H-Phosphonate Approach for Solid-Phase Synthesis of Oligodeoxyribonucleoside Boranophosphates and Their Characterization

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Received April 30, 1998

Abstract: Substitution of a borano (BH_3^-) group for nonbridging oxygen in the phosphate backbone of DNA results in a new class of isoelectronic and isoionic DNA analogues. An effective chemical method of synthesis of oligodeoxynucleoside boranophosphates (BH_3^-−ODNs) on a solid phase has been developed via an *H*-phosphonate chain elongation approach followed by boronation. The boronation procedure involves the intermediate conversion of an *H*-phosphonate to a phosphite triester group by silylation and subsequent oxidation by a borane−amine complex. The efficiency of the boronation procedure to form BH_3^-−ODNs is close to that of iodine oxidation to form phosphodiester ODNs. Oligothymidine boranophosphates of different lengths up to 12-mer have been readily synthesized, purified by HPLC and/or PAGE methods, and characterized by NMR spectroscopy and MS spectrometry. In physiologically relevant buffers the dodecathymidine boranophosphate hybridized with complementary dodecadeoxyadenylate and exhibited a cooperative melting transition (T_m = 14 °C). Studies of substrate properties showed that BH_3^-−ODNs are readily 5′-phosphorylated by T4 polynucleotide kinase. Boranophosphate analogues are much more resistant toward nuclease hydrolysis than phosphodiester ODNs, and more resistant to P_1 and S_1 nucleases and snake venom phosphodiesterase than phosphorothioate ODNs.

Understanding the principles of molecular recognition between nucleic acids, starting with the discovery of Watson and Crick,[1] gave rise to the possibility of directing highly specific interactions between a relatively short oligonucleotide molecule and targeted single- or double-stranded nucleic acid.[2] Practical applications of this idea emerged as antisense and antigene strategies for gene therapy.[2,3] As the concept developed, it became apparent that gene expression could be altered by exogenously administered oligodeoxynucleotides (O^-−ODNs). For therapeutics, however, O^-−ODNs had a number of disadvantages. Their high nuclease susceptibility[4] and low cell membrane permeability[5] was an impetus for the design and synthesis during the last two decades of a great variety of oligonucleotide analogues exhibiting different physicochemical and biochemical properties. Among the phosphodiester backbone analogues, the phosphorothioate (S^-−ODNs) and methylphosphonate (CH_3−ODNs) modified oligonucleotides were the first promising to emerge.[6] These modifications improved some of the antisense and/or antigene characteristics compared to natural oligonucleotides, but they also were accompanied by some undesirable properties. For example, the S^-−ODNs have a tendency to bind to proteins,[7] producing non-sequence-specific effects with accompanying toxicity.[8] The nonionic CH_3−ODNs are minimally water soluble and incapable of inducing RNase H cleavage of mRNA in heteroduplexes,[9] which limits their

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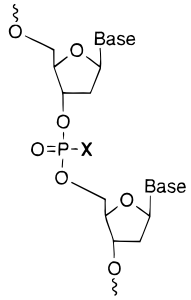
S0002-7863(98)01492-9 CCC: $15.00
Published on Web 09/09/1998

Scheme 1. Different Phosphate-Modified ODNs: X = S^-, Phosphorothioates; X = NH_2, Phosphoramidates; X = CH_3, Methylphosphonates; X = BH_3^-, Boranophosphates



utility as antisense agents since RNase H-mediated degradation is believed to be a primary mechanism of ODNs drug action.[10] Clearly, there is still a need to design more efficient and therapeutically suitable agents.

Replacing one nonbridging oxygen in the phosphate moiety by a borano (BH_3^-) group (Scheme 1) results in a new backbone modification of ODNs.[11] This surprisingly stable modification imparts unique properties to these analogues. The oligonucleoside boranophosphates[12] (BH_3^-−ODNs) are isosteric and isoelectronic to the CH_3−ODNs. Being sterically similar to a methylphosphonate group, the boranophosphate retains the negative charge of the phosphate or phosphorothioate groups, making the boranophosphate oligomers highly water soluble. Although their net charge is identical to those of analogous O^-−ODNs and S^-−ODNs, the boranophosphates are more hydrophobic. High stability of a single boranophosphate internucleotide linkage toward nuclease hydrolysis was demonstrated[11b,d,13] and successfully exploited for a one-step PCR sequencing method.[14] Another important and unique feature of the BH_3^-−ODNs involves possible use for boron neutron capture therapy (BNCT)[15] in combination with specific inhibition of gene expression to enhance their therapeutic efficiency.

Deoxyribonucleoside 5′-*O*-[α-BH_3]triphosphates are good substrates for a number of DNA polymerases.[13c,14,16] A template-directed primer extension reaction was successfully used to synthesize oligonucleotides containing a single boranophosphate insertion[13c] or an all-boranophosphate 44-nucleotide-long stretch.[16] However, chemical approaches for BH_3^-−ODNs synthesis based on phosphoramidite methodology have been limited so far to dimers and trimers.[11] Recently we described a new efficient procedure for converting an internucleoside *H*-phosphonate diester group into boranophosphate, using dithymidine *H*-phosphonate as a model compound.[16] In the present study, we demonstrate the applicability and efficiency of this method for the chemical synthesis of longer boranophosphate oligomers on solid support, utilizing an *H*-phosphonate approach.[17] The resulting BH_3^-−ODNs can be easily handled, purified, and analyzed by conventional HPLC and PAGE techniques. We also report some hybridization properties of the resulting oligonucleotides as well as their substrate properties toward a number of enzymes commonly used for oligonucleotide analysis.

Experimental Section

Materials and Methods. *N*,*O*-bis(trimethylsilyl)acetamide (BSA), *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA), heptamethyldisilazane (HMDS), chlorotrimethylsilane (CTMS), triethylamine (TEA), borane−*N*,*N*-diisopropylethylamine (BH_3·DIPEA), borane−pyridine (BH_3·Py), and dimethyl *H*-phosphonate were purchased from Aldrich Chemical Co. Borane−2-chloropyridine[18] (BH_3·CPy) and borane−aniline[19] (BH_3·An) were freshly prepared before use. Tetrahydrofuran (THF, Fisher Scientific) was dried prior to use by distillation from sodium−benzophenone. Reagents for *H*-phosphonate synthesis and 5′-*O*-(4,4′-dimethoxytrityl)thymidin-3′-yl *H*-phosphonate were purchased from Glen Research. For oligonucleotide synthesis the following 5′-*O*-(4,4′-dimethoxytrityl)thymidine-3′-succinoyl-derivatized solid supports were used: controlled pore glass (ABI, 29 μmol/g loading), low cross-linked polystyrene (Sigma, 280 μmol/g), high cross-linked LV polystyrene (Glen Research, 12 μmol/g), and silica gel (Sigma, 130 μmol/g).

T4 polynucleotide kinase (EC 2.7.1.78, New England BioLab, 10 U/μL), snake venom phosphodiesterase (SVP, EC 3.1.4.1, Sigma, 0.08 U/mg), bovine spleen phosphodiesterase (BSP, EC 3.1.16.1, Sigma, 7.5 U/mg), S_1 nuclease (EC 3.1.30.1, Promega, 260 000 U/mg), and P_1 nuclease (EC 3.1.30.1, Sigma, 407 U/mg) were used for enzymatic studies.

Oligonucleotide syntheses were carried out on a Cyclone Plus synthesizer (Milligen/Biosearch) via the *H*-phosphonate method. Phosphodiester oligothymidylates and their phosphorothioate analogues (2-, 4-, 8-, and 12-mers) were obtained by postsynthetic oxidation with 0.1 M iodine in pyridine/water (98:2, v/v; 10 min, rt)[20] and 0.2 M S_8 in CS_2/pyridine/triethylamine (12:12:1, v/v; 4 h, rt),[21] respectively. ^{31}P NMR analysis confirmed the purity of phosphorothioate analogues. Dodecathymidine methylphosphonate [$T(^mpT)_{11}$] was purchased from TriLink BioTechnologies, Inc.

High-performance liquid chromatography was performed on a Waters 600E controller system equipped with a 991 photodiode array detector. Samples were eluted at a flow rate of 3 mL/min using a gradient of solutions described as A and B below. For ion-exchange (IE) chromatography, a 10 × 250 mm column with strong anion-exchange resin Polysil CA 10 μm (Koltsovo, Russia) was used. Eluents were (A) 50% CH_3CN in water and (B) 30% CH_3CN in 0.3 M KH_2PO_4, pH 6.5. A linear gradient of 0−100% B was developed in 60 min. Reversed-phase (RP) chromatography was carried out on a 7.8 × 300 mm Delta Pak C18 (15 μm) column. Eluents were (A) 0.05 M triethylammonium acetate, pH 7.0 and (B) CH_3CN. A linear gradient of 0−45% B was developed in 45 min.

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Gel electrophoretic analysis was performed in a 20% polyacrylamide gel (19:1, acrylamide−*N*,*N*′-methylenebisacrylamide) under denaturing conditions (7 M urea, 0.09 M Tris−borate (pH 8.3), 40 °C). Visualization of unlabeled oligonucleotides was achieved by staining with Stains-all (Eastman Kodak Co.) or by autoradiography for 5′-^{33}P-labeled compounds. Quantification of radiolabeled material was carried out on a PhosphorImager (Molecular Dynamics).

