Hydrolysis of Thymidine Boranomonophosphate and Stepwise Deuterium Substitution of the Borane Hydrogens. ^{31}P and ^{11}B NMR Studies†

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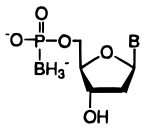
Abstract: The α-*P*-boranophosphate nucleosides comprise a new class of modified nucleotides that may find use as therapeutic and DNA diagnostic agents. Hydrolysis of thymidine 5′-boranomonophosphate, d(p^{B}T), has been studied in H_2O and D_2O using ^1H, ^{31}P, and ^{11}B NMR spectroscopies. Although d(p^{B}T) is quite stable at 25 °C, it hydrolyzes slowly at higher temperatures. At 50 or 60 °C, d(p^{B}T) hydrolyzes first into thymidine (dT) and boranophosphate ($O_3P-BH_3^{3-}$), followed by subsequent hydrolysis of the $O_3P-BH_3^{3-}$ to produce phosphonate and boric acid. A three-step deuterium substitution of the borane hydrogens in $O_3P-BH_3^{3-}$ was detected in D_2O by the presence of a ^{31}P isotope shift. The ^{31}P resonances shifted downfield by 0.14 ppm upon substitution of each of three ^1H atoms by ^2H. Exchange of the borane hydrogens with D_2O occurs as sequential processes superimposed upon hydrolysis of $O_3P-BH_3^{3-}$. The hydrolysis and deuteration steps were characterized in terms of pseudo-first-order rate constants. Hydrolysis of $O_3P-BH_3^{3-}$ is an order of magnitude slower than deuterium substitution.

Introduction

A series of α-*P*-boranomono- (**1**) or triphosphate (**2**) nucleosides designed for use as potential therapeutic and DNA diagnostic agents have been synthesized.[1−3] These compounds



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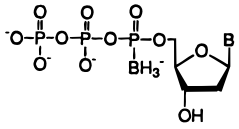
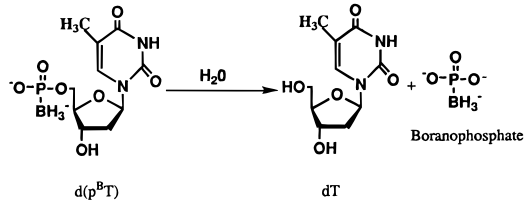


2

B = nucleobase

are distinctive in that one of the nonbridging oxygens in the phosphate diester is replaced by a $-BH_3$ borane moiety. Nucleoside 5′-α-*P*-boranotriphosphates **2** can substitute for normal dNTP and be successfully incorporated into DNA by DNA polymerase,[2,4,5] yet once in DNA, the boranophosphate linkage is more resistant to *exo* and *endo* nucleases than normal phosphate diesters.[3,6−9a]

Scheme 1. Phosphate Ester Hydrolysis Reaction of the d(p^{B}T) Nucleotide in H_2O



The hydrolytic stability of thymidine 5′-boranomonophosphate, d(p^{B}T), has been studied in solution under various pH and temperature conditions using reverse-phase HPLC.[9] The HPLC studies indicated that d(p^{B}T) undergoes slow hydrolysis in aqueous solution to produce thymidine (dT) (Scheme 1) with a rate constant of 10^{-5} s^{-1} at pH 7.4, 37 °C, analogous to the hydrolysis pathway of unmodified thymidine 5′-monophosphate, d(pT)[9]. Notably, hydrolysis cleaves the P−O−sugar linkage and not the P−B bond[9] (see Scheme 1).

Although HPLC methods were used to detect dT as one of the products, conventional HPLC methods cannot detect the other hydrolysis product, which is presumably boranophosphate ($O_3P-BH_3^{3-}$),[10] because this fragment does not absorb in the accessible UV range. Questions arise as to (a) whether $O_3P-BH_3^{3-}$ indeed forms as a direct product of d(p^{B}T) hydrolysis, (b) whether $O_3P-BH_3^{3-}$ undergoes further hydrolysis, and if

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† Abbreviations: dT; thymidine; d(pT), thymidine 5′-monophosphate; d(p^{B}T), thymidine 5′-boranomonophosphate; d(Tp^{B}T), dithymidine boranomonophosphate; EDTA, ethylenediaminetetraacetic acid; Et_2OBF_3, diethyl ether−boron trifluoride; HPLC, high-performance liquid chromatography; k_1, observed pseudo-first-order rate constant; NMR, nuclear magnetic resonance spectroscopy; ppm, parts per million; γ, magnetogyric ratio; TSP, 3-(trimethylsilyl)propionate-*2,2,3,3-d*$_4$ sodium salt.

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so, what the identities of the hydrolysis products are, and (c) whether stepwise hydrolysis and/or exchange of borane hydrogens with solvent occurs. These questions are important for assessing the toxicity of boranophosphate-containing nucleotides as therapeutic agents, as well as for enhancing our understanding of the chemistry of boron-containing phosphorus compounds. In order to address the fate of these B−P bond-containing compounds, we have employed ^{1}H, ^{11}B, and ^{31}P NMR to study the d(p^{B}T) hydrolysis. We describe new aspects of d(p^{B}T) hydrolysis, identify $O_3P-BH_3^{3-}$ as the main hydrolysis product, and document solvent exchange of the borane hydrogens.

Materials and Methods

Sample Preparation. Lyophilized d(p^{B}T) powder in ammonium form was graciously provided by Dr. J. Tomasz.[2] NMR samples, which typically contained about 85 mM d(p^{B}T), were made by dissolving the lyophilized d(p^{B}T) powder directly into H_2O or D_2O. The concentration of d(p^{B}T) was estimated from the UV absorbance at 260 nm using the molar extinction coefficient of unmodified thymidine monophosphate d(pT).[11] All samples were analyzed in 5 mm NMR tubes (Wilmad).

Since all samples were prepared in nonbuffered H_2O or D_2O solvents, a gradual decrease in pH was observed as hydrolysis proceeded. Initially, the pH was about 6; by the final stages of hydrolysis it had decreased to about 5. This pH change may affect the rate constants[12a] and chemical shifts[12b] to a limited extent.

