DISCLAIMER

K. DESCRIPTION/ABSTRACT

Final Technical Progress Report, DE-FG02-97ER62376, Period Covered: 2/15/97 – 11/30/01
"One-Step PCR Sequencing".
Barbara Ramsay Shaw, Department of Chemistry, Duke University, Durham, NC 27708-0346

We investigated new chemistries and alternate approaches for direct gene sequencing and detection based on the properties of boron-substituted nucleotides as chain delimiters in lieu of conventional chain terminators. Chain terminators, such as the widely used Sanger dideoxynucleotide truncators, stop DNA synthesis during replication and hence are incompatible with further PCR amplification. Chain delimiters, on the other hand, are chemically-modified, "stealth" nucleotides that act like normal nucleotides in DNA synthesis and PCR amplification, but can be unmasked following chain extension and exponential amplification. Specifically, chain delimiters give rise to an alternative sequencing strategy based on selective degradation of DNA chains generated by PCR amplification with modified nucleotides. The method as originally devised employed template-directed enzymatic, random incorporation of small amounts of boron-modified nucleotides (*e.g.*, 2'-deoxynucleoside 5'-alpha-[P-borano]-triphosphates) during PCR amplification. Rather than incorporation of dideoxy chain terminators, which are less efficiently incorporated in PCR-based amplification than natural deoxynucleotides, our method is based on selective incorporation and exonuclease degradation of DNA chains generated by efficient PCR amplification of chemically-modified "stealth" nucleotides. The stealth nucleotides have a boranophosphate group instead of a normal phosphate, yet behave like normal nucleotides during PCR-amplification. The unique feature of our method is that the position of the stealth nucleotide, and hence DNA sequencing fragments, are revealed at the desired, appropriate moment following PCR amplification. During the current grant period, a variety of new boron-modified nucleotides were synthesized, and new chemistries and enzymatic methods and combinations thereof were explored to improve the method and study the effects of borane modified nucleotides on polymerase and unmasking mechanisms.

This approach takes advantage of differences in reactivity of the normal and modified nucleotidic linkages to generate PCR sequencing fragments that terminate at the site of incorporation of the modified nucleotide. In principle, the position of the modified nucleotide in each PCR product can be revealed in two ways, either by enzymatic unmasking (as previously described) or by chemical unmasking. We identified reagent sets for enzymatic or chemical conversion of boronated PCR products into mono- and bidirectional sequencing fragments. (a) We developed a new modified cytidine boranophosphate analogue that is (i) compatible with PCR, but more resistant to exonuclease III read-through than unmodified cytidine and (ii) permits better base calling; (b) We developed chemical methods for DNA and RNA cleavage at boronated nucleotide sites; and (c) We developed methods to quantify and detect stealth boranophosphate groups in DNA and RNA. Key advantages of boranophosphates as sequence delimiters in PCR are that they (1) delineate the DNA sequence yet (2) do not obstruct exponential amplification, and they (3) permit direct PCR sequencing, cycle sequencing, or RNA sequencing. They are also compatible with most sequencing platforms.

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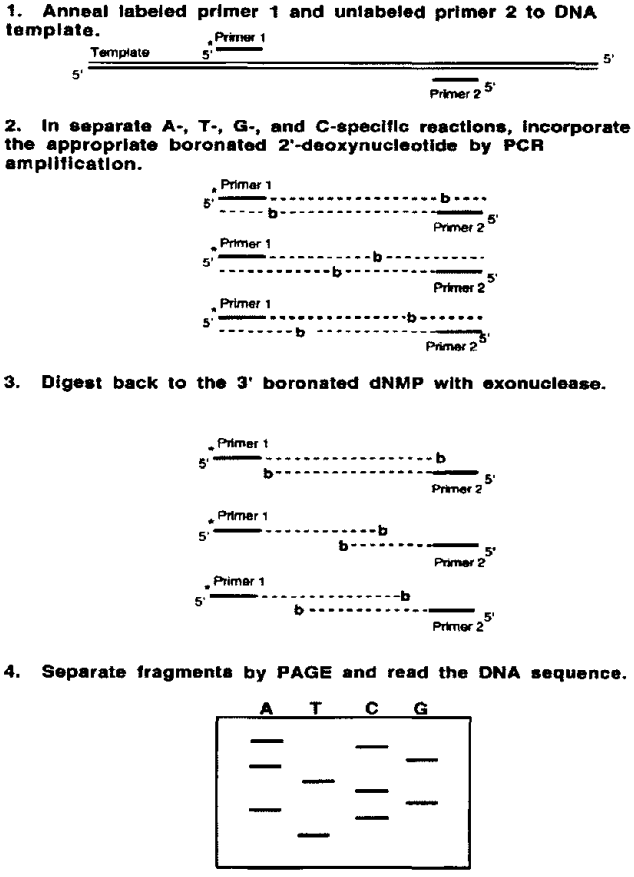
Abstract:

