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## Nucleosides, Nucleotides and Nucleic Acids

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### SYNTHESIS OF OLIGONUCLEOSIDE BORANOPHOSPHATES VIA AN H-PHOSPHONATE METHOD WITHOUT NUCLEOBASE PROTECTION

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## SYNTHESIS OF OLIGONUCLEOSIDE BORANOPHOSPHATES VIA AN H-PHOSPHONATE METHOD WITHOUT NUCLEOBASE PROTECTION

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### ABSTRACT

Short oligonucleoside boranophosphates containing *all four* nucleosides were synthesized on solid support using base-unprotected nucleoside H-phosphonate monomers. This strategy avoided irreversible base modifications during the boronation procedure. Structures of the boranophosphate oligomers were confirmed by  $^1\text{H}$ ,  $^{31}\text{P}$ ,  $^{10}\text{B}$  NMR and MS analysis as well as by enzymatic hydrolysis.

### INTRODUCTION

A great variety of oligonucleotide analogs have been synthesized in past decades with the hope of developing efficient antisense and antigene therapeutics, creating superior diagnostic probes, and improving existing oligonucleotide tools in biochemistry and molecular biology. Among these analogs are the oligonucleoside boranophosphates ( $\text{BH}_3^-$ -ODNs), an interesting class of backbone-modified oligonucleotides that are stable towards nucleases (1-3) and activate RNase H (4,5).  $\text{BH}_3^-$ -ODNs also display enhanced hydrophobicity (1,2) and altered metal-binding affinity (6) relative to normal oligonucleotides.

Significant progress was made toward the synthesis of the  $\text{BH}_3^-$ -ODNs in recent years, with several groups independently reporting synthesis of oligothymidine boranophosphate (up to 15 nt long) via an H-phosphonate method (2,5,7). Reports of mixed-base  $\text{BH}_3^-$ -ODNs synthesis, however, have so far been limited

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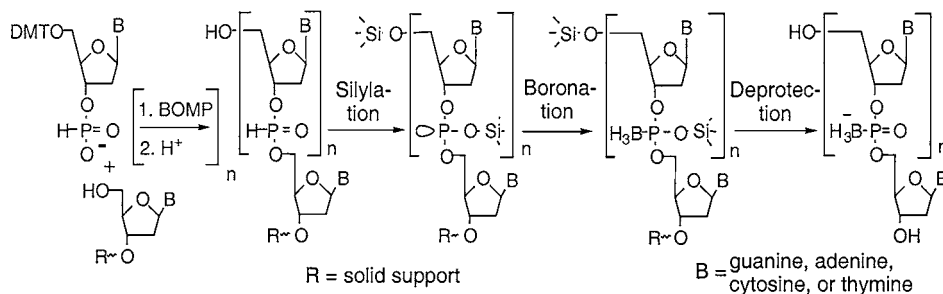
to enzymatic template-directed synthesis (8) and synthesis of boranophosphate DNA (9) and RNA (10) dimers. We report here a procedure for the synthesis of mixed-base  $\text{BH}_3^-$ -ODNs and their characterization.

## RESULTS AND DISCUSSION

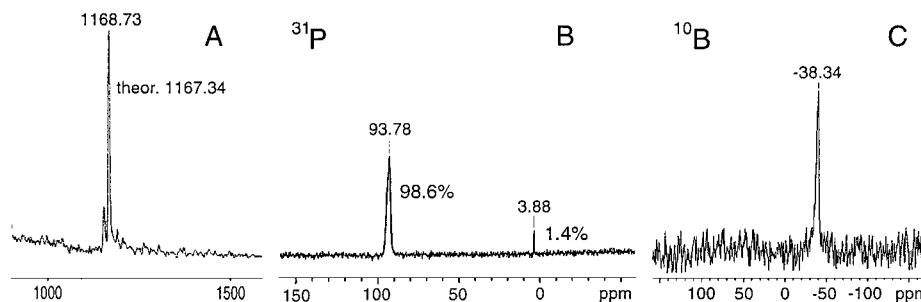
The standard H-phosphonate method has been successfully used for oligothymidine boranophosphate synthesis. Attempts to use it for mixed-base  $\text{BH}_3^-$ -ODNs synthesis gave rather complicated mixtures. Significant base modifications were observed during conversion of internucleoside H-phosphonate linkages to boranophosphate by the boronating agent (2). We discovered that conventional N-acyl protecting groups were reduced under mild conditions by different amine-borane complexes to the corresponding N-alkyl groups, quickly and with high yield (9,11,15). The resulting alkyl groups could not be removed by standard ammonia treatment, yielding base-modified oligonucleotide mixtures.