Hydrolysis and Solvent Exchange Reactions. Hydrolysis of d(p^{B}T) was studied in H_2O and D_2O by examining ^{1}H, ^{31}P, and ^{11}B NMR spectra at different incubation times. Hydrolysis and deuterium substitution rates were determined by measuring changes in the heights of the respective NMR peaks with time. Intensity changes measured as a function of time were fit to a pseudo-first-order reaction model. In general, the correlation coefficients (R^2) were better than 0.92. The beginning of each time-dependent acquisition is referred to as $t = 0$. True zero times were not available in this kind of experiment due to the elapsed time required for sample preparation and proper setup. However, calculation of a pseudo-first-order rate constant from reactant concentration changes does not require knowledge of the exact reaction initial time.

NMR Experiments. All ^{1}H, ^{31}P, and ^{11}B NMR experiments were performed on a Varian Unity-500 NMR spectrometer at the Duke NMR Center. Spectra were acquired in the X-nucleus channel of a 5 mm reverse-detect probe which was tuned for either ^{31}P or ^{11}B. D_2O was used as the lock signal. For experiments performed in H_2O, the D_2O was enclosed in a special NMR insert tube (Wilmad) to avoid unwanted deuterium exchange between the sample and solvent. ^{31}P and ^{11}B NMR spectra were recorded using enough transients to provide satisfactory signal-to-noise ratios. NMR spectra were processed and plotted using a Sun SPARC 2 computer with Varian NMR software. NMR peak intensities represent time-averaged values for a given acquisition period, which was much less than the total reaction time. Since the line widths did not change appreciably with time, NMR peak integrals could be determined using signal heights.

^{31}P NMR spectra were acquired at 202 MHz using 32k data points and a sweep width of 33 113 Hz. ^{31}P chemical shifts were referenced externally to a solution of 85% H_3PO_4. The ^{31}P spectra of d(p^{B}T) and $O_3P-BH_3^{3-}$ are complicated due to direct bonding of phosphorus to the boron atom. Naturally abundant boron consists of 80.4% ^{11}B (spin $I = 3/2$) and 19.6% ^{10}B (spin $I = 3$). When a ^{31}P ($I = 1/2$) atom is bonded to a single ^{11}B, as in the $-P-BH_3^{3-}$ linkage, four equally-spaced and equal-intensity lines (a quartet with a 1−1−1−1 pattern) are expected in the ^{31}P spectrum. If a ^{31}P is coupled to a ^{10}B atom, seven equally-spaced and equal-intensity lines (a septet with a 1−1−1−1−1−1−1 pattern) are expected. Thus, a P−B bond-containing sample with naturally abundant boron could be expected to show a complicated ^{31}P spectrum. The ^{31}P−^{10}B coupling constant is about one-third that of the ^{31}P−^{11}B coupling constant due to the difference in magnetogyric ratios ($\gamma(^{11}B)/\gamma(^{10}B) = 2.99$).[13a] The intensity of an individual ^{31}P peak coupled to a ^{10}B is hence about 0.14 times the intensity of a ^{31}P peak coupled to a ^{11}B in a naturally abundant boron compound.[13b] Thus, underlying the quartet due to ^{31}P coupling with ^{11}B in the ^{31}P spectrum is a septet due to ^{31}P coupling with ^{10}B at similar chemical shifts. Experimentally, the ^{31}P spectra usually appear as if the boron effects derive only from ^{11}B scalar coupling.[14] However, the intensities of the two middle peaks are often enhanced relative to the intensities of the two outside peaks. In this study, we consider only the ^{11}B coupling and neglect the ^{10}B coupling in the ^{31}P spectra.

^{1}H NMR spectra were collected at 499.843 MHz with a sweep width of 5498.3 Hz and 16k data points. ^{1}H chemical shifts were measured relative to TSP (3-(trimethylsilyl)propionate-*2,2,3,3-d*$_4$ sodium salt) as internal reference.

^{11}B NMR spectra were acquired at 160 MHz using 16k data points and a sweep width of 22 447 Hz. ^{11}B chemical shifts were referenced externally to a solution of diethylether−boron trifluoride, Et_2OBF_3. Although ^{11}B is a quadrupolar nucleus, line-broadening is not severe for the molecules studied. Boron in the glass NMR tube and the NMR probe produce a broad background ^{11}B signal, which did not obstruct our observations.

Results

^{1}H, ^{31}P, and ^{11}B Spectra of d(p^{B}T). The starting material used for these studies was the mononucleotide thymidine 5′-boranomonophosphate, d(p^{B}T). The 1.5−7 ppm range of the ^{1}H spectrum (Figure 1A) shows a peak pattern very similar to that of normal thymidine 5′-monophosphate, d(pT),[15] corresponding to the protons on the sugar and base of d(p^{B}T). An eight-line pattern centered at about 0.33 ppm corresponds to a 1−1−1−1 quartet (Figure 1A,B) from borane hydrogens which couple with ^{11}B and are further split by ^{31}P to produce a doublet in each peak of the quartet (Figure 1B). Couplings to both ^{11}B and ^{31}P were verified by ^{11}B- and ^{31}P-decoupling experiments, respectively (data not shown). The ^{11}B-decoupled spectrum shows only a doublet due to ^{31}P coupling with borane hydrogens; the ^{31}P-decoupled spectrum shows a 1−1−1−1 pattern due to ^{11}B coupling with borane hydrogens.

The ^{31}P NMR spectrum of d(p^{B}T) at 25 °C is a 1−1−1−1 quartet centered at about 85 ppm due to ^{11}B coupling (Figure 1C). Further splitting of the ^{31}P resonance by borane hydrogens was not resolved under these conditions.

(10) The actual ionization states of thymidine boranomonophosphate, boranophosphate, phosphonate (or phosphite), and boric acid in solution are both pH- and ionic strength-dependent. For convenience, we show thymidine boranomonophosphate, boranophosphate, and phosphonate in their fully deprotonated forms, and boric acid in its fully protonated form. This probably does not reflect the actual protonation states of these molecules at pH 5−6 (*vide infra*). The state of protonation is not the subject of the present paper.