We investigated new chemistries and alternate approaches for direct gene sequencing and detection based on the properties of boron-substituted nucleotides as chain delimiters in lieu of conventional chain terminators. Chain terminators, such as the widely used Sanger dideoxynucleotide truncators, stop DNA synthesis during replication and hence are incompatible with further PCR amplification. Chain delimiters, on the other hand, are chemically-modified, "stealth" nucleotides that act like normal nucleotides in DNA synthesis and PCR amplification, but can be unmasked following chain extension and exponential amplification. Specifically, chain delimiters give rise to an alternative sequencing strategy based on selective degradation of DNA chains generated by PCR amplification with modified nucleotides. The method as originally devised employed template-directed enzymatic, random incorporation of small amounts of boron-modified nucleotides (*e.g.*, 2'-deoxynucleoside 5'-alpha-[P-borano]-triphosphates) during PCR amplification. Rather than incorporation of dideoxy chain terminators, which are less efficiently incorporated in PCR-based amplification than natural deoxynucleotides, our method is based on selective incorporation and exonuclease degradation of DNA chains generated by efficient PCR amplification of chemically-modified "stealth" nucleotides. The stealth nucleotides have a boranophosphate group instead of a normal phosphate, yet behave like normal nucleotides during PCR-amplification. The unique feature of our method is that the position of the stealth nucleotide, and hence DNA sequencing fragments, are revealed at the desired, appropriate moment following PCR amplification. During the current grant period, a variety of new boron-modified nucleotides were synthesized, and new chemistries and enzymatic methods and combinations thereof were explored to improve the method and study the effects of borane modified nucleotides on polymerase and unmasking mechanisms.

This approach takes advantage of differences in reactivity of the normal and modified nucleotidic linkages to generate PCR sequencing fragments that terminate at the site of incorporation of the modified nucleotide. In principle, the position of the modified nucleotide in each PCR product can be revealed in two ways, either by enzymatic unmasking (as previously described) or by chemical unmasking. We identified reagent sets for enzymatic or chemical conversion of boronated PCR products into mono- and bidirectional sequencing fragments. (a) We developed a new modified cytidine boranophosphate analogue that is (i) compatible with PCR, but more resistant to exonuclease III read-through than unmodified cytidine and (ii) permits better base calling; (b) We developed chemical methods for DNA and RNA cleavage at boronated nucleotide sites; and (c) We developed methods to quantify and detect stealth boranophosphate groups in DNA and RNA. Key advantages of boranophosphates as sequence delimiters in PCR are that they (1) delineate the DNA sequence yet (2) do not obstruct exponential amplification, and they (3) permit direct PCR sequencing, cycle sequencing, or RNA sequencing. They are also compatible with most sequencing platforms.

Background:

Standard sequencing methods rely on base-modified chain terminating dideoxynucleoside triphosphates (ddNTPs). A relatively unexplored approach for improving genome sequencing has been the use of phosphate modified nucleotide analogs. We set out to **substitute chain delimiters for terminators** directly in the PCR amplification process. This alternate sequencing chemistry relies on the selective delineation of a boranophosphate in the DNA. It helps to avoids problems associated with sequencing of thermally stable and repetitive DNA sequences, allows direct bidirectional genomic sequencing, and permits direct loading of PCR products onto the separating system. Specifically, as seen in Figure 1, the method employs template-directed enzymatic (random but base-specific) incorporation of small amounts of boron-modified nucleotides (i.e. 2'-deoxynucleoside 5'-alpha-[P-borano]-triphosphates) (**dNTPαBs**) (see Fig. 2C during PCR amplification.



Direct PCR sequencing with boronated nucleotides involves three major processes. All but the last may be carried out in one vessel.

a) **Primer annealing** with **PCR amplification.** Boronated nucleotides are randomly, but base-specifically incorporated into the product DNA by the polymerase chain reaction and labeled primers.

b) **Chew-back with exonuclease.** Generate sequencing fragments that terminate at a boranophosphate. Base-specific 3'-termini are obtained by exonuclease digestion, because the 3'→5' exonuclease III -mediated degradation of DNA is efficiently blocked at the boranophosphate positions.

c) **Readout.** Fragments terminating in a boranophosphate are separated using standard sequencing techniques. The method permits direct loading of PCR products onto the separating system.

Figure 1. Direct PCR Sequencing [publication 64]

Direct PCR sequencing with boronated nucleotides involves three processes.

(a) Boronated nucleotides are randomly, but base-specifically, incorporated into the product DNA by the polymerase chain reaction.

(b) PCR fragments are cleaved at the site of base-specific modification to generate sequencing fragments. In our original strategy, cleavage was accomplished enzymatically using exonucleases. Boranophosphate 3'-termini were obtained by an exonuclease "chew-back" digestion from the 3'termini. The chew-back terminates at boron-specific positions because the 3'-to-5' exonuclease III-mediated degradation of DNA is efficiently blocked at the boranophosphate positions, but not at normal phosphates. In our newer approach, sequencing fragments are generated by chemical cleavage.

(c) Sequencing fragment separation and analysis.