^{31}P NMR spectra were recorded on a Varian Inova 400 spectrometer at 161.9 MHz frequency and 12 h acquisition times (30 000 accumulations). Samples contained 0.73−1.0 mM oligonucleotide in 0.1 M $NaDCO_3$ (pH 8.0), 0.1 mM EDTA in D_2O. Chemical shifts were referenced to external 85% H_3PO_4 in H_2O as a standard. ^{1}H and ^{11}B NMR spectra were recorded with the same samples on a Varian Inova 500 spectrometer at 499.9 and 160.4 MHz frequencies, respectively. Acquisition times were 1 h for ^{1}H and 3 h for ^{11}B spectra, and chemical shifts were referenced to external 3-(trimethylsilyl)propionic acid and BF_3·Et_2O, respectively.

High-resolution fast atom bombardment mass spectrometry (HR FAB MS) data were obtained on a JEOL-300 mass spectrometer in negative ion mode with polyethylene glycol-600 as a matrix. Electrospray ionization (ESI) technique was applied in the cases of 4-, 8-, and 12-meric BH_3^-−ODNs. Measurements were made on a Micromass-VG Quattro BQ triple quadrupole mass spectrometer equipped with a pneumatically assisted electrostatic ion source operating at atmospheric pressure. Samples were introduced by loop injection into a stream of 50% aqueous acetonitrile containing 1% formic acid flowing at 8 μL/min. Mass spectra were acquired in negative ion mode as continuum mode from *m*/*z* 225−1200 with a scan time of 5 s.

The thermal denaturation experiments were carried out on an AVIV DS14 spectrophotometer equipped with a thermoprogrammer using 0.2 mm quartz cuvettes and 1×10^{-4} M complex concentration. The concentration was determined spectrophotometrically on the basis of calculated molar absorptivity coefficients: $\epsilon_{260} = 97\ 800\ M^{-1}\cdot cm^{-1}$ for all dodecathymidylates and $147\ 000\ M^{-1}\cdot cm^{-1}$ for dodecadeoxyadenylate.[22] The samples were prepared by mixing single-stranded oligonucleotides at a 1:1 ratio in 0.15 M KCl, 0.02 M KH_2PO_4 (pH 7.0) buffer solution followed by heating to 65 °C and gradually cooling (0.5 °C/min) to 4 °C. After overnight incubation at 4 °C, $MgCl_2$ was added to the sample to give 10 mM concentration. The sample was degassed, and the absorbance was registered at 260 nm at temperatures between 1 and 65 °C at a heating rate of 0.33 °C/min. The integrity of the samples containing oligothymidine boranophosphates was confirmed after the melting by IE HPLC analysis.

^{31}P NMR Studies of Conversion of an *H*-Phosphonate Group into Boranophosphate. A. Solution Studies. To a 5 mm NMR tube containing 9.2 μL of dimethyl *H*-phosphonate (0.1 mmol) in a mixture of 700 μL of anhydrous THF and 200 μL of DMSO-d_6, we added 0.3 mmol of silylating agent (79.7 μL of BSTFA, 74.1 μL of BSA, 66.0 μL of HMDS, or 38.1 μL of CTMS together with 76.7 μL of TEA), and after determined time intervals ^{31}P NMR spectra were recorded. After complete silylation, the borane exchange reaction was initiated by addition of 0.5 mmol of borane−amine complex (63.7 mg of BH_3·CPy, 87 μL of BH_3·DIPEA, 53.5 mg of BH_3·An, or 62.5 μL of BH_3·Py), and the progress of the reaction was monitored. After total disappearance of the phosphite triester signal and formation of the boranophosphate triester signal, 0.5 mL of concentrated ammonia solution was added to the reaction mixture. It resulted in quick hydrolysis of trimethylsilyl ester (1 h at rt), yielding boranophosphate dimethyl ester.

B. Studies on Solid Support. A 200 mg portion of 5′-*HO*-thymidine-3′-*O*-succinoyl polystyrene resin (0.28 mmol/g loading) in 2 mL of anhydrous pyridine−acetonitrile (1:1) solution was coupled with 0.56 mL of 0.2 M 5′-*O*-(4,4′-dimethoxytrityl)thymidin-3′-yl *H*-phosphonate in the presence of 0.56 mmol of pivaloyl chloride. After 2 min of coupling, the solid support was washed three times with 2 mL of acetonitrile and the 5′-*O*-(4,4′-dimethoxytrityl) group was removed by 2.5% dichloroacetic acid in methylene chloride (1 min). The resulting 5′-*HO*-thymidin-3-yl 3′-*O*-succinoylthymidin-5′-yl *H*-phosphonate (dithymidine *H*-phosphonate) tethered to solid support was washed exhaustively with methylene chloride and then acetonitrile and dried in vacuo prior to ^{31}P NMR experiments.

Table 1. Yields of the Oligothymidine Boranophosphates and Their Phosphodiester Counterparts Obtained in Parallel Synthesis (Scheme 2)[a]

	yields on different solid supports, %		
oligonucleotide	CPG	polystyrene resin	silica gel
TpT	90	94	92
T^{b}pT	83	83	70
$T(pT)_3$	82	86	76
$T(^bpT)_3$	69	69	58
$T(pT)_7$	66	84	57
$T(^bpT)_7$	63	68	45
$T(pT)_{11}$	55	63	45
$T(^bpT)_{11}$	55	53	37

[a] The boronation procedure was performed manually. Yields were calculated on the basis of chromatographic profiles (absorbance at 260 nm). For chromatographic conditions see the Experimental Section.

A 100 mg portion of the solid support (about 25 μmol of dithymidine *H*-phosphonate) was placed in a 5 mm NMR tube filled with argon, and 1.5 mL of anhydrous THF and 0.4 mL of DMSO-d_6 were added. After incubation for 5 min to allow the polystyrene to swell, 75 μmol of the silylating agent was added (19.9 μL of BSTFA, 18.5 μL of BSA, 16.5 μL of HMDS, or 9.5 μL of CTMS together with 19.2 μL of TEA), and the mixture was shaken well. The same experiments were done with addition of 0.3 mmol of the silylating agent. After ^{31}P NMR analysis showed complete disappearance of *H*-phosphonate signal and formation of phosphite triester signal, 1 mmol of a borane−amine was added (127.4 mg of BH_3·CPy, 174 μL of BH_3·DIPEA, 107.0 mg of BH_3·An, or 125 μL of BH_3·Py). After the exchange reaction was completed, yielding boranophosphate triester, the solid support was washed with THF (2 × 2 mL), followed by acetonitrile (2 × 2 mL), after which 1 mL of concentrated ammonia was added. After 2 h at rt, a complete conversion to boranophosphate diester along with cleavage from the solid support occurred. The resulting dithymidine boranophosphate was purified by RP HPLC and characterized by NMR and MS methods.

Synthesis of the Oligothymidine Boranophosphates. *H*-Phosphonate synthesis was performed on a 5.6 μmol scale (silica gel solid support), a 2.0 μmol scale (CPG), and a 0.2 μmol scale (low-volume polystyrene resin). A 2-fold excess of *H*-phosphonates and 3 min of coupling were employed for synthesis on silica gel and CPG supports and 4-fold excess and 1 min for synthesis on polystyrene support.[23] After removal of the final DMT group, the solid support was divided. A portion of the solid support was oxidized by 0.1 M I_2 in pyridine−water (98:2) (10 min, rt) to yield natural oligothymidylates. The boronation procedure was performed with the remaining solid support either on a DNA synthesizer or manually.

On the synthesizer, the solid support was treated with 0.3 M BSTFA in anhydrous THF for 20 min to ensure complete silylation. After washing with CH_3CN for 30 s, 0.5 M BH_3·CPy in anhydrous THF was delivered and allowed to react for 30 min at rt. After washing with CH_3CN for 30 s, the solid support was treated with concentrated ammonia solution for 2 h at rt to deprotect and cleave the product. The yield for $T(^bpT)_{11}$ (dodecathymidine boranophosphate) was 46% based on the IE HPLC profile registered at 260 nm, equal to that for the corresponding $T(pT)_{11}$ oligomer after iodine oxidation.