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(12) (a) The rate−pH profile of the thymidine boranomonophosphate hydrolysis in ref 9 indicated that the rate constants change by less than 25% over the pH range from 3 to 6.5. (b) Small but observable changes in ^{31}P chemical shifts were observed during hydrolysis of d(p^{B}T) mononucleotide to dT and hydrolysis of $O_3P-BH_3^{3-}$ in H_2O or D_2O, in addition to intensity changes. The ^{31}P chemical shifts of both the reactant d(p^{B}T) and product boranophosphate decreased with time (e.g., by −0.15 and −0.11 ppm, respectively, in H_2O at 50 °C in 35 min). During subsequent hydrolysis of boranophosphate, the ^{31}P chemical shifts of non-deuterated and partially and fully deuterated species I−IV increased with time in both D_2O and H_2O (e.g., by +0.06 ppm for intermediates II−IV in 3 h at 60 °C), while the ^{11}B chemical shift decreased (e.g., by −0.06 ppm in 9 h at 60 °C). The reason for the small observed ^{31}P chemical shift changes is not apparent. These changes could be due to a pH change as a result of hydrolysis, chemical exchange among different species, or an equilibrium isotope effect.

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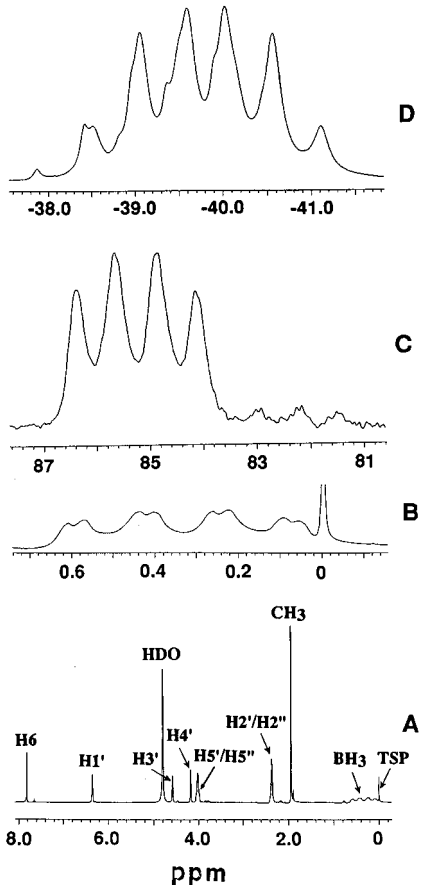


Figure 1. ^{1}H, ^{31}P, and ^{11}B NMR spectra of d(p^{B}T) at 25 °C: (A) ^{1}H spectrum, 512 scans at 10 Hz line-broadening; (B) expanded region from panel A for the $-$P$-$BH$_3$ linkage; (C) ^{31}P spectrum, 1024 scans at 10 Hz line-broadening; peaks in the 81−83 ppm range are due to the hydrolysis product of d(p^{B}T); (D) ^{11}B spectrum, 512 scans at 10 Hz line-broadening.

In the ^{11}B NMR spectrum of d(p^{B}T) at 25 °C (Figure 1D), two partially overlapped 1−3−3−1 patterns centered at about −40.1 ppm are seen due to coupling with both the single ^{31}P and three equivalent hydrogens in the $-$P$-$BH$_3$ linkage. Upon ^{1}H decoupling of the ^{11}B spectrum, a doublet was observed at this chemical shift (data not shown), confirming the ^{11}B assignments in the $-$P$-$BH$_3$ linkage.

Phosphate Ester Hydrolysis of d(p^{B}T) in H$_2$O. Hydrolysis of d(p^{B}T) in H$_2$O was studied by ^{1}H, ^{31}P, and ^{11}B NMR at higher temperatures in order to accelerate the reaction for suitable NMR observation. When d(p^{B}T) was incubated at 50 °C and the ^{1}H-decoupled ^{31}P spectrum was monitored with time, a new 1−1−1−1 quartet (centered at *ca.* 83.5 ppm) was seen upfield from the quartet of the starting d(p^{B}T) material (centered at 87.1 ppm) (Figure 2). The intensity of the new upfield quartet increased with time as the intensity of the d(p^{B}T) quartet decreased. This is consistent with previous HPLC experiments, which indicated that the d(p^{B}T) mononucleotide undergoes slow phosphate ester hydrolysis.[9] The decomposition products are proposed to be dT and O$_3$P$-$BH$_3^{3-}$ as shown in Scheme 1. The upfield quartet centered at 83.5 ppm indicates that the hydrolysis product retains the P$-$B bond, since the chemical shift is nearly equal to that of the d(p^{B}T) quartet (only a 3.6 ppm difference) and both peaks had the same splitting pattern.

Additional evidence that hydrolysis proceeds as shown in Scheme 1 comes from an examination of the spectra of the hydrolysis products. The ^{11}B spectrum of O$_3$P$-$BH$_3^{3-}$ in H$_2$O shows a doublet of quartets with 1−3−3−1 patterns (centered at *ca.* 39.3 ppm at 25 °C). (The 25 °C spectrum is not shown, but is similar to the 50 °C spectrum shown in Figure 4). The ^{11}B pattern can be attributed to ^{11}B coupling with a single ^{31}P and three equivalent hydrogens, indicating the presence of a $-$P$-$BH$_3$ linkage in the hydrolysis product.

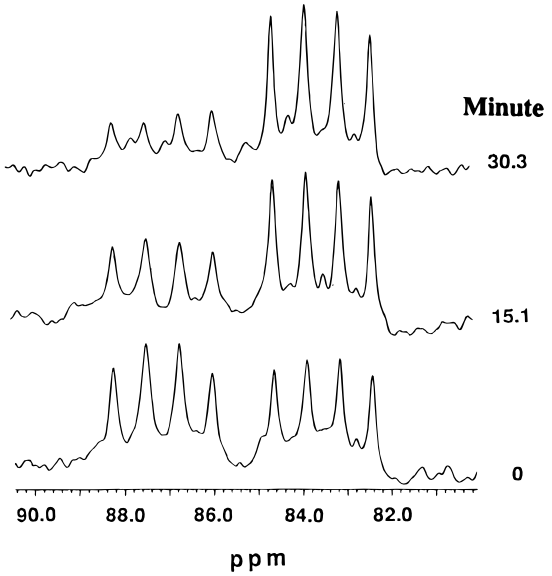


Figure 2. ^{1}H-decoupled ^{31}P spectra during d(p^{B}T) hydrolysis in H$_2$O at 50 °C. The downfield quartet centered at *ca.* 87.1 ppm corresponds to d(p^{B}T) resonance; the upfield quartet centered at *ca.* 83.5 ppm is from O$_3$P$-$BH$_3^{3-}$ resonance. A total of 52 scans were acquired for each spectrum in about 1.5 min, and a line-broadening of 30 Hz was applied. The exact initial time is not available but refers to the time of the first measurement of the set of data (see the Materials and Methods).