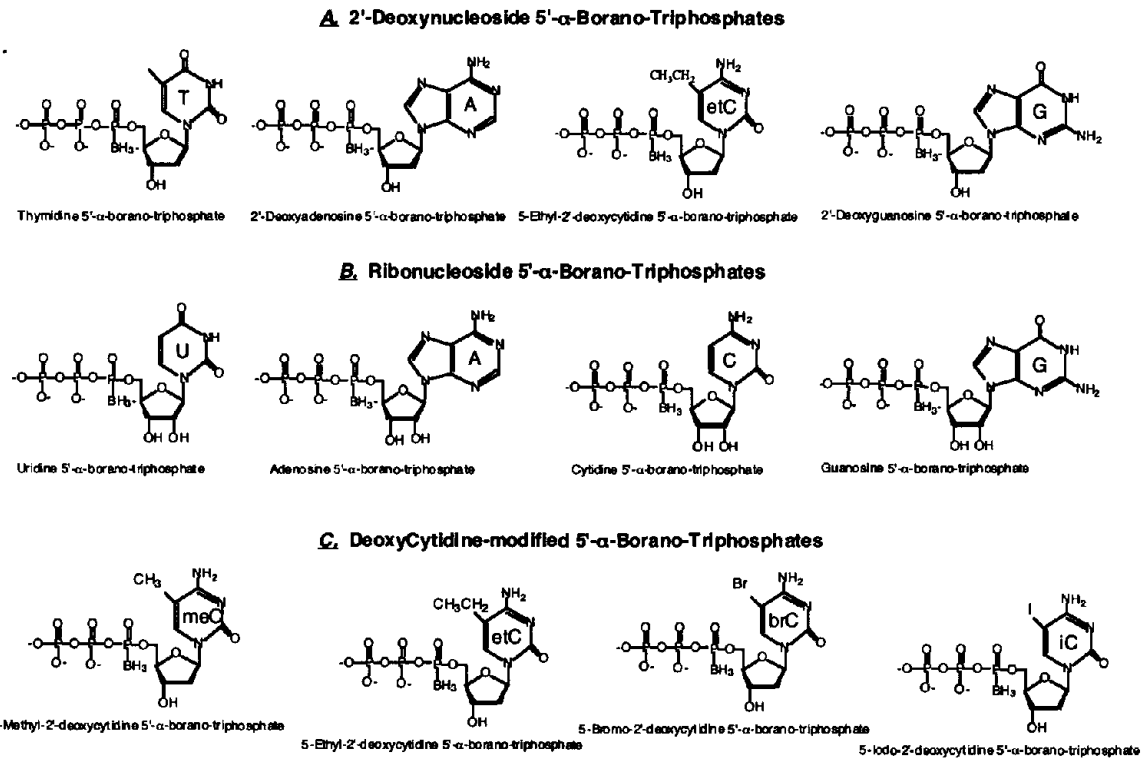


Figure 2. New compounds synthesized (partial listing). *[publications #70, 71, 76]*

Current Project Results:

Goals. In the current project, we introduced modifications to the nucleotides, the sample preparation steps, the cleavage step, and the base calling software in order to make boranophosphate sequencing more generally useful. Most of these approaches were carried out concurrently. We extended and optimized the Direct PCR sequencing method with boranophosphates, and we developed new strategies for sequencing single and double-stranded DNA and RNA.

PART I. Variations and optimization of the direct PCR sequencing method.
The standard protocol in Figure 1, which employs exonuclease III chew-back to reveal the boron stealth nucleotides, was improved considerably by streamlining sample preparation, PCR, and digestion conditions, by developing modified boranophosphate nucleotides, and by examining different polymerases and exonucleases.

A. **Sample preparation and reactions were streamlined** by digesting the PCR products directly after amplification. The digestion buffer was augmented with the disaccharide, trehalose, which has several advantages. Specifically, trehalose stabilizes the exonuclease III (exo III), allows us to reduce the amount of exo III, and eliminates phosphodiesterase I from the protocol.

B. **Creating new base-modified and ribose-modified α-boranophosphates for optimization of the Direct Sequencing method.** The utility of a unique modification like the α-boranophosphate derives from its potential use as a chain delimiter, rather than chain terminator. We examined here the chemical and substrate properties of new analogues and their compatibility with the Direct PCR sequencing methods.

Selection of the optimal chemical modification required: (1) new methods of synthesis to prepare novel boranophosphate compounds and separate their *Sp* and *Rp* stereoisomers; (2) careful analysis and optimization of their efficiency and stereoselectivity of incorporation by polymerases, and (3) analysis of their resistance to nucleases and their stability (or lability) to chemical cleavage.

New chemistries were explored and novel sets of borane-modified deoxyribo- and ribonucleotides were introduced. These included new base-modified, ribose-modified, and 5'-fluorescently labeled nucleosideα-boranophosphates. Some of the most useful compounds and chemistries resulting from this DOE-sponsored study (see publications 70, 71, 76) include those in Figure 2A, 2C.

C. Sequencing optimization with new 2'-deoxycytidine (dC) analogues. Although stealth sequencing with boranophosphates as previously presented was shown to be feasible, there was a systematic sequencing error at some neighboring C positions. This problem was traced to an intrinsic property of exonuclease III, *e.g.*, more rapid chew-back of C relative to A, T and G nucleotides.