Concurrently, we found that base-*un*protected nucleosides underwent a complexation with borane under the boronation procedure conditions. Presumably, the complexes are formed at N7 of dG, N1 or N7 of dA, and N3 of dC as was shown for nucleoside complexes with cyanoborane (12,13). However, these borane complexes are unstable, reversible, and completely dissociate during concentrated ammonia (55°C, 12 h) or brief hydrazinehydrate (5 min, r.t.) treatments (11), yielding the *un*modified nucleosides as the sole products.

These findings encouraged us to look into the synthesis of the  $\text{BH}_3^-$ -ODNs without base protection. Among several reports of H-phosphonate oligonucleotide syntheses with unprotected bases, the recent method utilizing a phosphonium condensing agent seemed to be the most promising (14). Indeed, our first synthesis of a boranophosphate tetramer ( $^b\text{GACT}$ ) performed manually at 7.0  $\mu\text{mol}$  scale was quite successful (Scheme 1). Condensations were carried out on a 500 Å T-CPG (35  $\mu\text{mol/g}$ ) solid support with 0.05 M H-phosphonate (10 eq.) and 0.2 M 2-(benzotriazol-1-yloxy)-1,3-dimethyl-2-pyrrolidin-1-yl-1,3,2-diazaphospholidinium hexafluorophosphate (BOMP) as condensing agent in pyridine for 2.5 min. The boronation was performed as reported previously for  $\text{BH}_3^-$ -oligoT



**Scheme 1.** Synthesis of  $\text{BH}_3^-$ -ODNs via H-phosphonate method with unprotected bases.



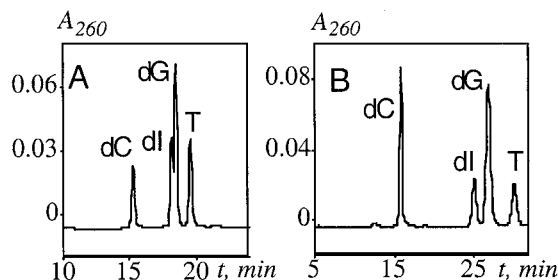
**Figure 1.** Characterization of  $^b\text{GACT}$ : (A) MALDI MS, (B)  $^{31}\text{P}$  NMR, and (C)  $^{10}\text{B}$  NMR.

synthesis (2), i.e., by 0.3 M bis(trimethylsilyl)acetamide (BSA) followed by 0.5 M borane- $N,N$ -diisopropyl- $N$ -ethylamine ( $\text{BH}_3$ -DIPEA) in THF for 2 h. Cleavage from the solid support and deprotection were completed in concentrated ammonia during 12 h at 55°C.

After ion-exchange (IE) and reverse-phase (RP) HPLC separations we obtained about 130 OD (48%) of the desired  $^b\text{GACT}$ . MALDI mass spectrometry confirmed the identity of the product (Fig. 1). The  $^{31}\text{P}$  NMR spectrum was composed of a broad signal centered at 93.8 ppm, distinctive for boranophosphate diesters, and a singlet at 3.88 ppm due to minor phosphate diester impurity (1.4%). In  $^{10}\text{B}$  NMR the typical boranophosphate signal at  $-38.3$  ppm was detected and no borane-nucleobase species (around  $-20$  ppm) were found (Fig. 1). Tetramer  $^b\text{GACT}$  had essentially the same UV absorbance spectrum and mobility on 20% denaturing PAGE as the normal phosphodiester counterpart.

To confirm the base composition of the synthesized  $\text{BH}_3^-$ -ODNs, we developed a procedure for complete enzymatic hydrolysis. As shown previously (2,13), bovine spleen phosphodiesterase (BSP) is capable of slowly hydrolyzing the boranophosphate bond of both  $S_p$  and  $R_p$  configurations. At high enzyme concentration we were able to achieve complete hydrolysis of  $^b\text{GACT}$  into nucleosides after 48 h incubation at 37°C (Fig. 2a). Concurrently, we observed complete conversion of dA to dI by adenosine deaminase which is inevitably present as an impurity in commercial preparations of BSP. Deamination was observed for normal oligonucleotide samples as well as for the  $\text{BH}_3^-$ -ODNs. Nucleoside 3'-boranophosphates were not observed in the final hydrolysis mixture due to their lability (3 h half-life at these conditions) (13). The resulting nucleosides were directly analyzed by RP HPLC (Fig. 2). The nucleoside ratio for  $^b\text{GACT}$  was dC(1.02): dI(0.98): dG(1.11): T(0.97), in good agreement with theory. No base-modifications were detected.