In the manual boronation procedure, 15−40 mg of the solid support was incubated in 0.5 mL of 0.3 M BSA or BSTFA in anhydrous THF under argon at rt for 2 h or 20 min, respectively. After washing with THF (1 × 1 mL), BH_3·Py complex was added to 0.5 M concentration and the reaction mixture was incubated under argon for 12 h at 50 °C. Alternatively, 1 mL of 0.5 M BH_3·DIPEA or BH_3·An complex was added and the reaction mixture was incubated under argon at rt for 1.5 h. Excess of the borane−amine complex was removed by washing with CH_3CN (2 × 1 mL), and oligonucleotides were deprotected and

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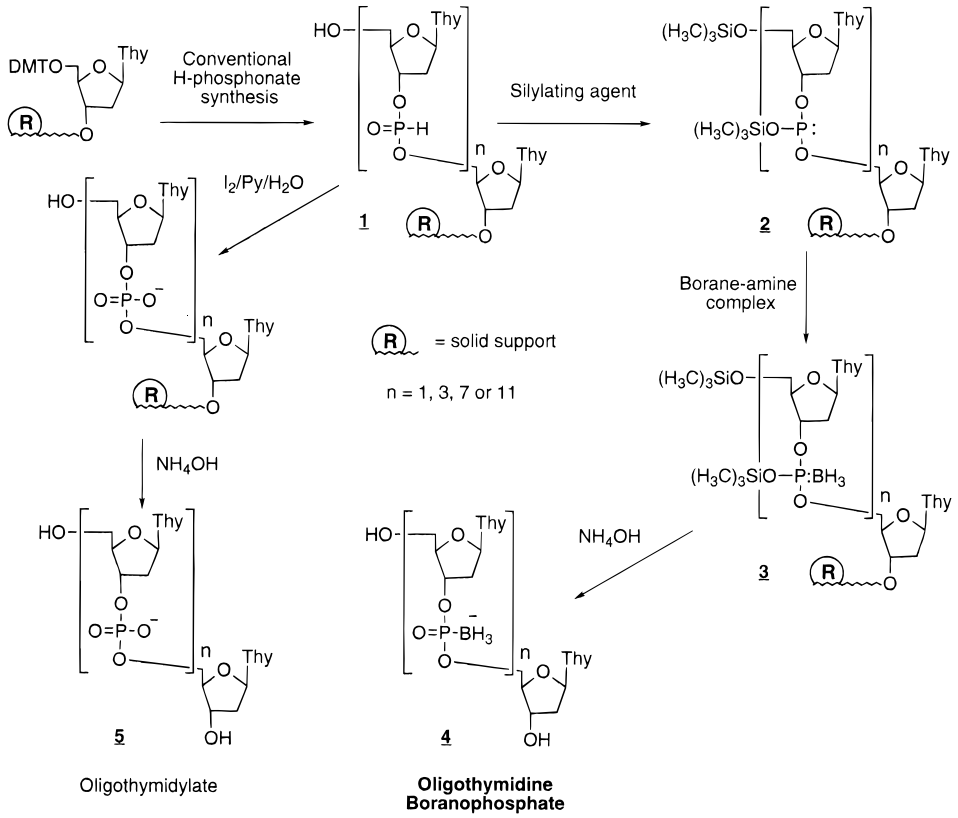
Scheme 2. Synthesis of the Oligothymidine Boranophosphates via the *H*-Phosphonate Approach



cleaved from the solid support by concentrated ammonia treatment at rt for 2 h. For yields see Table 1.

NMR and MS data for the oligothymidine boranophosphates after HPLC purification were as follows: ($T^{b}pT$ first eluted isomer) ^{31}P NMR $\delta = 93.3$ (q, $J_{P(H)-B} = 130$ Hz); ^{11}B NMR $\delta = -40.9$ br; ^{1}H NMR $\delta = 7.673$ (s, H6, 1 H), 7.646 (s, H6, 1 H), 6.339 (t, H1′, 1 H), 6.243 (t, H1′, 1 H), 4.83 (br, H3′, 1 H), 4.594 (2t, H3′, 1 H), 4.156 (m, H4′, 2 H), 4.105 (m, H5′, 2 H), 3.854 (2d, H5′, 1 H), 3.778 (2d, H5′, 1 H), 2.511 (m, H2′, 1 H), 2.353 (m, H2′, 3 H), 1.930 (s, CH_3, 3 H), 1.891 (s, CH_3, 3 H), 0.38 (br, BH_3, 3 H). ($T^{b}pT$ second eluted isomer) ^{31}P NMR $\delta = 93.5$ (q, $J_{P(H)-B} = 130$ Hz); ^{11}B NMR $\delta = -40.8$ br; ^{1}H NMR $\delta = 7.717$ (s, H6, 1 H), 7.662 (s, H6, 1 H), 6.309 (t, H1′, 1 H), 6.241 (t, H1′, 1 H), 4.90 (br, H3′, 1 H), 4.571 (2t, H3′, 1 H), 4.167 (m, H4′, 2 H), 4.096 (m, H5′, 2 H), 3.844 (2d, H5′, 1 H), 3.782 (2d, H5′, 1 H), 2.517 (m, H2′, 1 H), 2.367 (m, H2′, 3 H), 1.923 (s, CH_3, 3 H), 1.890 (s, CH_3, 3 H), 0.36 (br, BH_3, 3 H); HR FAB MS (mixture of the isomers) found 543.1679, calcd for $C_{20}H_{29}BN_4O_{11}P$ 543.1663. ($T^{b}pT)_n$, $n = 3$, 7, or 11) ^{31}P NMR $\delta = 93.8$ br; ^{11}B NMR $\delta = -40.8$ br; ^{1}H NMR $\delta = 7.73-7.63$ (m, H6), 6.29 (m, H1′), 4.8 (br, H3′), 4.57 (m, H3′), 4.34 (m, H4′), 4.23−4.02 (m, H4′, H5′), 3.81 (m, H5′), 2.54−2.44 (br, H2′), 2.38−2.25 (br, H2′), 1.92 (br, CH_3), 0.69−0.01 (br, BH_3); ESI MS analysis for $n = 3$ 1148.3 ± 0.4 (calcd 1148.4), $n = 7$ 2357.3 ± 3.7 (calcd 2356.6), $n = 11$ 3564.5 ± 3.0 (calcd 3564.9).

Studies of the Substrate Properties. Standard conditions have been chosen for 5′-phosphorylation of BH_3^-−ODNs by T4 polynucleotide kinase.[24] The reaction mixture (10 μL) contained 10 pmol of HPLC-purified oligonucleotide, 10 pmol of [γ-^{33}P]ATP (>2500 Ci/mmol, Amersham), and 10 units of T4 polynucleotide kinase in 50 mM Tris−HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.1 mM spermidine−HCl, and 0.1 mM EDTA. The reaction mixture was incubated at 37 °C for 30 min and then analyzed by 20% denaturing PAGE. Alternatively, at the same buffer conditions 1 nmol of $T(^{b}pT)_{11}$ or $T(pT)_{11}$ and 5 nmol of ATP were incubated with 10 units of T4 polynucleotide kinase in 20 μL total volume, and aliquots were withdrawn at appropriate times and analyzed by IE HPLC.

For nuclease resistance studies, HPLC-purified $T(^{b}pT)_{11}$, $T(^{s}pT)_{11}$, or $T(pT)_{11}$ was used at 10 μM concentration. SVP hydrolysis was carried out at 37 °C in 50 mM NaCl, 50 mM Tris−HCl (pH 9.0), 15 mM $MgCl_2$, 100 μg/mL bovine serum albumin, and 2 or 20 μg/mL (0.0006 or 0.006 U/mL) enzyme. For BSP hydrolysis 5 or 50 μg/mL (0.037 or 0.37 U/mL) enzyme in 0.13 M sodium succinate (pH 6.5) at 37 °C was used. For P_1 nuclease hydrolysis, 0.01 or 10 μg/mL (0.004 or 4.07 U/mL) enzyme in 30 mM sodium acetate (pH 5.3), 0.2 mM $ZnSO_4$ at 37 °C was used. For S_1 nuclease hydrolysis, 0.01 or 5 μg/mL (0.0026 or 1.3 U/mL) enzyme in 80 mM NaCl, 30 mM sodium acetate (pH 4.6), 1 mM $ZnSO_4$ at 37 °C was used. At appropriate times, aliquots were withdrawn from the reaction mixtures and immediately analyzed by IE HPLC. The control experiments without the enzyme were carried out at the same conditions.

Results

Synthesis of Oligothymidine Boranophosphates via the *H*-Phosphonate Approach. *H*-Phosphonate methodology consists of assembling an oligonucleotide chain bearing internucleoside *H*-phosphonate diester linkages and subsequently converting all linkages to stable phosphate, phosphorothioate, phosphoroamidate, etc. diesters.[25] We used a similar strategy for the synthesis of BH_3^-−ODNs by boronation of the assembled oligonucleotide chain (Scheme 2), but through an intermediate silylated phosphite triester species. It has been shown elsewhere that boronation of phosphite triester species is accomplished as a borane (BH_3) exchange reaction with borane−sulfide or borane−amine complexes.[11,16,17,26] A phosphorus atom in the

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H-phosphonate form, however, lacks a free electron pair and is not a suitable donor for the BH_3 group. The boronation procedure, therefore, requires an intermediate conversion of the *H*-phosphonate diester group (**1**, Scheme 2) to a phosphite triester (**2**), which is a typical Lewis base. This conversion was readily achieved by silylation with different agents. Along with the previously used chlorotrimethylsilane[27] and *N*,*O*-bis(trimethylsilyl)acetamide,[16,17a,28] we tested heptamethyldisilazane and *N*,*O*-bis(trimethylsilyl)trifluoroacetamide.

Silylation of *H*-phosphonate was studied in solution with *H*-phosphonate dimethyl ester and on a solid phase with dithymidine *H*-phosphonate (**1**, $n = 1$, Scheme 2) tethered to polystyrene resin.[29] The course of the reaction was monitored by ^{31}P NMR spectroscopy and revealed that silylation of *H*-phosphonate was readily achieved by all tested agents, being the fastest for BSTFA. Conversion of half of the *H*-phosphonate ($\delta = 11.61$ ppm) to phosphite ($\delta = 128.44$ ppm) in solution with 0.3 M silylating agent (3-fold excess) took <5 min (time required for recording the NMR spectrum) for both BSTFA and for CTMS. Under similar conditions the $t_{1/2}$ for BSA and HMDS was 10 and 30 min, respectively. On the polystyrene resin the conversion proceeded at approximately the same rate with 0.04 M silylating agent (3-fold excess relative to the bound *H*-phosphonate dimer) and was even faster with 0.3 M silylating agent. Complete conversion of *H*-phosphonate (**1**, $\delta = 8.9$ and 10.3 ppm) to phosphite species (**2**, $\delta = 128.4$ and 128.8 ppm) occurred in 5 min for 0.3 M BSTFA, in 10 min for CTMS in the presence of TEA, and in 30 min for BSA and HMDS. No other byproduct signals were observed in ^{31}P NMR spectra, except for a signal at −9.0 ppm due to dithymidine trimethylsilyl phosphate triester. From the NMR integration, the intensity of the signal was always less than 5% of the total phosphorus species and depended on how strictly anaerobic conditions were maintained during preparation of the NMR sample.