In order to overcome the susceptibility of C residues to digestion by exo III, we reasoned that judicious ring substitution of cytosine could eliminate the read-through problem. Therefore, we synthesized a new series of 2'-deoxycytidine 5'-triphosphate analogues, each containing a α-*P*-boranophosphate group and an alkyl or halogeno substitution at the 5-position of cytosine. (Fig 2C) *[publication #76]* The first eluted diastereromers (putatively Rp) of each dCTP analogue were isolated. These analogues were examined for their (1) utility as substrates for DNA polymerases, and (2) their resistance to exonucleases after incorporation into DNA.

1) **Utility of dC-analogues as substrates for DNA polymerase: Primer extension with 5-substituted dC analogues**: The (*Rp*) diastereomer of each dCTP analog in Fig. 2C was a substrate for T7 DNA polymerase (Sequenase) as seen in Fig. 3 and had an incorporation efficiency similar to that of normal dCTP and dCTPαB, with the 5-iodo-dCTPαB analog having the poorest incorporation efficiency. The second eluted (*Sp*) diastereomers were not

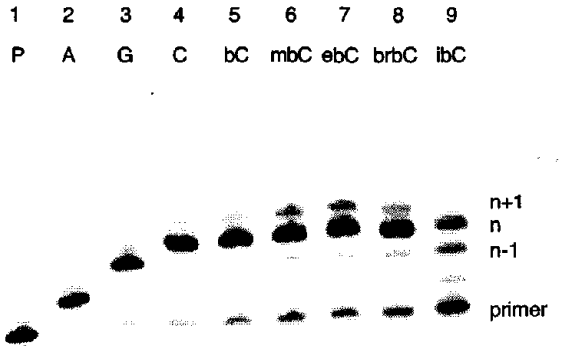
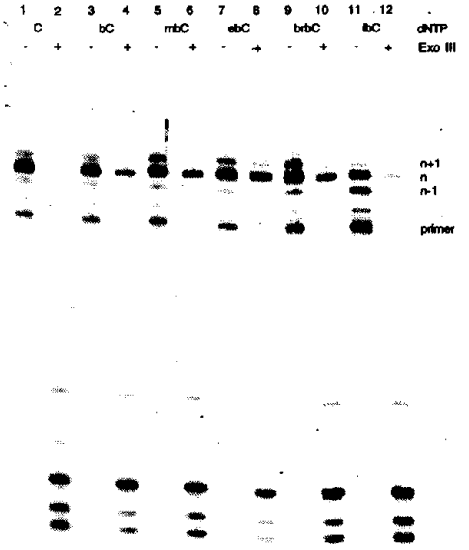


Figure 3 legend. Primer extension with dCTPαBs. Primers were extended by dATP (lane 2), dATP + dGTP (lane 3), dATP + dGTP + dCTP (lane 4), dATP + dGTP + dCTPαB (lane 5), dATP + dGTP + 5-Me-dCTPαB (lane 6), dATP + dGTP + 5-Et-dCTPαB (lane 7), dATP + dGTP + 5-Br-dCTPαB (lane 8), dATP + dGTP + 5-I-dCTPαB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

Figure 3. Fluorescently labeled primer {[5'-HEX-d(CAGGAACAGCTA-TGGCCTC)-3']; 10 pmol} was annealed to unlabeled template {30 pmol [5'-d(GTGTAGCTGAGGCCATAGCTGTTCCTG)-3']}. Extension was accomplished with modified T7 DNA polymerase (Sequenase; 0.5 U) in the presence of various dNTPs and a modified dCTPαB (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 exo-nuclease 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)]. *[publication #76]*

substrates. With the exception of the 5-iodo derivative, we concluded that the *Rp* stereoisomer of 5-bromo, 5-methyl, and 5-ethyl derivatives of 2'-deoxycytidine 5'-(α-P-borano)-triphosphates are good substrates for T7 DNA polymerase, as seen in Figure 3.

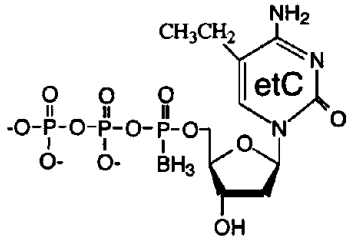
2) **Improving the resistance to digestion by exonuclease.** In the enzymatic approach for revealing a stealth nucleotide in DNA, substituting a phosphate by a boranophosphate inter-nucleotidic linkage prolongs its lifetime toward degradation by nucleases, thus making it more resistant to cleavage than a normal phosphate linkage in DNA. The sequential hydrolytic reactions by 3'-5' exonuclease III are thereby blocked by a boranophosphate, resulting in fragments that all terminate in a nucleoside boranophosphate. However, in earlier studies we found that normal and borano-phosphate linkages with a 3'-*cytosine* were more susceptible to exonuclease degradation than other purines and pyrimidines, which reduces band uniformity. The series of base-modified cytosine derivatives synthesized in Fig. 2C were tested for nuclease resistance (see Fig. 4). Substitution at the C-5 position of cytosine by alkyl groups (ethyl and methyl) markedly enhanced the cytidine boranophosphate resistance towards exonuclease III (i.e., 5-ethyl-dC > 5-methyl-dC > dC ≈ 5-bromo-dC > 5-iodo-dC). The best analog, 5-ethyl-α-borano-dCTP, not only showed an increased resistance to exonuclease III compared to the α-borano-dCTP used previously in our method, but did so without affecting incorporation, and resulted in more even banding patterns. Analysis of ABI 394 data with Basefinder software (M. Giddings) takes into account any mobility changes due to base modification, permitting increased consistency and accuracy of base calling. 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. **Thus, resistance followed the order: 5-Et-dCTPαB > 5-Me-dCTPαB > 5-Br-dCTPαB > dCTPαB > 5-I-dCαB >> dCTP.** *[publication #76]*