Formation of dT, as the other d(p^{B}T) hydrolysis product, was also detected by following the ^{1}H NMR spectrum. As expected, the integrals of the sugar and base ^{1}H reasonances of dT increased with time while those of d(p^{B}T) decreased (data not shown).

The intensity changes of the ^{31}P peaks with time, demonstrated in Figure 2, were fit to a pseudo-first-order exponential decay rate equation with respect to d(p^{B}T) concentration. The calculated hydrolysis rate constant k_1 determined at 50 °C with 21 data points acquired over 30 min is 4×10^{-4} s^{-1} (R^2 = 0.98; data not shown).

Since phosphate ester hydrolysis of d(p^{B}T) to produce dT and O$_3$P$-$BH$_3^{3-}$ (Scheme 1) has been studied under various conditions using quantitative HPLC methods, those results will be reported elsewhere.[9c] Our emphasis in this work is to describe further reactions of O$_3$P$-$BH$_3^{3-}$ in aqueous solution.

O$_3$P$-$BH$_3^{3-}$ Hydrolysis in H$_2$O. The same solutions described above, which had undergone nearly complete phosphate ester hydrolysis of d(p^{B}T) mononucleotide (lacking the 87 ppm quartet in the ^{31}P spectrum), were used to investigate further hydrolysis of O$_3$P$-$BH$_3^{3-}$. Upon monitoring the ^{1}H-decoupled ^{31}P spectrum in H$_2$O at 50 °C, a singlet was seen at *ca.* 3.6 ppm whose intensity increased with time while that of the O$_3$P$-$BH$_3^{3-}$ quartet centered at about 83.5 ppm decreased (Figure 3). For the same solution, the *intensity* of the O$_3$P$-$BH$_3^{3-}$ ^{11}B spectrum (having two sets of quartets with a 1−3−3−1 pattern centered at *ca.* −38.8 ppm, Figure 4) decreased with time. Concomitantly, a singlet appeared at about 19.5 ppm which increased in intensity. However, neither the ^{31}P nor ^{11}B spectral *patterns* changed over time in H$_2$O. Such spectral behaviors are consistent with the proposed hydrolysis reaction of O$_3$P$-$BH$_3^{3-}$ as depicted in Scheme 2, where the P$-$B bond is broken accompanied by formation of phosphonate (corresponding to a singlet at 3.6 ppm in the ^{1}H-decoupled ^{31}P

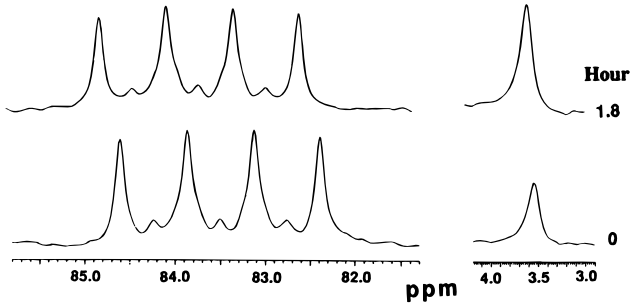


Figure 3. ^{1}H-decoupled ^{31}P spectra during $O_3P-BH_3^{3-}$ hydrolysis in H_2O at 50 °C. The major quartet centered at *ca.* 83.5 ppm is the $O_3P-{}^{11}BH_3^{3-}$ resonance, the singlet at about 3.6 ppm is the phosphonate resonance, and the three minor peaks centered at *ca.* 83.5 ppm are due to the resonance of the ^{10}B-form of $O_3P-BH_3^{3-}$. A total of 175 scans were acquired in each spectrum in about 5 min, and a line-broadening of 20 Hz was applied (see Figure 2 for $t = 0$).

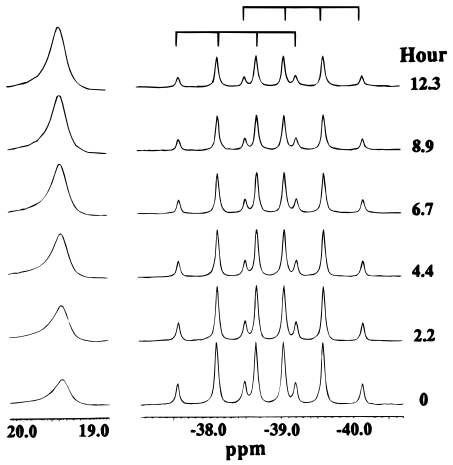
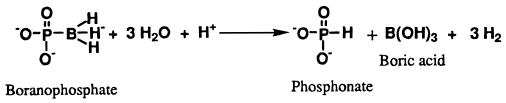


Figure 4. ^{11}B spectra during $O_3P-BH_3^{3-}$ hydrolysis in H_2O at 50 °C. The peaks centered at −38.8 ppm are from the $O_3P-BH_3^{3-}$ resonance, and the singlet at about 19.5 ppm is from the resonance of boric acid. A total of 136 scans were collected in each spectrum in about 5 min, and a line-broadening of 5 Hz was applied (see Figure 2 for $t = 0$).

Scheme 2. Hydrolysis of $O_3P-BH_3^{3-}$ in H_2O



spectrum, Figure 3) and boric acid (a singlet at *ca.* 19.5 ppm in the ^{11}B spectrum, Figure 4, close to the literature value of 18.8 (±1.0) ppm at 25 °C[14,16]). When the ^{31}P spectrum was collected without ^{1}H decoupling, a doublet was observed at *ca.* 3.6 ppm with a coupling constant of 618 Hz instead of a singlet (data not shown), confirming that a P−H bond exists in the phosphonate, as expected for the $O_3P-BH_3^{3-}$ hydrolysis product.