Automated synthesis of  $\text{BH}_3^-$ -ODNs using a Cyclone Plus synthesizer required reconfiguration to minimize mixing volumes of monomers and BOMP condensing agent. We found that preincubation of the H-phosphonate and BOMP for 30 s resulted in complete loss of coupling activity, probably due to H-phosphonate oxidation (14). The synthetic cycle followed the original procedure (14) except for minor modifications of deblocking and coupling steps. The deblocking was



**Figure 2.** Base composition analysis of <sup>b</sup>GACT (A) and <sup>b</sup>GCAGCT (B) after extensive BSP hydrolysis (2 units/ml enzyme, 10  $\mu$ M BH<sub>3</sub><sup>-</sup>-ODN, pH 6.5, 48 h, 37°C). Different gradients were used in A and B.

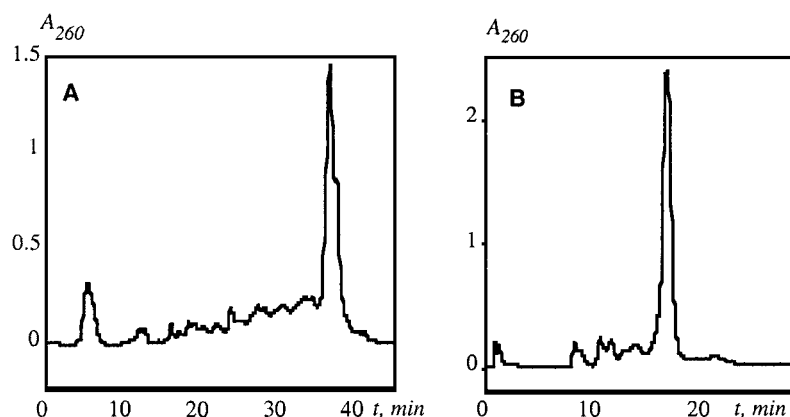
carried out with 3% dichloroacetic acid for 90 s and the coupling with 0.05 M H-phosphonate and 0.2 M BOMP in pyridine for 30 s (repeated 3–4 times). Coupling yields were investigated on the dimer level for a variety of solid supports. Based on RP HPLC analysis of TT dimer synthesis, the best yield (97%) was achieved on highly cross-linked polystyrene (22  $\mu$ mol/g, Primer Support, Amersham Pharmacia Biotech), slightly lower yield (95%) was obtained on polymethacrylate resin (28  $\mu$ mol/g, BioMac, Biosearch Technologies), and only 90–92% yields on different types of CPG support (500–2000 Å, 11–35  $\mu$ mol/g, Glen Research). The oligomer syntheses on polystyrene (PS) and polymethacrylate (MA) supports, however, were less efficient. Although the DMT cation release analysis indicated efficient coupling (95–100%), the IE HPLC analysis showed significant amounts of truncated sequences for both normal phosphodiester and boranophosphate ODNs synthesis. The relative amount of truncated sequences increased with oligonucleotide length. Thus, boranophosphate 6-mers, <sup>b</sup>GCAGCT and <sup>b</sup>ACAATT, were synthesized with 34–46% and 69% yields, correspondingly (Fig. 3); the 7-mer, <sup>b</sup>GCAGCTT, with 39% yield; and the 10-mer, <sup>b</sup>TCAACGTTGA, with only 20–30% yield. The presence of the truncated sequences, jointly with high coupling efficiency assessed from DMT cation release analysis, could point to some degradation of the H-phosphonate backbone during the synthetic cycle. A similar problem of inherent instability of the H-phosphonate backbone during oligonucleotide chain assembly was mentioned in the original article (14).