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	

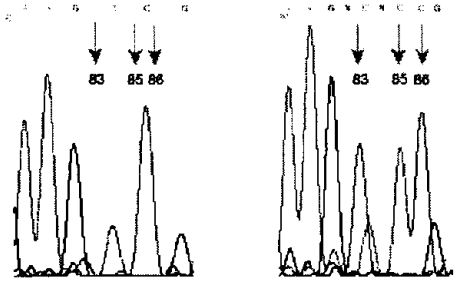
FIGURE 4: Exonuclease Digestion of dC analogues: Substitution at the C-5 position of cytosine by alkyl groups (ethyl and methyl) markedly enhanced the resistance of boranomonophosphate dCTPαB towards exonuclease III. *[publication #76]*

3) **5-Ethyl-Boronated dCTP Improves Boronated PCR Sequencing:** As the 5-ethyl analogue of boronated dCTP (Fig. 5a) was one of the best substrates for DNA polymerase (Figure 3), and also the most resistant to exonuclease (Figure 4), it was used to replace dCTP in a series of PCR sequencing experiments to determine whether it permitted recovery of the missing peaks at C positions (that had been observed in sequencing with unsubstituted boronated dCTP, for example, in Figure 5a (5'-A80AGCTCCG87-3' where the peak for C 83 is completely missing and the peaks for C 85 and C 86 are coalesced into a single peak (see arrows)). As hypothesized, sequencing with 5-ethyl boronated dCTP resulted in consistent peaks for all of the bases, including the previously troublesome Cs. For example, in Figure 5b, C positions 83, 85, and 86 are each defined by distinct, uniform peaks. The introduction of with 5-ethyl boronated dCTP into boronated PCR sequencing thus generates good sequencing peaks for C residues. The slightly altered mobility due to the ethyl substitution can be corrected for and called correctly by the base-calling software, BaseFinder.



5-Ethyl-2'-deoxycytidine 5'-α-borano-triphosphate

Figure 5a



A. Unsubstituted b-dC B. 5-Ethyl b-dC

Figure 5b

Summary: Introducing 5-ethyl boronated dCTP into the sequencing protocol effectively eliminated the error of most C positions. The peaks at C positions were now the equivalent of those produced for the other three bases. The slight mobility shift produced by the 5-ethyl boronated dC can be corrected for by BaseFinder and the sequence can be called accurately.

Part II. We explored new strategies for boranophosphate sequencing that capitalize on new boranophosphate chemistries, with a focus on devising a chemical method for generating sequencing fragments as an alternative to exonuclease chew-back.

The studies described above use enzymatic cleavage to unmask (by chew-back) the position of a stealth boranophosphate in DNA. We also explored chemical cleavage methods to unmask the boranophosphate position. This requires a modification of a phosphate linkage that would make the stealth nucleotide more sensitive to cleavage by a specific chemical. With chemical cleavage, the access of the small molecule to overall tertiary structures of the DNA (or RNA) structure should be less sensitive and sequence specific than access of larger molecules like enzymes. A chemical cleavage method might also be more amenable to one-pot/four-base incorporation of

fluorescently labeled boranophosphate nucleotides that could be unmasked after PCR, resulting in a more direct and efficient method of sequencing.

A. Chemical Conversion of Boronated PCR Products into Bidirectional Sequencing Fragments. Barbara Ramsay Shaw, Kenneth W. Porter, Ahmad Hasan, Kaizhang He and Jack Summers. *[abstract A185, A197, manuscript in preparation]*

In the chemical cleavage approach of stealth nucleotides in DNA, we examined several methods for generating sequencing fragments, as an alternative to exonuclease chew-back. The use of a small molecule reagent that could cleave modified DNA site-specifically would have advantages over enzyme reagents in (a) cost; (b) convenience of use (such as greater stability of the chemical cleaver); (c) reproducibility of cleavage (enzymes are more sensitive to other chemicals, such as metal ions and denaturants, present in the reaction mixtures); (d) steric accessibility of the cleavage reagent to the target DNA (which is known to form triplex, quadruplex and other higher order structures that could block enzyme access to the DNA; and (e) retention of activity at higher temperatures where secondary structure should be disrupted.