After most of the d(p^BT) had hydrolyzed (as in Scheme 1), changes in $O_3P-BH_3^{3-}$ peak intensities observed by ^{1}H-decoupled ^{31}P and ^{11}B NMR experiments gave reasonable pseudo-first-order kinetics fits. Equal hydrolysis rate constants of 2×10^{-5} s^{-1} ($R^2 = 1.00$) were calculated from ^{31}P and ^{11}B NMR data at 50 °C (16 and 31 points, respectively; data not shown). There is a 20-fold difference in the rates between d(p^BT) and $O_3P-BH_3^{3-}$ hydrolysis (Schemes 1 and 2).

$O_3P-BH_3^{3-}$ Hydrolysis and Solvent Exchange of Borane Hydrogens in D_2O. To further understand the chemical reactions of $O_3P-BH_3^{3-}$ in aqueous solution, and to elucidate

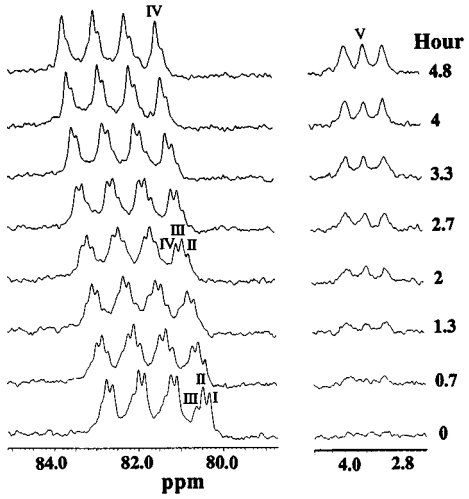


Figure 5. ^{1}H-decoupled ^{31}P spectra during $O_3P-BH_3^{3-}$ hydrolysis in D_2O at 60 °C. The multiple quartets centered at about 81.7 ppm are from boranophosphate resonances, and the triplet centered at about 3.4 ppm is from deuterated phosphonate. Subquartets I−IV correspond to the ^{31}P resonances of non-deuterated, partially deuterated, and fully deuterated boranophosphate populations $O_3P-BH_3^{3-}$, $O_3P-BH_2D^{3-}$, $O_3P-BHD_2^{3-}$, and $O_3P-BD_3^{3-}$, respectively, in Scheme 3. A total of 344 scans were collected in each spectrum in about 10 min, and a line-broadening of 10 Hz was applied (see Figure 2 for $t = 0$).

the hydrolysis mechanism, we determined whether the borane hydrogens exchanged with solvent. When the time course of $O_3P-BH_3^{3-}$ incubation in D_2O at 50 or 60 °C was followed by ^{1}H-decoupled ^{31}P NMR, the peak intensity of $O_3P-BH_3^{3-}$ (centered at *ca.* 81.7 ppm; Figure 5) decreased, accompanied by an increase in peak intensity of phosphonate (at *ca.* 3.4 ppm; Figure 5). This behavior was similar to that in H_2O solvent (Figures 3 and 4); however, the peak patterns differed. Specifically, the $O_3P-BH_3^{3-}$ quartets (centered at about 81.7 ppm) measured in D_2O at 60 °C (Figure 5) consisted of two or more subquartets (labeled I, II, III, and IV) each having a 1−1−1−1 pattern. The total intensity of the quartets decreased with time; simultaneously the relative intensities within each of the subquartets changed. The results in Figure 5 show that the intensity of subquartet II increases and the intensity of subquartet I decreases with time in the early phase of the reaction. Then, subquartet III starts to appear, and its intensity increases with time. At the same time, subquartet I starts to disappear and the intensity of subquartet II decreases. As the reaction proceeds, subquartet IV starts to appear while the intensity of subquartet III decreases and subquartet II disappears. Finally, only quartet IV exists, and its intensity decreases further with time (data not shown). Intensity changes of subquartets I−IV are plotted in Figure 6; the relative ^{31}P NMR intensity changes of subquartets I, II, III, and IV increase through a maximum, and then decay. This behavior is characteristic of a reaction consisting of four sequential pseudo-first-order steps.

The ^{31}P subquartet II is 0.14 ppm downfield from subquartet I. Downfield shifts of this magnitude were also observed for subquartet III relative to subquartet II, and for subquartet IV relative to subquartet III. The shift values are independent of reaction time and insensitive to temperature changes in the 25−60 °C range (data not shown). Since subquartets I−IV are 1−1−1−1 quartets and have chemical shifts very similar to those of $O_3P-BH_3^{3-}$ (quartet I), subquartets II, III, and IV must correspond to species which all retain P−B bonds (as proposed in Scheme 3).

In the same ^{1}H-decoupled ^{31}P spectra (Figure 5), a triplet with a 1−1−1 pattern (peak V) centered at about 3.4 ppm in

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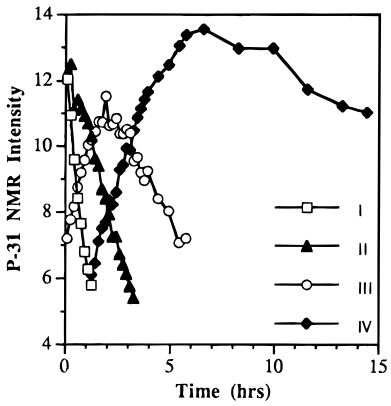
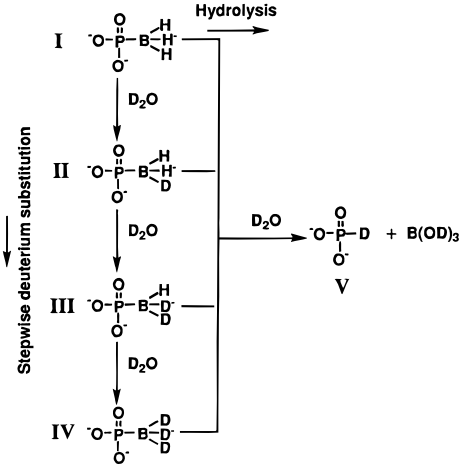


Figure 6. Time dependence of ^{31}P NMR intensities for species I−IV during $O_3P-BH_3^{3-}$ hydrolysis in D_2O at 60 °C obtained from experiments illustrated in Figure 5.

Scheme 3. Hydrolysis of $O_3P-BH_3^{3-}$ in D_2O Superimposed upon Stepwise Deuterium Substitution in D_2O[a]



[a] Stepwise deuteration produces boranophosphate intermediates II−IV. Hydrolysis of intermediates I−IV produced deuterated phosphonate (V). The $-BH_3$ group carries one negative charge.