Exchange of a borane group between the phosphite triesters (**2**) and borane−amine complex produced boranophosphate triesters (**3**, Scheme 2). We examined the ability of three different types of amines (aliphatic, borane−*N*,*N*-diisopropylethylamine; aromatic, borane−aniline; and heterocyclic, borane−pyridine and borane−2-chloropyridine) to transfer a BH_3 group to compound **2**. All of the studied amine complexes underwent exchange of the borane group with phosphite triesters in quantitative yields, although reaction rates differed. ^{31}P NMR data testified that the most active boronating agent was BH_3·CPy complex. In less than 5 min, 0.1 M trimethylsilyldimethyl phosphite triester ($\delta = 128.44$ ppm) was completely converted to trimethylsilyldimethyl boranophosphate ($\delta = 105.6$ ppm) by reaction with 0.5 M boronating agent. Less active were the BH_3·DIPEA and BH_3·An complexes, which required 10 min for 50% boronation at the same conditions, and 40 min for complete conversion. The BH_3·Py complex turned out to be the least reactive boronating agent. Boronation of trimethylsilyldimethyl phosphite by BH_3·Py proceeded very slowly, if at all, at ambient temperature; only at 50 °C was an appreciable reaction rate achieved, requiring 130 min for the half-reaction. On polystyrene resin with 0.5 M boronation agent (50-fold excess) the exchange reaction was complete in 10 min for BH_3·CPy complex and 15 min for BH_3·DIPEA and BH_3·An complexes. The BH_3·Py complex converted 50% of dithymidine trimethylsilyl phosphite triester (**2**) to the corresponding boranophosphate (**3**, $\delta = 103.5$ ppm) in 100 min at 50 °C, and complete conversion was achieved in 12 h. For all boronating agents the exchange reaction with the phosphite triesters proceeded smoothly, producing only boranophosphate triesters according to ^{31}P NMR.

Treatment with concentrated ammonia (Scheme 2) easily hydrolyzed the silyl group of the boranophosphate triester (**3**) to give diester (**4**, $n = 1$, $\delta = 93.6$ ppm)[30] and simultaneously cleaved the dithymidine boranophosphate from the solid support. The overall yield of the desired product starting with *H*-phosphonate was >95% based on integrated ^{31}P NMR data, with the phosphate diester ($\delta = 0.55$ ppm) being the main byproduct (2−3%).

Using the synthetic scheme and reagents described above, di- [T^bpT], tetra- [$T(^bpT)_3$], octa- [$T(^bpT)_7$], and dodecathymidine [$T(^bpT)_{11}$] boranophosphates were synthesized, performing the boronation procedure either manually with different combinations of reagents, or by DNA synthesizer with BSTFA and BH_3·CPy reagents. Three different types of solid supports were tested for the synthesis: CPG, silica gel, and a highly cross-linked polystyrene resin. After complete chain elongation (**1**), the solid support was divided in order to estimate the effectiveness of the boronation procedure. One part was treated with iodine[20] to obtain O^-−ODNs (**5**), and the other was boronated (Scheme 2). Yields of boranophosphate analogues constituted 80−100% of those for natural oligothymidylates (Table 1). Boronation was performed most efficiently on CPG support. Slightly lower efficiency was found for boronation on polystyrene resin, and silica gel seemed to be the least favorable support for the boronation procedure.

To investigate the content of phosphate diester impurities possibly introduced during boronation, the crude reaction mixtures from the dodecathymidine boranophosphate syntheses after ammonia deprotection were monitored by ^{31}P NMR. In manual synthesis up to 4% of the total phosphorus signals were phosphate diester species, whereas in machine-assisted synthesis the phosphodiester signal dropped to less than 1%.

HPLC Purification and Characterization of the Synthesized BH_3^-−ODNs. The synthesized BH_3^-−ODNs were purified by ion-exchange (IE) and reversed-phase (RP) HPLC. Using IE chromatography, we observed the boranophosphate dodecamer (**4**, $R_t = 33$ min) as the main peak (Figure 1), surrounded with minor peaks associated with failed sequences as expected for typical *H*-phosphonate synthesis. The IE purification profile of the phosphodiester dodecathymidylate **5** ($R_t = 27$ min) obtained in the parallel synthesis was very similar to that of the boranophosphate analogue (not shown). The primary difference was that the boranophosphate analogues had greater retention times and broader peaks. Similar behavior was observed for phosphorothioates[31] and might be explained by hydrophobic interactions of the diastereomeric BH_3^-−ODNs with the stationary ion-exchange matrix. Introduction of acetonitrile to the eluant diminished these nonionic interactions, improving peak width and shape.

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(29) The polystyrene resin (Sigma) has been chosen for these experiments due to its high loading index (280 μmol/g) that is necessary to provide a reliable signal-to-noise ratio in ^{31}P NMR experiments for reasonable time (see the Supporting Information). The silica gel and CPG solid supports gave too weak signals in similar experiments.

(30) Thymidin-3′-yl thymidin-5′-yl boranophosphate (T^bpT) comprising a mixture of stereoisomers gave a broad signal centered at 93.6 ppm in the ^{31}P NMR spectrum (reaction mixture after final ammonia deprotection). After RP HPLC separation, pure stereoisomers of T^bpT appeared as broad quartets due to ^{11}B splitting centered at 93.3 ppm (first eluted) and 93.5 ppm (second eluted) in 80 mM Na_2HPO_4 (pH 7.0), 0.4 mM EDTA.

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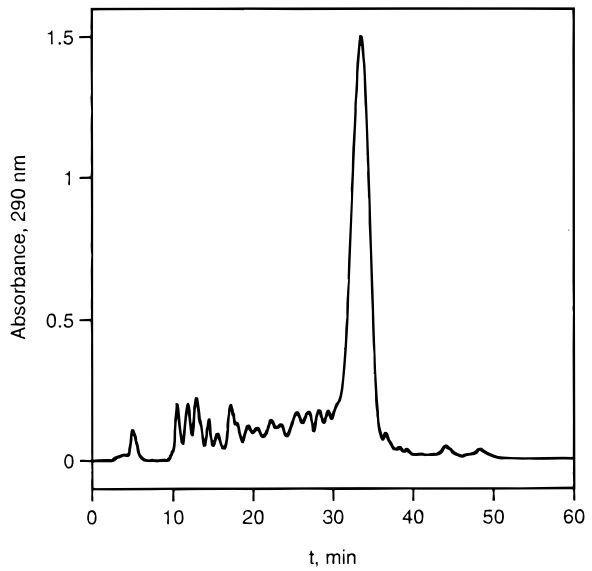

Figure 1. Ion-exchange HPLC profile of the reaction mixture of $T(^{b}pT)_{11}$ synthesis on CPG on a 2 μmol scale. A 10 × 250 mm Polysil CA 10 μm column and gradient (50−30% CH_3CN, 0.0−0.3 M KH_2PO_4 (pH 6.5)) in water for 60 min at a flow rate of 3 mL/min were used.

Table 2. Retention Times and Peak Widths on RP HPLC for the Different Backbone-Modified Oligothymidylates[a]

oligonucleotide	retention time, min	peak width, min
TpT	19.6	0.25
$T^{s}pT$	23.8 and 24.8[b]	0.29 and 0.30
$T^{b}pT$	26.6 and 27.0[b]	0.37[c]
$T(pT)_3$	22.8	0.35
$T(^{s}pT)_3$	29.2	1.67
$T(^{b}pT)_3$	33.2	0.82
$T(pT)_7$	23.3	0.35
$T(^{s}pT)_7$	31.6	1.19
$T(^{b}pT)_7$	35.9	0.97
$T(pT)_{11}$	24.7	0.34
$T(^{s}pT)_{11}$	33.0	1.01
$T(^{b}pT)_{11}$	38.0	0.72
$T(^{m}pT)_{11}$	42.1	0.62

[a] Chromatographic conditions: Nucleoside/Nucleotide (Waters) 7 μm (4.6 × 250 mm, 11 000 plates/m) column; eluant A, 50 mM triethylamine acetate (pH 7.0); eluent B, CH_3CN; linear gradient 5−40% B in 60 min at a flow rate of 1.0 mL·min^{-1}. Peak widths were measured at half-height. [b] Two stereoisomers; for backbone-modified tetra-, octa-, and dodecathymidylates the retention times reflect the average for stereoisomers which are eluted as a broad peak. [c] Average width for both peaks.