Deoxy cleavage. We identified chemical reagents that selectively cleave the backbone of the PCR product at *deoxy* boranophosphate linkages, while leaving the normal phosphodiester linkages intact. This was done by studying the chemistry of boranophosphates and identifying possible reagents that selectively react with boranophosphate relative to the phosphate. With DNA, formaldehyde at specific pH cleaved at stealth boranophosphate linkages but not at normal DNA linkages. The overall patterns over several hundred bases were encouragingly similar to those of base-specific Maxim-Gilbert type cleavage ladders. However, closer examination of short oligonucleotides showed that two new bands appeared in comparable intensities indicating that hydrolysis occurred with little selectivity for cleavage at the 5' versus 3' side of a boranophosphate linkage. (A concurrent attempt to develop new boranophosphate linkages amenable to only 3' or 5'selective cleavage was begun (see Section C), but applications to sequencing fell beyond the scope of this grant.) While it is conceivable that treatment of DNA with formaldehyde followed by ammonia borane might cause base modification, we saw no evidence of such a reaction occurring. Treated (and cleaved) DNA could re-hybridize and be extended enzymatically (unpublished). Although the formaldehyde method looked encouraging, with termination of funding we were unable to examine further the effect of double banding on sequencing long DNA fragments, where there seemed to be most interest in applications.

B. Utility of Boranophosphate Ribonucleotides as Sequencing Delimiters. In another modification of the chemical approach, sequencing of DNA might be done through incorporation of a boranophosphate modified ribonucleotide that is highly susceptible to chemical cleavage. RNA is well-known to be cleaved by high concentrations of bases like sodium hydroxide, but subsequent neutralization results in high concentrations of salt that must be removed before any kind of fragment size analyses. We hypothesized that, if boranophosphate ribonucleotides were good substrates for RNA polymerase (and possibly DNA polymerase) and readily cleavable at neutral pH, they might be useful stealth nucleotides for sequencing. Therefore, to examine the substrate properties with polymerases and nucleases, boranophosphate nucleoside triphosphates and small RNA fragments were synthesized chemically. We then explored the chemical structure and reactivity of ribonucleoside boranophosphates and their interaction with polymerases,

nucleases, and chemical cleavage reagents.

1). Boronated nucleotides as substrates for T7 RNA polymerase. K. He synthesized for the first time the ribonucleoside 5'-(α-P-borano)triphosphates (NTPαB, where N = A, U C G) (Figure 2B). He then showed that these were efficiently incorporated into short and long (2,000 nt) RNA transcripts by T7, Sp6, and T3 RNA polymerases. Only the *Rp* diastereomers of NTPαB serve as substrates for such transcription.[publications 69, 71, 81]

2). Boranophosphate modification increases RNA reactivity to base and formaldehyde. We developed new methods to synthesize a boranophosphate RNA dimer analogue[publications 73, 77, 80, 92] and used it to find conditions under which the *ribo* boranophosphate linkage is considerably more susceptible to cleavage than a normal ribo phosphodiester linkage. [manuscript in preparation]

- Boranophosphate RNA, but not DNA, can be cleaved in mild base. Under alkaline conditions (pH ≥ 10), the ribonucleotide boranophosphate linkage in Up(BH_3)A was completely hydrolyzed.
- Formaldehyde cleaves boranophosphate RNA. The boranophosphate RNA was cleaved completely by formaldehyde, while the normal RNA was intact under the same conditions.

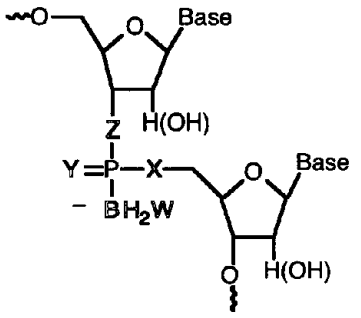
3). Boranophosphates increase the nuclease resistance of RNA. [K. He, Ph.D. dissertation, Duke University 2000; manuscript in preparation] The Sp isomer of the boronated RNA dimer, Up(BH_3)A, was found to be 2000-fold more resistant to hydrolysis by snake venom phosphodiesterase (SVPDE, 3'-exonuclease) than the natural RNA dimer UpA. The Rp isomer of boronated dimer Up(BH_3)A is not a substrate at all. Both diastereomers of Up(BH_3)A are more resistant to SVPDE than the corresponding diastereomers of the phosphorothioate dimer Up(S)A. Further, *Rp*- and *S*p-isomers of Up(BH_3)A or Up(S)A are very poor substrates for bovine spleen phosphodiesterase (BSPDE, 5'-exo-nuclease). Thus relative to natural RNA, the boranophosphate RNA linkage exhibits a greater selectivity of resistance to these nucleases than do the DNA counterparts. It is thus possible to propose that (i) DNA might be better sequenced with the ribo-boranophosphate-NTPs in an enzymatic chew-back method (variation of Figure 1) provided a mutant DNA polymerase able to incorporate boranophosphate ribonucleotides can be found. (ii) It might be possible to develop an exonucleolytic chew-back or chemical sequencing method for RNA if an RNA polymerase could be found that would incorporate a fluorescent-base-labeled ribonucleoside 5'-(α-*P*-borano)triphosphates (NTPαB, where N = A, U C G). This would require establishing the feasibility of synthesizing fluorescent NTPαBs (section D).