D_2O is observed, instead of a singlet as previously seen in H_2O (Figure 3); the peak intensity increases with time (Figures 5 and 6). Removal of ^{1}H decoupling did not change the nature of the triplet (data not shown), suggesting that a D nucleus (spin $I = 1$) is coupled to a ^{31}P. Therefore, peak V was assigned to a deuterated phosphonate which contains a P−D bond (see Scheme 3). The measured coupling constants $J(^{31}P-^1H)$ and $J(^{31}P-D)$ for the ^{1}H- and D-forms of phosphonate species were 584.5 and 89.9 Hz, respectively, giving a $J(^{31}P-^1H)/J(^{31}P-D)$ ratio of 6.50. Agreement with the theoretical value of $\gamma^H/\gamma^D = 6.514$[17] for the ^{1}H- and D-forms of phosphonate species confirms the assignment. Thus, the data in D_2O solvent are consistent with $O_3P-BH_3^{3-}$ undergoing hydrolysis to produce the deuterated phosphonate species V (as proposed in Scheme 3).

We also observed evidence for deuterium substitution of the borane hydrogens in the ^{11}B spectra. Upon ^{1}H decoupling, the ^{11}B resonance of $O_3P-BH_3^{3-}$ in H_2O at 50 °C became a doublet (data not shown), due to coupling between bonded ^{11}B and ^{31}P atoms. However, each peak of the doublet developed a complicated splitting pattern and changed with time upon incubation in D_2O (data not shown). The ^{11}B doublet is the result of coupling between the ^{11}B and ^{31}P atoms due to the existence of a P−B bond. The changing peak pattern in the

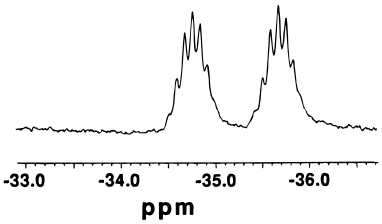


Figure 7. ^{11}B spectra of $O_3P-BD_3^{3-}$ at 25 °C. A total of 1024 scans were acquired, and a line-broadening of 2 Hz was applied.

doublet can be attributed to further coupling between ^{1}H/D and ^{11}B during stepwise deuterium substitution of the borane hydrogens. After extensive incubation in D_2O, when the ^{31}P spectrum only contains quartet IV, the corresponding ^{11}B spectrum (Figure 7) contains a doublet and each peak of the doublet has a 1−3−5−6−5−3−1 pattern. This ^{11}B spectrum is unaffected by ^{1}H decoupling (data not shown). The ^{11}B pattern shown in Figure 7 is due to the presence of three equivalent deuterium atoms bonded to boron, as in the $-P-BD_3$ linkage (species IV in Scheme 3). The ^{11}B NMR results hence corroborate the conclusion obtained from the ^{1}H-decoupled ^{31}P NMR studies that the borane hydrogens undergo stepwise deuterium substitution.

Discussion

Hydrolysis of Boranophosphate Mononucleotide Involves P−O−R Bond Cleavage in H_2O. ^{1}H, ^{31}P (Figure 2), and ^{11}B NMR studies indicate that d(p^BT) is hydrolyzed to produce thymidine dT and boranophosphate $O_3P-BH_3^{3-}$ in aqueous solution at 50 °C as shown in Scheme 1. This result is in agreement with previous HPLC experiments which showed that dT, not thymidine monophosphate d(pT), was the observed product at 260 nm.[9] From these results, it can be concluded that the $P-BH_3$ bond in deoxythymidine borano-monophosphates is hydrolyzed less readily than the P−O−R bond.

It is interesting to compare these experimental results with conclusions from *ab initio* SCF calculations by Thatcher and Campbell,[20] who assumed that phosphate hydrolysis proceeds *via* a trigonal bipyramidal pentacoordinate intermediate in which incoming and leaving groups occupy the apical position.[18,19] These calculations predicted that the P−O bond would be more labile to hydrolysis than the P−B bond.[20] This is consistent with our experimental results. It remains to be determined whether hydrolysis of the P−O bond in boranophosphate nucleotides proceeds *via* a similar intermediate.

Hydrolysis of Boranophosphate, $O_3P-BH_3^{3-}$, in H_2O. Hydrolysis of $O_3P-BH_3^{3-}$, as followed by ^{31}P and ^{11}B NMR with time, indicated that $O_3P-BH_3^{3-}$ decomposes into phosphonate and boric acid in H_2O at 50 °C as shown in Scheme 2. Does $O_3P-BH_3^{3-}$ hydrolyze in a stepwise manner? Several researchers have proposed that stepwise hydrolysis of tetrahydroborate (BH_4^-) is possible *via* the hydrated intermediates $BH_{4-n}(OH)_n^-$.[21−26] We attempted to find evidence for analogous $O_3P-B_{3-n}(OH)_n^{3-}$ intermediates; however, the ^{31}P or ^{11}B

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peak patterns and *J* coupling constants of $O_3P-BH_3^{3-}$ did not change with time during $O_3P-BH_3^{3-}$ hydrolysis in H_2O (shown in Figures 3 and 4). This suggests that either the hydrated intermediates $O_3P-BH_2(OH)^{3-}$, $O_3P-BH(OH)_2^{3-}$, and $O_3P-B(OH)_3^{3-}$ do not form or they are too unstable to be detected by NMR.

