δ) dA_{14}:BH_3dT_{14}.

The hybridization properties of a boranophosphate dT_{14} (BH_3dT_{14}) with complementary rA_{14} and dA_{14} were determined by thermal denaturation studies. Figure 1 shows that at low ionic strength (0.1 M KCl, 0.1 M phosphate buffer pH 7), BH_3dT_{14} hybridizes with rA_{14} and dA_{14}. The binding affinity is less than the unmodified phosphodiester control, with both the BH_3dT_{14}:dA_{14} and BH_3dT_{14}:rA_{14} duplexes having Tm depressions of 29 °C. High ionic strength conditions (1 M KCl, 0.1 M phosphate buffer pH 7) increased the Tm of all duplexes by approximately 5 °C. The ability of boranophosphate DNA to direct *E. coli* RNase H cleavage was also examined at 4 °C. Under these conditions, fully modified BH_3dT_{14} was indeed active in stimulating RNase H but there was about a 10 fold reduction in the rate of cleavage compared to dT_{14}.

Our preliminary results suggest that thymine protection is required under all boronation conditions tested in order to prevent base modification as observed by reduction in UV absorbance. Boronated DNA prepared in this manner forms duplexes with reduced stability, relative to native oligomers, and is RNase H active. Using a similar chemistry but without base protection, Zhang et al.[11] report no detectable duplex formation with boronated dT_{15}. However since data collection did not continue below 15 °C, it is difficult to judge whether results similar to ours would have been possible using the Zhang et al. chemistry.

The observation that boranophosphate DNA is RNase H active is indeed encouraging, especially when coupled with the nuclease resistance of these compounds.[3] However the significant reduction in Tm with this linkage needs further examination. Perhaps diastereomeric mixtures of boranophosphates leads to these Tm reductions and must be replaced with a stereocontrolled chemical synthesis strategy. Perhaps, however, boranophosphate may introduce far less instability when used in combination with normal phosphate linkages. Certainly this is the case with dithioate DNA.[12] Further research addressing these issues as well as developing appropriately protected derivatives of the remaining bases is currently underway.

Acknowledgements: This research was supported by the NIH (Grant GM25680). This is number 47 in a series on nucleotide chemistry. Paper 46 is Seeberger et al., *J. Am. Chem. Soc.* **118**, 9562 (1996).

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