We screened a number of borane complexes in different solvents to optimize boronation conditions for the mixed-base sequences (Table 1). Quick and relatively efficient boronation on the synthesizer could be achieved with borane-dimethylsulfide (BH<sub>3</sub>-DMS) in tetrahydrofuran (THF) or dichloromethane (DCM). Previously used boronating agents, BH<sub>3</sub>-DIPEA and borane-2-chloropyridine (BH<sub>3</sub>-CPy) in THF (2), required longer reaction times and gave lower yields. Increased boronation time resulted in reduced yields, probably due to side reactions. Manual boronation with borane-pyridine complex (BH<sub>3</sub>-Py) in THF gave repeatedly better yields (>3–6%) compared to the other borane complexes, yet



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**Figure 3.** IE HPLC analysis of the synthesis of boranophosphate GCAGCT (A) and ACAATT (B). Conditions: (A) Mono Q  $10 \times 100$  column, gradient 0–1.5 M NaCl, 50–25%  $\text{CH}_3\text{CN}$ , 10 mM NaOH, flow rate 1.5 mL/min; (B) Polysil CA  $4.6 \times 50$  mm column, gradient 0–0.3 M  $\text{KH}_2\text{PO}_4$  and 50–30%  $\text{CH}_3\text{CN}$  (pH 6.5), flow rate 1.5 mL/min.

required stringently anaerobic conditions to avoid phosphite oxidation. HPLC-purified oligonucleotides showed no signs of base modifications, regardless of the boronating agent choice.

The integrity of the HPLC-purified  $\text{BH}_3^-$ -ODNs was confirmed by 20% denaturing PAGE analysis where the boranophosphates appeared as single bands almost comigrating with their natural counterparts. Their structure was confirmed

**Table 1.** Yields of the Boranophosphate GCAGCT at Different Boronation Conditions<sup>a</sup>

Boronating Agent	Solid Support	Time	Yield, %	Comparative Yield, % <sup>f</sup>
$\text{BH}_3$ -DMS	CPG	10 min	40	69
$\text{BH}_3$ -DMS <sup>b</sup>	PS	20 min	37	68
$\text{BH}_3$ -DIPEA	PS	80 min	36	56
$\text{BH}_3$ -DIPEA	CPG	70 min	34	58
$\text{BH}_3$ -DIPEA	MA	70 min	34	56
$\text{BH}_3$ -DIPEA <sup>c</sup>	PS	2 h	43	80
$\text{BH}_3$ -CPy	CPG	1 h	35	60
$\text{BH}_3$ -Py	PS	12 h <sup>e</sup>	43	80
$\text{BH}_3$ -Py	US-PS <sup>d</sup>	7.5 h <sup>e</sup>	46	79

<sup>a</sup>The solid support was first silylated with 0.3 M BSA for 15 min, then 1 M boronating agent and 0.3 M BSA in THF were codelivered.

<sup>b</sup>In DCM.

<sup>c</sup>In DMF.

<sup>d</sup>Polystyrene universal support (Biosearch Technologies).

<sup>e</sup>Manual boronation at 50°C.

<sup>f</sup>Ratio of the boranophosphate/phosphodiester ODN, which was synthesized from the same portion of solid support by oxidation with 0.2 M (10-camphorsulfonyl)oxaziridine (14).



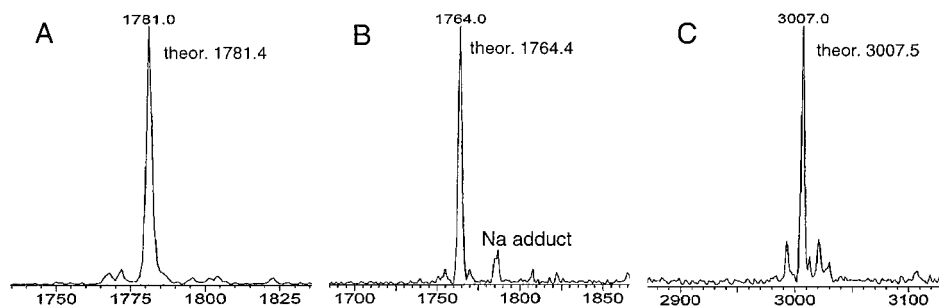


Figure 4.

by electrospray ionization (ESI) MS (Fig. 4). The base compositions determined by enzymatic degradation (Fig. 2b) were close to the theoretical ones (<12% deviation).

In conclusion, short ( $\leq 10$  nt) mixed-base sequence  $\text{BH}_3^-$ -ODNs were successfully synthesized and characterized. Base modifications during the boronation procedure were avoided using the N-unprotected bases and H-phosphonate chain assembly.

### ACKNOWLEDGMENT

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