4). DNA sequencing by chemical cleavage. Relative to normal phosphodiester linkages in RNA, enzymatic cleavage is inhibited at a ribo-boranophosphate linkage, but greatly accelerated by base and formaldehyde. A kinetic study of the normal UpU dimer and boronated Up(BH_3)U dimer revealed that base (pH 11) hydrolysis results in almost no hydrolysis of UpU (a few % each of products 3'-UMP, 2'-UMP, 2',3'-UMP , and lesser uridine), but results in complete hydrolysis for Up(BH_3)U (giving 3'-UMPB, 2'-UMPB, 2',3'-UMPB, and lesser uridine). The high pH method is thus not sufficiently selective. Hydrolysis with formaldehyde, however, is even faster and more selective, resulting in no cleavage of the normal UpU dimer, but in complete hydrolysis of the boronated Up(BH_3)U, and one main product. Products

appear to be the same with the Rp and Sp isomers. Direct sequencing of PCR products by cleavage at stealth ribo-boranophosphates might simplify mono- and bidirectional sequencing and provide a simple, direct, and complementary method to cycle sequencing.

C. We synthesized heretofore unknown members of the boranophosphate family, as a means to better understand and tune the chemical stability and reactivity of boranophosphate nucleotides and oligonucleotides.

The class of inorganic boranophosphates, originally discovered by our lab, was expanded into a family of boranophosphates by a graduate student, Jin-lai Lin. [publications 89,95, 98, 99]. Hundreds of thousands of reactions have been described for other first row elements like carbon, hydrogen, oxygen, and nitrogen, but only a handful of reactions are known for preparation and reaction of boranophosphate-type compounds. Nearly all of these have been described in our laboratory since the first report in 1990. Exploration of newer chemistries for developing better synthetic methods and understanding reactivity of boranophosphates was needed in order to move forward in this project. New boranophosphate family members in Figure 6 provided new synthetic pathways into boranophosphates, and enable tuning of chemical reactivity and nuclease resistance.



BH_2W	X	Y	Z
BH_3	O	S	O
BH_3	S	O	O
BH_3	O	O	S
BH_3	NH	O	O
BH_3	O	O	NH
$^+BH_3$	O	$-CH_3$ (Y=)	O
BH_2CN	O	O	O

Figure 6. New boranophosphate family members enable tuning of chemical reactivity and nuclease resistance.

D. Based on exploration of new boranophosphate chemistries, several methods were explored to detect the final sequencing products. We devised a colorimetric chemical method to quantify and carry out in situ quantitative labeling of boranophosphates, and developed synthetic schemes to synthesize fluorescently labeled boronated dNTPαBs.

1). Colorimetric method for in situ microdetermination of boranophosphates [publication 81]. We identified agents that can result in colorimetric detection of boranophosphate. While the boranophosphates have unusual hydrolytic stability ($t_{1/2}$ = 40 years at pH 7) they retain some characteristics of hydroborating agents. They react with aldehydes at pH below 4 and with iminium salts, resembling cyanoborohydrides [Borch et al., J. Am. Chem. Soc. 93, 2897 (1971)] in their functional group selectivity, but differing from cyanoborohydrides by a markedly decreased reactivity. For instance, boranophosphates themselves appear not to react with

transition metal ions in high oxidation states; however, an ion reduction can be promoted by carbonyl containing compounds in diluted acids. The particular color reactions of boranophosphate with Mo^{+6} of molybdic (735 nm) or phosphomolybdic (845 nm) acid in the presence of acetone and sulfuric acid were found practically useful for qualitative detection and quantitative determination of boranophosphates by infrared spectroscopy. [publication 81] The molar absorptivity, 20.1×10^3 $M^{-1}cm^{-1}$, specific for the molybdate method (centered in a distinctly visible window around 735 nm), is 2-fold higher than that averaged characteristic for nucleotides at 260 nm. The molybdic method avoids the expense, disadvantages, and inconvenience of employing fluorescent markers or radioactivity. Thus, this method can be recommended for precise determination of oligonucleoside boranophosphate molar absorptivities. The phosphomolybdate method, although 4-fold less sensitive, has its own advantages being insensitive to contamination like methanol and acetonitrile (up to 10%, v/v), and to some buffer components including phosphate, etc. This makes the method especially valuable in detection and quantification of boranophosphates in complex mixtures like chromatographic fractions or biochemical mixtures. The value for chip detection sequencing was not examined.

2). In an attempt to make the direct sequencing method compatible with chip-based one-pot amplification and capillary electrophoresis systems, new synthetic schemes for the synthesis of several fluorescent dNTPαBs (N = pyrimidine) were developed. We demonstrated the compatibility of boranophosphates with chemistries used to synthesize fluorescently labeled boronated dNTPs, thus establishing the proof of principle.[abstract A163] This pointed to a feasibile entry into direct sequencing of one PCR reaction containing mixtures of four-color fluorescent boranophosphate. We anticipated that chemical cleavage following incorporation of fluorescently labeled borano-dNTPs could result in a more efficient method of mono- and bidirectional sequencing. However, the compounds we made were poor substrates for a number of polymerases. Matching the appropriate fluorescent label and linker lengths with the appropriate polymerase while maximizing the efficiency of boranophosphate incorporation is a daunting task that remains to be explored.