IE purification was followed by RP HPLC for all of the BH_3^-−ODNs except the dimer $T^{b}pT$, which was purified only by RP HPLC. The synthetic scheme yielded oligothymidine boranophosphates as mixtures of diastereomers. Subsequently, the dimer was resolved by RP HPLC into two peaks corresponding to R_p and S_p isomers (Table 2). Longer BH_3^-−ODNs (2^{n-1} isomers for an *n*-meric oligomer) were eluted as one broad peak. Comparative RP HPLC peak width data for different backbone-modified oligothymidylates are given in Table 2. The diastereomeric dodecathymidine boranophosphate displayed a width intermediate between that of the wider peak of the analogous phosphorothioate dodecamer and that of the narrower peak of the methylphosphonate dodecamer, being more similar to the latter. Likewise, the RP HPLC retention time values exhibited by the BH_3^-−ODNs are intermediate between S^-− and CH_3−ODNs, providing evidence that boranophosphate analogues are more hydrophobic than charged phosphorothioates yet less hydrophobic than neutral methylphosphonates.

After HPLC purification the structures of the BH_3^-−ODNs were confirmed by several spectroscopic techniques and by denaturing PAGE analysis. An important verification of the purity of backbone-modified oligonucleotides is the absence of different phosphorus species impurities, especially phosphate diester. For all synthesized BH_3^-−ODNs, the ^{31}P NMR spectra registered only a broad signal centered at 93.8 ppm which corresponds to the boranophosphate moiety.[11,16,17] No other ^{31}P signals were observed. Proton spectra of $T^{b}pT$ coincided with previous data[11b,33] for the dithymidine boranophosphate synthesized by the phosphoramidite method.[11a] The proton spectra of the tetra-, octa-, and dodecamers closely resembled that of the dimer. In all spectra the protons of the BH_3 group appeared as a broad signal centered at 0.36 ppm. The ratio of signal intensity of the BH_3 protons to the base and/or deoxyribose protons is close to theoretical. The ^{11}B spectra for all oligothymidine boranophosphates consisted of a single broad signal centered at −40.8 ppm.

Molecular mass data obtained by mass spectrometry techniques (HR FAB for the boranophosphate dimer and electrospray ionization for boranophosphate 4-, 8-, and 12-mers) were practically identical to those theoretically calculated (±1 Da). In conjunction with supporting NMR data, one may conclude that the resulting oligonucleotides contained no boronated bases or base/sugar modifications.

Since the BH_3^-−ODNs are negatively charged, a gel electrophoretic analysis proved to be effective for determining their composition and length. Denaturing (20%) PAGE revealed only one band for each of the HPLC-purified oligothymidine boranophosphates (staining with Stains-all), confirming the homogeneity of the compounds. As expected for isoionic compounds, electrophoretic mobilities of the boranophosphate analogues were very similar to those of the natural oligothymidylates.

Thermal Denaturation Studies. Hybridization properties of the BH_3^-−ODNs were studied as a function of temperature using the dodecathymidylate−dodecadeoxyadenylate model system, 1:1 molar ratio. We compared the melting temperatures, widths of melting transition, and changes in hyperchromicity of complexes formed between $dA(pdA)_{11}$ and analogous boranophosphate, phosphorothioate, methylphosphonate, and natural phosphodiester $T(pT)_{11}$. At physiologically relevant buffer conditions, the $T(^{b}pT)_{11}$·$dA(pdA)_{11}$ complex exhibited a cooperative transition with T_m = 14 °C as estimated on the basis of the differential melting curve (Figure 2). By comparison, the closest analogues, phosphorothioate and methylphosphonate dodecamers, formed more stable complexes with $dA(pdA)_{11}$ at the same conditions (T_m = 28 and 32 °C, respectively), while the most stable complex with T_m = 47 °C was formed by natural dodecathymidylate. Under these conditions, the broadest melting transition among studied complexes was registered for the $T(^{m}pT)_{11}$·$dA(pdA)_{11}$ complex (Figure 2). The width of the melting transition for the boranophosphate complex fell between those of methylphosphonate and phosphorothioate complexes, being closer to the latter. Assuming that the change in enthalpy (ΔH) for the complex formation correlates with transition width,[32] the relative ΔH values would be methylphosphonate < boranophosphate < phosphorothioate < natural phosphodiester complex.

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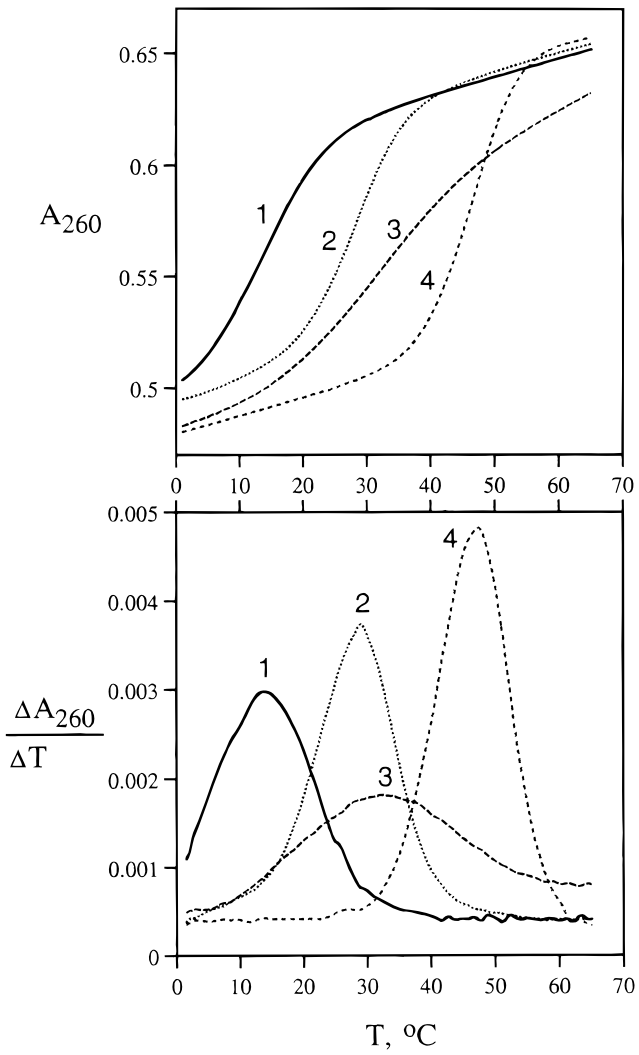

Figure 2. Thermal denaturation curves for the $dA(pdA)_{11}$ complexes with boranophosphate $T(^{b}pT)_{11}$ (**1**), phosphorothioate $T(^{s}pT)_{11}$ (**2**), methylphosphonate $T(^{m}pT)_{11}$ (**3**), and $T(pT)_{11}$ (**4**) at 1×10^{-4} M concentration in 150 mM KCl, 20 mM KH_2PO_4 (pH 7.0), 10 mM $MgCl_2$ buffer. The rate of the thermal ramping was 0.33 °C/min.

The observed hyperchromicity in the 1−65 °C interval was the highest for the unmodified $T(pT)_{11}$·$dA(pdA)_{11}$ complex ($\Delta A_{260} = 0.177$ or 36.8%), followed by the phosphorothioate complex ($\Delta A_{260} = 0.16$ or 32.3%), and the lowest for methylphosphonate and boranophosphate complexes ($\Delta A_{260} = 0.149$ (30.9%) and 0.148 (29.2%), respectively). Taking into account the possibility that the optical density increase was underestimated for $T(^{b}pT)_{11}$·$dA(pdA)_{11}$ complex melting [since the low-temperature baseline was not established (Figure 2)], one may conclude that the complementary complex formed with dodecathymidine boranophosphate reveals hyperchromicity comparable with its closest analogues, the methylphosphonate and phosphorothioate complexes.

When the ionic strength was increased, the $T(^{b}pT)_{11}$ dodecamer formed a more stable complex with $dA(pdA)_{11}$, as expected for negatively charged compounds. Increasing the KCl concentration from 0.15 to 1.0 M shifted the melting curve to higher temperatures ($T_m = 19$ °C). A similar shift ($\Delta T_m = 5$ °C) was observed for the $T(pT)_{11}$·$dA(pdA)_{11}$ complex upon 1 M KCl addition (data not shown).