Natural boron consists of about 80% ^{11}B and 20% ^{10}B. This can be detected by the presence of major and minor peaks in the ^{31}P spectrum of $O_3P-BH_3^{3-}$. The major peak patterns (Figure 3) can be explained by the proposed hydrolysis reaction of $O_3P-BH_3^{3-}$ shown in Scheme 2. However, three additional minor ^{31}P peaks of equal intensity are present throughout the hydrolysis time course, superimposed upon the major $O_3P-BH_3^{3-}$ quartet (Figure 3). While the pattern of these peaks did not change with time, their intensities did, similar to the observed intensity decrease of the major quartet corresponding to $O_3P-BH_3^{3-}$. The three minor peaks are adequately explained as corresponding to the ^{10}B-form of $O_3P-BH_3^{3-}$, while the major quartet is due to the ^{11}B-form of $O_3P-^{11}BH_3^{3-}$. Since ^{10}B has a nuclear spin of 3, it is expected that (1) the ^{31}P spectrum of the ^{10}B-form of $O_3P-BH_3^{3-}$ should be a septet with equal intensities (a 1−1−1−1−1−1−1 pattern), (2) the individual ^{31}P peak intensity of the ^{10}B-form in $O_3P-BH_3^{3-}$ should be about 0.14 times that of the ^{31}P peak in the ^{11}B-form in the same solution (see Materials and Methods), and (3) the coupling constant ratio $J(^{31}P-^{11}B)/J(^{31}P-^{10}B)$ should be about 3.[13b,27] Experimentally, only three minor peaks with equal intensities are resolved in Figure 3. Upon closer inspection, one can see that the two middle peaks in the major quartet have broader base regions than those of the two outside peaks. This suggests that the four hidden resonances of the septet from the ^{10}B-form are probably buried under the two middle peaks of the major quartet of the ^{11}B-species. Under this assumption, parameters for the resolved minor peaks in Figure 3 can be estimated: (1) The ^{31}P chemical shifts of the ^{10}B- and ^{11}B-forms of $O_3P-BH_3^{3-}$ are both *ca.* 83.5 ppm. (2) The *J* coupling constants $J(^{31}P-B)$ are 149.8 and 50.0 Hz for the ^{11}B- and ^{10}B-forms, respectively, giving an experimental $J(^{31}P-^{11}B)/J(^{31}P-^{10}B)$ ratio of 3.0. (3) The estimated individual ^{31}P peak intensity ratio for the ^{10}B-form relative to the ^{11}B-form is 0.16. Thus, the experimental *J* coupling ratio ($J(^{31}P-^{11}B)/J(^{31}P-^{10}B)$) and ^{31}P intensity ratio (^{10}B-form/^{11}B-form) are in good agreement with theoretical values of 2.99 and 0.14, respectively.[13b,27] This confirms our assumptions that the three minor ^{31}P peaks in Figure 3 arise from the ^{10}B-form of $O_3P-BH_3^{3-}$ and that the other four peaks of the ^{10}B-form septet overlap with the two middle peaks of the major quartet of the ^{11}B-form. By following the decrease in ^{31}P intensities of the minor peaks illustrated in Figure 3, we calculated a pseudo-first-order rate constant of 2×10^{-5} s^{-1} at 50 °C (with 21 data points acquired over 1.8 h; data not shown), which is the same as that observed for the ^{11}B-form in the same experiments.

The three minor ^{31}P peaks from the ^{10}B-form of $O_3P-BH_3^{3-}$ seen in H_2O (Figure 3) were not observed in D_2O (Figure 4). This is presumably because the four subquartets of stepwise D-substituted ^{11}B-forms of $O_3P-BH_3^{3-}$ broaden the ^{31}P resonances and thereby obscure observation of the minor ^{31}P septet from the ^{10}B-form of $O_3P-BH_3^{3-}$.

Hydrolysis and Deuterium Exchange of $O_3P-BH_3^{3-}$ in D_2O. Incubation of $O_3P-BH_3^{3-}$ in D_2O gave ^{31}P (Figure 5) and ^{11}B spectral patterns different from those found when the same reaction was followed in H_2O (Figures 3 and 4). The $O_3P-BH_3^{3-}$ ^{31}P spectrum in D_2O consists of two or more subquartets (Figure 5), while only one quartet was observed in H_2O (Figure 3) throughout the reaction period. We propose that deuterium substitution of the borane hydrogens in $O_3P-BH_3^{3-}$ in D_2O occurs in a three-step manner with three different rate constants. The observed multiple subquartets noted in ^1H-decoupled ^{31}P spectra at different stages in D_2O can be explained as "isotope shifts" (see below) due to individual stepwise D substitution of ^1H atoms in the $-P-BH_3$ linkage. Boranophosphate hydrolysis and deuterium substitution reactions in D_2O are summarized in Scheme 3, in which I, II, III, and IV correspond to stepwise deuterated boranophosphate intermediates $O_3P-BH_3^{3-}$, $O_3P-BH_2D^{3-}$, $O_3P-BHD_2^{3-}$, and $O_3P-BD_3^{3-}$, respectively. From these data we propose that hydrolysis of $O_3P-BH_3^{3-}$ occurs superimposed upon deuterium exchange. Borane hydrogens exchange with deuteriums in D_2O to produce deuterated borane; at the same time, hydrolytic P−B bond cleavage occurs, producing phosphonate and boric acid.

To further confirm deuterium substitution of the borane hydrogens in $O_3P-BH_3^{3-}$ in D_2O, a reverse exchange experiment was conducted by utilizing the reaction mixture which had been hydrolyzed in D_2O to give fully deuterated $O_3P-BD_3^{3-}$ (determined by ^{11}B and ^{31}P NMR), followed by lyophilizing and transferring the molecule into H_2O. Phosphorus-31 and ^{11}B spectra both showed the expected peaks (data not shown) due to stepwise hydrogen substitution intermediates as depicted in Scheme 3.

We think it likely that the ^{31}P multiplets at *ca* 81.7 ppm in Figure 5 arise as a result of stepwise hydrolysis or deuterium exchange. If stepwise hydrolysis were to occur instead of the three-step deuterium substitution reaction in D_2O as proposed in Scheme 3, formation of partially or fully hydrated intermediates (e.g., $O_3P-B(OD)_3^{3-}$) would result. The deuterium in the −B−OD linkage is expected to be in fast exchange with D_2O, so coupling of ^{11}B to D may not be observable in the ^{11}B NMR spectrum with the 1−3−5−6−5−3−1 pattern shown in Figure 7. In this case, the 1−3−5−6−5−3−1 pattern obtained in the ^{11}B spectrum provides further evidence that the species corresponding to subquartets I, II, III, and IV in ^1H-decoupled ^{31}P spectra (Figure 5) are due to intermediates in stepwise deuterium substitution of the borane hydrogens in D_2O, and are not produced by intermediates in stepwise hydrolysis of $O_3P-BH_3^{3-}$. We concluded above that stepwise hydrolysis of borane hydrogens in $O_3P-BD_3^{3-}$ was not observable in H_2O. The D_2O results are consistent with this conclusion.