Summary and Prospective:

- We described an alternative sequencing strategy to dideoxy chain termination — one based on selective degradation of DNA chains generated from PCR amplification. This simplifies mono- and bidirectional sequencing and provides a one-step direct sequencing of PCR products from both strands simultaneously. By employing labeled PCR primers, the original PCR products can be converted directly into bidirectional sequencing fragments.
- We can use enzymatic cleavage or chemical cleavage to reveal the position of a stealth boranophosphate nucleotide in DNA.
- Earlier studies with the enzymatic (chew-back) method were extended by developing a new modified cytidine boranophosphate analogue that is (i) compatible with PCR, but more resistant to exonuclease III read-through than unmodified cytidine and (ii) permits more uniform intensities and better base calling. The enzymatic approach may find use in applications where high resolution of longer fragments requires stronger signals at longer read lengths, because the distribution of fragments produced by nuclease digestion is skewed to long fragments. It is also compatible with bi-directional sequencing.

- We discovered reagent sets for chemical conversion of boronated PCR products into sequencing fragments. This involved developing chemical methods for cleavage at boronated nucleotide sites, and introducing a new set of boronated nucleotides. We identified a class of reagents that selectively cleave the backbone of the PCR product or an RNA molecule at stealth boranophosphate linkages *under relatively mild conditions*, while leaving the normal phosphodiester linkages intact. Practically, chemical cleavage is less likely than enzymatic cleavage to be influenced by the sequence or secondary structure of the substrate DNA, and it has a number of other advantages, for example, generating a product that may be detectable with IR. The disadvantage, that most chemical methods result in two cleavage products differing slightly in mobility, may be able to overcome with further study.
- Chemical cleavage of a boronated-RNA linkage can be accomplished by treatment with either mild base or formaldehyde. Further, cleavage results in retention of the phosphate on the 3'-hydroxyl of the cleavage product. Boronated ribo-NTPs thus provide a better chemistry for differential cleavage of boronated linkages over normal RNA, or DNA The ability to specifically cleave DNA or RNA at a boronated DNA or RNA linkage provides new methods for generating PCR or other sequencing fragments. Boranophosphates may find use in sequencing by chip technology and mass spectroscopy. However, further development of the method requires new polymerases that can incorporate the boronated ribo-NTPs into DNA as efficiently as boronated deoxy-NTPs.
- Boronated ribonucleotides (NTPαB) can be efficiently incorporated into RNA transcripts by T7 and other RNA polymerases. Only the first HPLC-eluted diastereomers of each NTPαB (putatively Rp) are substrates; the second HPLC-eluted isomers (putatively Sp) are not. The main drawbacks of a direct RNA sequencing method come from 3'end heterogeneity due to the over-extension run-off products, incomplete extensions, the poly G ladders, general lability of RNA, and no obvious way to generally label the 5'end. Boranophosphate delimiters can reduce poly G ladders.
- RNA can be copied with reverse transcriptase and complementary boron-modified NTPαB to give boronated sequencing fragments [publication 106].
- A colorimetric method for in situ micro determination of boranophosphates was developed.
- Fluorescently labeled boronated dNTPs were synthesized, and the compatibility of borane chemistry with fluorescently labeling of the nucleoside was established.

Graduate students trained, degrees granted and post-doctoral tenures completed.

Dr. Kenneth Porter, Sr. Research Associate and Co-investigator	100% 3/97 to 6/98 75% 7/98 to 2/99	25% 3/99 to 6/99
Dr. Ahmad Hasan, Sr. Research Associate,	100% 4/97 to 6/97	
Mr. David Briley, Sr. Res. Tech	100% 6/97-7/97	
Dr. Jack Summers, Sr. Research Associate	100% 11/97 to 12/98	10% 5/99 to 8/99
Dr. Vladimir Rait, Sr. Research Associate	100% 2/98 to 6/98	10% 11/98 to 12/99
Dr. Dmitri Sergueev, Sr. Research Assoc.	100% 3/97 to 9/97	100% 2/98 to 10/98
Dr. Bozenna Krzyzanowska, Research Associate	100% 5/97 to 12/97	
Dr. Karl Base, Research Associate	70% 12/99 to 9/00	50% 9/00 to 11/00
Dr. Mikhail Dobrikov, Sr. Research Associate	100% 11/99 to 10/01	
Mr. Kaizhang He, graduate student awarded Ph.D. Duke Univ 2001	100% 6/97 to10/98	100% 1/00 to 8/00
Mr. JinLai Lin, graduate student awarded Ph.D. Duke Univ 2001	100% 6/97 to 8/99	100% 9/00 to 11/00
Mr. Ping Li, graduate student, anticipate Ph.D. Dec 2004	100% 5/00 to 11/00	
Mr. Fred Wilson, undergraduate student awarded B.S. Duke Univ. 2000	100% 5/97 to 8/97	

PUBLICATIONS

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Support not listed. Discusses sequencing. Should acknowledge partial support from DOE ER62376

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ABSTRACTS PRESENTED AT CONFERENCES:

The following abstracts have resulted from this current DOE grant (**ER62376**) and its predecessor (**ER61882**) of the same title.

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