Some Substrate Properties of BH_3^-−ODNs. The synthetic oligothymidine boranophosphates were reasonable substrates for T4 polynucleotide kinase. At standard conditions in the presence of [γ-^{33}P]ATP the oligothymidylate BH_3^-−ODNs were readily 5′-labeled, though less efficiently than their natural counterparts (Figure 3). The 12- and 8-meric oligothymidine boranophosphates were labeled at approximately 25−30% of the natural phosphodiesters, whereas the 4-mer was labeled at 10−15% of the natural tetrathymidylate. Individual isomers of the T^{b}pT dimer also were 5′-^{33}P-phosphorylated by T4 polynucleotide kinase, but with quite different efficiencies. Isomer II (having greater retention time on RP HPLC and tentatively assigned as R_p)[13] was labeled at 10% of the TpT, whereas isomer I (tentatively S_p) incorporated only 2−3% of radioactivity compared to TpT at the same conditions (Figure 3). IE HPLC was used as an alternative method to follow the progress of the phosphorylation, allowing direct detection of original and 5′-phosphorylated species. At 50 μM oligonucleotide, 500 μM ATP, and 0.5 U/μL enzyme it took 40 min to achieve 50% phosphorylation of $T(^{b}pT)_{11}$ and 180 min to

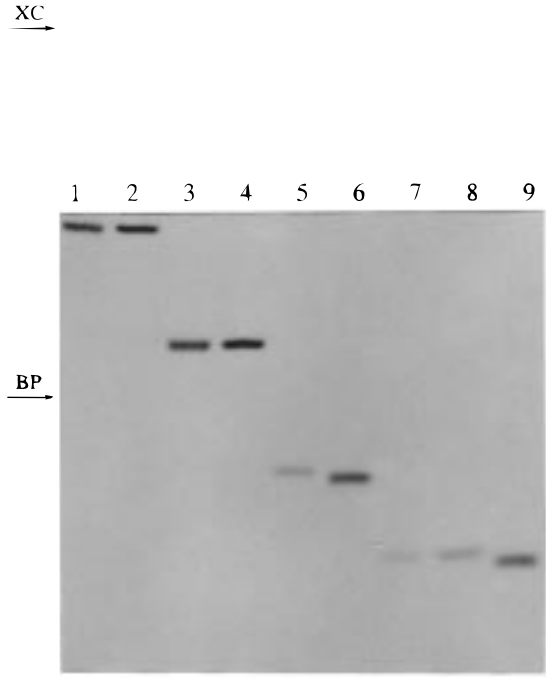

Figure 3. Denaturing (20%) PAGE analysis of 5′-phosphorylation of oligothymidine boranophosphates and their natural counterparts by T4 polynucleotide kinase and [γ-^{33}P]ATP: lanes 1 and 2, [5′-^{33}P]$T(^{b}pT)_{11}$ and [5′-^{33}P]$T(pT)_{11}$; lanes 3 and 4, [5′-^{33}P]$T(^{b}pT)_7$ and [5′-^{33}P]$T(pT)_7$; lanes 5 and 6, [5′-^{33}P]$T(^{b}pT)_3$ and [5′-^{33}P]$T(pT)_3$; lanes 7 and 8, [5′-^{33}P]T^{b}pT (I and II isomers); lane 9, [5′-^{33}P]TpT. BP and XC are Bromophenol Blue and Xylene cyanole FF markers.

Table 3. Comparative Stability of Dodecathymidylates $T(pT)_{11}$, $T(^{s}PT)_{11}$, and $T(^{b}PT)_{11}$ toward Nucleases and Phosphodiesterases[a]

enzyme concn, μg/mL	SVP		BSP		P_1 nuclease		S_1 nuclease	
	2	20	5	50	0.01	10	0.01	5
$T(pT)_{11}$	15 min	n/a[b]	20 min	n/a	<5 min	n/a	<5 min	n/a
$T(^{s}pT)_{11}$	n/a	2 h[c]	n/a	3 h	2 h	<5 min	40 min	<5 min
$T(^{b}pT)_{11}$	n/a	7 h[c]	n/a	1 h	stable	6 h	stable	70 h

[a] The table presents half-hydrolysis times calculated on the basis of the IE HPLC profiles. Presented half-hydrolysis times are the incubation times that are necessary to convert 50% of a full-length oligonucleotide to its lower molecular weight fragments upon integration of the corresponding HPLC peaks. A typical 100 μL reaction mixture containing 10 μM oligonucleotide was incubated at 37 °C for different periods of time; 20 μL aliquots were analyzed on a Polysil CA 10 μm 4 × 50 mm column with a 0.0−0.3 M KH_2PO_4 gradient in 30% CH_3CN at a flow rate of 1 mL/min. [b] Data not available. [c] Times that are necessary to reach half of the terminal hydrolysis, which corresponded to 42% for $T(^{s}pT)_{11}$ and 33% for $T(^{b}pT)_{11}$.

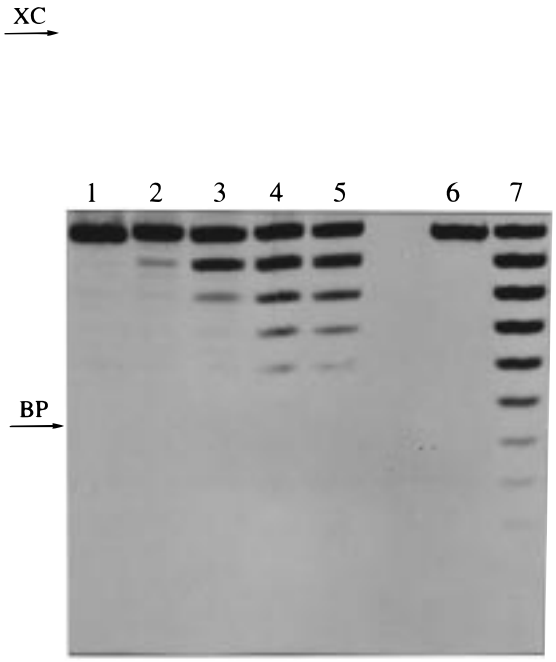

Figure 4. Denaturing (20%) PAGE analysis of the snake venom phosphodiesterase (SVP) hydrolysis of dodecathymidine boranophosphate (lanes 1−5) and its natural counterpart (lanes 6 and 7). Hydrolysis was carried out at 37 °C in 50 mM NaCl, 50 mM Tris−HCl (pH 9.0), 15 mM $MgCl_2$, 100 μg/mL bovine serum albumin, and 10 μM oligonucleotide. Key: lane 1, [5′-^{33}P]T(^{b}pT)$_{11}$ in the digestion buffer for 32 h; lanes 2−4, [5′-^{33}P]T(^{b}pT)$_{11}$ in the presence of 20 μg/mL SVP for 30 min, 7 h, and 24 h; lane 5, as in lane 4 plus addition of a fresh portion of the enzyme (total of 40 μg/mL SVP) and incubation for 8 h more; lane 6, [5′-^{33}P]T(pT)$_{11}$ in the digestion buffer for 30 min; lane 7, [5′-^{33}P]T(pT)$_{11}$ in the presence of 2 μg/mL SVP for 30 min.

achieve 85% phosphorylation. Under the same conditions, natural T(pT)$_{11}$ was 95% phosphorylated in 10 min.

The gel-purified 5′-^{33}P-labeled and original unlabeled boranophosphate dodecamers were tested as substrates for a number of exo- and endonucleases. For comparative purposes, the same experiments were done with natural and phosphorothioate counterparts. In general, the boranophosphate dodecamer was much more resistant to nuclease hydrolysis than T(pT)$_{11}$ and even superior to T(^{s}pT)$_{11}$ (Table 3). Specifically, with SVP, the boranophosphate analogue was 140 and 3.5 times more resistant than the natural and phosphorothioate counterparts. Notably, the [5′-^{33}P]T(^{b}pT)$_{11}$ dodecamer was not hydrolyzed completely by SVP, even after a long incubation period and addition of a fresh portion of enzyme (lane 5, Figure 4), while the natural dodecamer degraded substantially at lower enzyme concentration and shorter incubation time (lane 7). In the converse experiment with unlabeled T(^{b}pT)$_{11}$ and [5′-^{33}P]T(pT)$_{11}$, complete degradation of the natural dodecamer was observed (not shown). The distribution of products seen upon terminal digestion of [5′-^{33}P]T(^{b}pT)$_{11}$ (lane 5, Figure 4) indicated that only one stereoisomeric configuration of the boranophosphate bond was susceptible to SVP hydrolysis. It is well-known that S$^-$−ODNs also exhibit stereospecificity for SVP hydrolysis.[6a−c] The pattern obtained for T(^{s}pT)$_{11}$ hydrolysis was very similar to that for T(^{b}pT)$_{11}$ (IE HPLC data, not shown).

In contrast to SVP, BSP hydrolyzed the boranophosphate linkage of both configurations in the T^{b}pT dimers, as reported previously,[13a] as well as in the synthetic T(^{b}pT)$_{11}$ dodecamer. Moreover, the BH_3^-−ODNs were hydrolyzed most easily by BSP among the studied enzymes. Yet, the boranophosphate analogue was 30 times more resistant than the natural dodecamer, but less resistant than the phosphorothioate counterpart toward this enzyme (Table 3). At high enzyme concentration (100 μg/mL), complete hydrolysis of T(^{b}pT)$_{11}$ was achieved in 48 h.

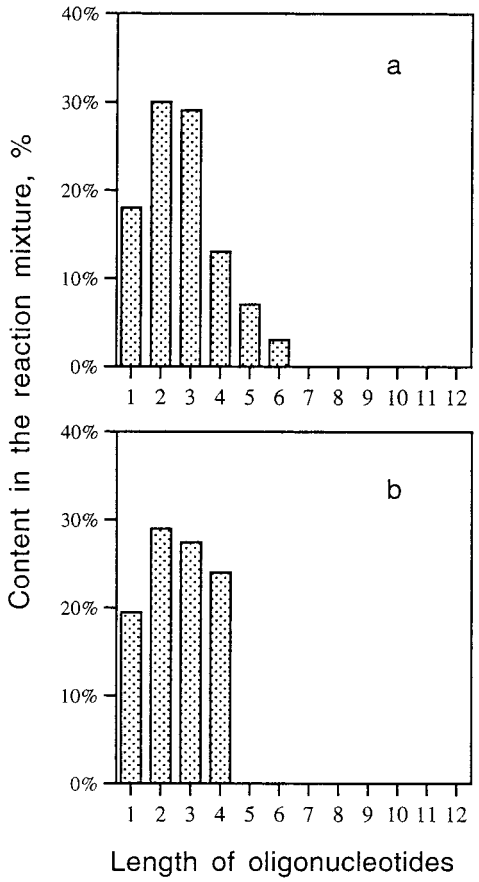

Figure 5. Final distribution of products after hydrolysis of (a) T(^{b}pT)$_{11}$ and (b) T(^{b}pT)$_3$ by P_1 nuclease. Reaction mixtures contained 10 μM oligonucleotide and 100 μg/mL (40.7 U/mL) enzyme in 30 mM sodium acetate (pH 5.3), 0.2 mM $ZnSO_4$ and were incubated at 37 °C for 144 h. In control samples where enzyme was not added, oligonucleotide degradation was <5%.

The BH_3^-−ODNs are highly resistant toward P_1 and S_1 nucleases (Table 3). T(^{b}pT)$_{11}$ was absolutely stable in the presence of 0.01 μg/mL P_1 or S_1 nuclease while the natural and phosphorothioate dodecamers were completely hydrolyzed within 10 min and 7 h, respectively. At very high enzyme concentrations and prolonged incubation periods, however, we were able to observe partial hydrolysis of boranophosphate dodecamer. With P_1 nuclease, complete disappearance of original dodecamer and formation of cleavage products were observed after 24 h with 10 μg/mL enzyme. Nevertheless, even with 100 μg/mL P_1 nuclease and after 144 h of incubation, some oligomeric hydrolysis products remained. The distribution of the cleavage products is shown in Figure 5, with the mono- to tetranucleotides being the main products. Interestingly, the treatment of T(^{b}pT)$_3$ with P_1 nuclease even at very harsh conditions (100 μg/mL enzyme, 144 h) did not result in complete hydrolysis of the original tetramer and produced a similar distribution of cleavage products (Figure 5).

Discussion

In previous studies, the synthesis of the dithymidine boranophosphate was carried out in solution using a modification of the conventional phosphoramidite method.[11] However,

several problems were encountered using that chemistry for solid-phase synthesis. The phosphoramidite methodology of oligonucleotide synthesis requires an oxidation (or boronation in our case) reaction in each chain elongation cycle that must be quantitative for efficient synthesis. Although powerful boronating agents, like dimethyl sulfide−borane, allow rapid and quantitative boronation, they also cause base damage.[11b,c,17a] Another problem arose from incompatibility of the boranophosphate triester group and the 4,4′-dimethoxytrityl (DMT) group. During the regular DMT-deprotection step a partial deboronation was observed.[11b,34]

***H*-Phosphonate Synthesis.** As an alternative, we investigated the *H*-phosphonate methodology of oligonucleotide synthesis, which is widely used for preparation of backbone-modified DNA analogues.[25] This method requires only a single boronation reaction after complete chain elongation, avoiding the problems associated with the removal of DMT groups and allowing milder boronating conditions. However, unlike the oxidation, thiolation, and amidation of an *H*-phosphonate diester (**1**, Scheme 2) that proceed directly to afford the desired P−O-, P−S-, and P−NH_2-modified ODNs, the boronation procedure required formation of an intermediate nucleophilic phosphite triester species (**2**). Silylation is an efficient method to afford such conversion.[27] All tested silylating agents, including chlorotrimethylsilane, provided smooth and relatively fast conversion of **1** to **2**. For automated synthesis the most reactive silylating agent, BSTFA, was chosen because it provided quantitative conversion in 20 min for all tested solid supports.

Until now, borane−dimethyl sulfide complex (BH_3·DMS) has been the only reagent reported for the borane exchange reaction with an internucleoside phosphite triester group.[11,17a] Whereas it provided fast and complete boronation, yielding boranophosphate triester, BH_3·DMS causes extensive base modification, especially for bases other than T[11b,c,17a] (our unpublished observations). We looked for a milder boronating agent that would be compatible with automated DNA synthesis and would have a diminished reactivity with bases. Different borane−amine complexes were tested as possible boronating agents. In contrast to the data published by Zhang et al.,[17a] we found that all tested complexes, including BH_3·Py, exchange a borane group onto an internucleoside phosphite triester **2**, yielding **3** (Scheme 2). A similar exchange reaction for trialkyl phosphite was reported earlier by Reetz.[26a] In general, a borane exchange reaction between two Lewis bases, such as amine and phosphite, leads to an equilibrium,[26] but the basicity, steric factors, solvent, etc.[35] can drive the equilibrium in favor of one product. Under the conditions we employed for the boronation of phosphite triester, either in solution or on solid supports, the reaction went to completion with all studied borane−amine complexes. ^{31}P NMR data showed the complete disappearance of phosphite triester **2** species with concomitant formation of boranophosphate triesters during the coarse of the reaction. Even the relatively inert complex, BH_3·Py, resulted in near quantitative boronation (see Table 1). However, this reagent required elevated temperature (50 °C) to accelerate the process, making it inconvenient for application in DNA synthesizers. *o*-Chloropyridine (CPy) formed a less stable complex with borane due to the electron-withdrawing and steric effects of the chlorine atom[18,36] and much more easily transferred the borane group than did BH_3·Py. This BH_3·CPy complex, in conjunction with a DNA synthesizer, ensured complete boronation of oligonucleotide backbones in 30 min at ambient temperature for all tested solid supports.

Among the tested solid supports, CPG provided the best yields for the BH_3^-−ODNs, while polystyrene resin yields were slightly lower (Table 1). The silica gel based solid support gave the poorest yields of desired products, possibly because of the presence of residual water trapped in the gel which affects the boronation procedure. The intermediate phosphite triester **2** species may be easily hydrolyzed[27] especially in the presence of a base, like the amine that is liberated during the borane exchange reaction. Hydrolysis of silyl esters results in *H*-phosphonate species **1** which do not participate in the exchange reaction. Subsequent ammonia treatment will hydrolyze any remaining *H*-phosphonate bonds, producing chain cleavage and diminishing the yield of the desired product. Thus, stringent anhydrous conditions are required for an efficient boronation procedure. It is known that silica gel can retain residual water even after washing with anhydrous solvents, especially after acid treatment.[37] If so, this may explain a drop in the yield during the boronation procedure on this support that would be independent of the length of the synthesized oligonucleotides. Table 1 data are consistent with the suggestion, giving preference for CPG and polystyrene resin as supports for BH_3^-−ODNs synthesis.

Conventional purification of phosphate-modified ODNs requires separation from truncated sequences and from any undesired oxygenated (i.e., partially natural phosphodiester) products, if present. Our proposed method for BH_3^-−ODN synthesis requires formation of an intermediate phosphite triester species that is easily oxidized.[27,38] Even though the boronation procedure was carried out under anaerobic as well as anhydrous conditions, minor amounts of phosphodiester species appeared in the crude reaction mixtures (≤1% for automated and 2−4% for manual syntheses of $T(^bpT)_{11}$ upon ^{31}P NMR analysis). To ensure that BH_3^-−ODNs were free from partially oxygenated species, purification and subsequent ^{31}P NMR analysis were applied.

Being negatively charged, the synthetic BH_3^-−ODNs were easily purified from truncated sequences by IE HPLC (Figure 1). This type of chromatography was also successfully used for the separation of all-S^-−ODNs from their analogues containing one or more phosphodiester bonds.[31b,c] Since the BH_3^-−ODNs are retained on strong ion-exchange matrix significantly longer than their phosphodiester counterparts, it was anticipated that IE HPLC should separate the fully substituted boranophosphate product from any molecules containing one or more natural phosphodiester linkages. In addition to IE, subsequent RP HPLC purification also facilitated separation of all-boronated ODNs from partially oxygenated analogues due to significant differences in the retention times between O^-−ODNs and BH_3^-−ODNs (Table 2). As a result, no signals other than that of boranophosphate (δ = 93.8 ppm) were detected in the ^{31}P NMR spectra of chromatographically purified oligothymidine boranophosphates (Supporting Information). Denaturing PAGE analysis confirmed the length homogeneity of the isolated BH_3^-−ODNs (Figure 3 and Supporting Informa-

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tion). Thus, HPLC purification is sufficient to ensure a length homogeneity and an absence of oxidized phosphate species.

Hybridization Properties. The first insight into hybridization properties of the BH_3^-−ODNs was made by Li et al.[13c] with a 14-meric oligonucleotide containing a single, enzymatically incorporated, boranophosphate linkage tentatively in the S_p configuration. They found a $\Delta T_m = -0.7$ °C between parent and modified DNA duplexes and concluded that this modification effected only a minor change in the stability of the complementary complex.[13c] In another study,[14,16] data for template-directed primer extension also suggested that the boranophosphate backbone modification did not significantly disturb hybridization ability. In the current study, however, a dodecathymidine boranophosphate synthesized as a diastereomeric mixture formed a relatively weak complex with complementary dodecadeoxyadenylate (Figure 2). There might be several reasons for this difference: (1) All phosphate-modified ODNs (such as methylphosphonates, phosphorothioates, phosphorodithioates, or phosphoramidates) perturb the structure of the DNA helix (and/or solvation environment) to some extent and form less stable complementary complexes compared to their natural counterparts.[39] The same effect is expected for boranophosphate analogues. Since the boranophosphate linkage is isomorphic to methylphosphonate yet bears a negative charge, the electrostatic repulsion between strands will add to the negative influence of steric distortion on complex stability. Electrostatic considerations imply that the boranophosphate analogues will probably form less stable complexes than methylphosphonates, which is what we observed. (2) The $(T)_n$·$d(A)_n$ family of duplexes, which would include the $T(pT)_{11}$·$dA(pdA)_{11}$ complex studied here, adopts a helical conformation slightly different from the canonical B-form.[40] We suggest that this atypical conformation might accentuate the steric hindrance of the phosphate substituents on the pyrimidine strand. In general, oligonucleotide homopolymer tracts having phosphate modifications on the pyrimidine strand are greatly destabilized compared to tracts having modifications on the purine strand or tracts with mixed sequences.[39c,41] For example, we observed a significant difference in melting temperature of complexes formed by natural dodecathymidylate and their phosphorothioate counterpart (ΔT_m = 19 °C, or 1.73 °C per linkage), in good agreement with previous data.[39c,41a,b] By contrast, for mixed-sequence S^-−ODNs much less duplex destabilization was observed.[39c,42] Further, abnormal melting characteristics of the duplex formed by a 19-meric oligo*thymidine* methylphosphonate and natural oligo*deoxyadenylate* also were reported.[41c] This duplex exhibited a broader melting transition and reduced melting temperature value compared to a 19-mer oligoA·oligoT duplex having a oligo*deoxyadenosine* methylphosphonate strand and a natural oligo*thymidylate* strand. It thus seems possible that in our case increased steric effects of the boranophosphate pyrimidine strand led to decreased complex stability. (3) The fact that synthetic oligothymidine boranophosphates were prepared as a mixture of stereoisomers must also be considered when trying to explain differences between this and previous[13c] complex stability data. For phosphate analogues, especially methylphosphonates, one stereoisomer typically disturbs complementary complexes less than the other.[43] One may assume that the enzymatically synthesized boranophosphate linkages[13c,14,16] are stereoregular and that the selected isomer provides greater stability to complementary complexes relative to the other isomer. This topic is the subject of our ongoing studies.

In the only other report about hybridization properties of a fully substituted oligonucleoside boranophosphate,[17a] Zhang et al. failed to observe hybridization of their 15-meric oligothymidine boranophosphate with complementary dA_{15} (or A_{15}) strands. By comparison, we observed hybridization with a shorter (12-mer) boranophosphate analogue. Our finding is not directly contradicted by the results of Zhang et al. since the melting conditions and oligomers were different; however, our conclusion disagrees with theirs. At physiologically relevant buffer conditions, even a 12-meric oligothymidine boranophosphate can hybridize with complementary oligo(dA). There are solid grounds for believing that longer (about 17−20-meric) mixed-sequence BH_3^-−ODNs should form sufficiently stable complementary complexes to target nucleic acids at 37 °C. Thus, the pessimistic forecast given for BH_3^-−ODNs in the above-mentioned article[17a] based on the assumption that the "more stable complex provides greater effect" seems to be a misleading generalization.

Hybrid Character of Boranophosphates (as Enzyme Substrates). As demonstrated *vide supra*, the BH_3^-−ODNs display properties of neutral methylphosphonate and charged phosphorothioate and phosphodiester ODNs to varying extents. This hybrid character is further revealed in its interactions with enzymes. Oligothymidine boranophosphates, unlike methylphosphonates, are able to be 5′-phosphorylated by T4 polynucleotide kinase. Postsynthetic phosphorylation provides a convenient method for labeling BH_3^-−ODNs and may allow subsequent derivatization of the 5′-phosphate. Phosphorylation of BH_3^-−ODNs supports speculations that a negative charge is an important factor for the kinase activity, perhaps contributing to the binding affinity of a substrate. Both stereoisomers of T^bpT are substrates for T4 polynucleotide kinase; however, the later eluting isomer (II isomer) was 5′-phosphorylated significantly faster.

Enhanced resistance of the boranophosphate bond toward nuclease hydrolysis has been reported previously.[13,14] However, in those studies the substrates contained only a single boranophosphate diester bond, or a boranophosphate dimer was used as substrate. Here we extended the work to cover oligonucleotides comprised of all boranophosphate bonds. Our findings proved consistent with the previous data (Table 3). Boranophosphate dodecathymidylate was more than 2 orders of magnitude more resistant toward SVP (phosphodiesterase I) than the natural congener. Ultimately, only 34% of full-length product was hydrolyzed by SVP regardless of the incubation period and enzyme concentration (Figure 4). The hydrolytic patterns are typical for hydrolysis of only one stereoisomer of

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boranophosphate linkage, and are in good agreement with previously reported data[13a] showing that only one stereoisomer (presumably S_p) of T^{b}pT was a substrate for SVP. Analogously, only the R_p stereoisomer of phosphorothioate linkage can be hydrolyzed by SVP,[6a−c] giving essentially the same pattern we observed for SVP hydrolysis of both T(^{b}pT)$_{11}$ and T(^{s}pT)$_{11}$.

BSP (phosphodiesterase II) was the single enzyme among those tested that hydrolyzed both stereoisomers of the internucleoside boranophosphate linkage at an appreciable rate. Yet T(^{b}pT)$_{11}$ was hydrolyzed 30 times slower than the natural dodecamer. Previously reported data for the T^{b}pT dimer showed a 30-fold decrease in rate for one isomer and an 80-fold decrease for the other isomer, compared to TpT.[13a] BSP might be useful for base composition analysis of synthetic BH_3^-−ODNs. The T(^{b}pT)$_{11}$ dodecamer was completely hydrolyzed to nucleotide and nucleoside monomers within 48 h at 37 °C by 100 μg/mL BSP. In contrast, oligonucleoside methylphosphonates are completely resistant toward phosphodiesterase II hydrolysis.[44]

BH_3^-−ODNs appear to be much more resistant than O^-− and S^-−ODNs toward P_1 and S_1 nucleases (Table 3). This stability correlated with data where neither isomer of the dithymidine boranophosphate was hydrolyzed at all by either nuclease.[13a] The hydrolysis of T(^{b}pT)$_{11}$ might yet be achieved at very high enzyme concentration and long incubation time (Table 3, Figure 5). These findings do not contradict each other since it was shown for nuclease S_1 that the rate of hydrolysis significantly increased with the length of the oligonucleotide.[45] The trimer TpTpT, for example, was hydrolyzed 18 times faster than TpT. The extremely low T(^{b}pT)$_{11}$ hydrolysis rate by P_1 and S_1 nucleases is consistent with the boranophosphate dimer being inert even under harsh conditions.[13a] The hydrolytic behavior of tetrathymidine boranophosphate when treated with 100 μg/mL P_1 nuclease at 37 °C lends favor to a length-dependent rate of hydrolysis (Figure 5). After 6 days of incubation we observed only 19% of the monomer and a roughly equal distribution (24−29%) of di-, tri-, and tetramers. Only slight ($\leq$5%) hydrolysis was observed in the absence of the enzyme. This extreme resistance of BH_3^-−ODNs to P_1 and S_1 nucleases precludes us from drawing any conclusions about stereoselectivity of boranophosphate linkage hydrolysis by these enzymes.

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In conclusion, the *H*-phosphonate approach for BH_3^-−ODNs synthesis allows a single borane transfer step to be carried out after the desired sequence has been assembled. This method proved to be a successful alternative to the previously used phosphoramidite approach. Silylation and subsequent boronation with a borane−amine complex ensure the mild conversion of an *H*-phosphonate internucleoside bond to a boranophosphate, with an efficiency comparable to that of O^-−ODN synthesis through iodine oxidation. The synthetic oligothymidine boranophosphates were purified by techniques regularly used for O^-−ODNs, e.g., IE and/or RP HPLC and PAGE. The dodecathymidine boranophosphate hybridized with the complementary dodecadeoxyadenylate and the resulting complex melted in a cooperative manner, although its stability was less than that of complexes formed by methylphosphonate and phosphorothioate counterparts. The oligothymidine boranophosphates, unlike neutral methylphosphonates, are reasonable substrates for T4 polynucleotide kinase, allowing for their 5′-labeling. Further, the borane modification of the phosphate group results in considerably enhanced stability toward nucleases. The BH_3^-−ODNs, like S^-−ODNs, are hydrolyzed by snake venom phosphodiesterase in a stereoselective manner but are much more stable toward P_1 and S_1 nuclease hydrolysis than their natural and phosphorothioate counterparts.

Acknowledgment. This work was supported by Grants NIH 5U01-CA60139 and DOE DE-FG05-94ER61882 to B.R.S. We thank Dr. A. Ribeiro (NMR Center, Duke University) for assistance in recording proton and boron NMR spectra, Drs. T. Oas, C. Fierke, and M. Been for use of the Aviv Model 14DS spectrophotometer, and Drs. J. S. Summers and V. K. Rait for particularly helpful comments and discussion on the manuscript. We further thank Dr. Rait for insightful observations on the chemistry of boranophosphates, thereby facilitating the purification, analysis, and melting experiments.

Supporting Information Available: ^{31}P NMR analysis of the boronation reaction of dithymidine *H*-phosphonate tethered to polystyrene resin, electrospray ionization mass spectrometry data for T(^{b}pT)$_{11}$, ^{31}P NMR spectrum of T(^{b}pT)$_{11}$, and 20% denaturing PAGE analysis of tetra-, octa-, and dodecathymidine boranophosphates and their natural counterparts (staining with Stains-all) (7 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

JA9814927