Isotope Shifts in the $O_3P-BH_3^{3-}$ NMR Spectra. Changes in chemical shifts and *J* coupling constants can occur upon isotopic substitution. Factors that influence the magnitude of the isotope shift include the number of intervening bonds between sites of isotopic substitution and the nucleus of interest, the fractional change in mass upon isotopic substitution, and the number of atoms in the molecule that are substituted by isotopes. Larger shifts occur when the substitution is linked directly to the nucleus, when a larger mass change occurs, and with a larger number of substitutions. Although the isotope shift leads to complications in interpreting NMR results, it can provide useful information regarding molecular structure and reaction mechanism, as shown here.

^{31}P isotope shifts provide additional, quantitative evidence for the premise of Scheme 3 that the subquartet patterns in ^1H-decoupled ^{31}P spectra of $O_3P-BH_3^{3-}$ in D_2O are the result of a three-step deuterium substitution of the borane hydrogens. The ^{31}P isotope shift is 0.14 ppm for each deuterium substitution of the borane hydrogens, and the shifts are additive; subquartets II ($O_3P-BH_2D^{3-}$), III ($O_3P-BHD_2^{3-}$), and IV ($O_3P-BD_2^{3-}$)

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shift downfield relative to subquartet I ($O_3P-BH_3^{3-}$) by 0.14, 0.28, and 0.42 ppm, respectively. Even though the phosphorus atom is separated by two bonds from the substituted position in the $-P-B(H/D)_3$ linkage, the observed ^{31}P isotope shift is quite large, possibly due to the large relative mass difference between ^1H and ^2H (a 100% change).

One would expect to see ^{11}B isotope shifts due to $^1H/D$ substitution. These were in fact observed, but the ^{11}B isotope shift values from each deuterium substitution were obscured by broadening and severe overlap of the ^{11}B resonances from the stepwise deuterated intermediates (data not shown). Yet these results showed clearly that deuterium substitution shifts the ^{11}B resonance upfield, as expected on the basis of zero-point vibrational energy arguments.[28] In contrast, the ^{31}P resonances shifted downfield upon deuterium substitution. It is possible that factors other than mass changes dominate in determining the ^{31}P isotope shift. One such possibility is that pH changes occur as a result of $O_3P-BH_3^{3-}$ hydrolysis to form phosphonate and boric acids under unbuffered conditions (see Materials and Methods).

A ^{31}P isotope shift due to $^{10}B/^{11}B$ substitution is expected theoretically; however, the observed ^{31}P chemical shifts of the ^{10}B-forms of $O_3P-BH_3^{3-}$ have almost the same values as the ^{11}B-forms in H_2O at 50 °C (in Figure 3). Thus, the secondary ^{31}P isotope shift due to $^{11}B/^{11}B$ substitution is very small, even though the boron is only one bond removed from the phosphorus atom. This is not surprising since the mass change due to $^{10}B/^{11}B$ substitution is only *ca.* 10%.

Influence of Electronic Environment on Spectral Line-Broadening. The ^1H, ^{31}P, and ^{11}B peaks of $O_3P-BH_3^{3-}$ (Figures 2 and 4; ^1H spectrum not shown) are considerably narrower than those of d(p^BT) mononucleotide (Figures 1 and 2). The broadened resonance of d(p^BT) might be ascribed to a less symmetric electronic environment around the ^{11}B atom. The line width of a quadrupolar nucleus is largely determined by its electric field gradient, which is related to the local electronic symmetry about the nucleus.[13a,29] Tetrahedral, octahedral, cubic, or spherical binding sites, in principle, are electronically symmetric and thus have zero field gradients; under these circumstances, sharp lines are observed for symmetric simple ions in aqueous solutions (e.g., BH_4^- and BF_4^-). Lower local asymmetry can cause profound line-broadening. The ^{11}B nucleus in the d(p^BT) nucleotide is in a less symmetric geometric environment than in the simpler $O_3P-BH_3^{3-}$, and thus broader peaks would be expected for d(p^BT), as was observed here.

Rates of $O_3P-BH_3^{3-}$ Hydrolysis and Deuterium Substitution. The ^{31}P NMR experiments provided evidence that stepwise deuterium substitutions involving four intermediates occur and that each intermediate undergoes hydrolysis concurrently in D_2O as in Scheme 3. Intermediate concentrations of $O_3P-BH_2D^{3-}$, $O_3P-BHD_2^{3-}$, and $O_3P-BD_3^{3-}$ first increased and then decreased with time during ^1H-decoupled ^{31}P NMR experiments (Figure 6). From these data, rate constants for hydrolysis and deuterium substitution steps can be estimated from the changing concentrations of reactants in situations in which the previous reaction is finished and the concentration of the species of interest decreases monotonically with observation time. For example, after the d(p^BT) phosphate ester is completely hydrolyzed to form $O_3P-BH_3^{3-}$ (species I, Scheme 3) in D_2O, the latter species undergoes hydrolysis to produce phosphonate (species V) and boric acids; deuterium substitution also occurs to produce species II ($O_3P-BH_2D^{3-}$). The concentration of species I decreases monotonically with time due to the two concurrent processes at this stage. Assuming that hydrolysis occurs more slowly than the rate of deuterium substitution, and that the reverse exchange reaction is negligible at early stages in the reaction, the rate of transformation from species I into species II can be calculated from pseudo-first-order plots of the changes in species I intensity with time. Rate constants were 1×10^{-4} and 2×10^{-4} s^{-1} at 50 and 60 °C, respectively (data not shown). Since the hydrolysis and deuterium substitution reactions occur concurrently, the calculated substitution rate constants are only upper limit values.

Table 1. Hydrolysis and Deuterium Substitution Rate Constants for d(p^BT)[a] and $O_3P-BH_3^{3-}$

	k_1 (s^{-1}) in H_2O	k_1 (s^{-1}) in D_2O	temp (°C)
Phosphate Ester Hydrolysis of d(p^BT)			
d(p^BT) → dT + $O_3P-BH_3^{3-}$	4×10^{-4}		50
Hydrolysis of $O_3P-BH_3^{3-}$			
$O_3P-BH_3^{3-} \rightarrow O_3P-H^{2-} + B(OH)_3$	2×10^{-5}		50
	7×10^{-5}		60
$O_3P-BD_3^{3-} \rightarrow O_3P-D^{2-} + B(OD)_3$		8×10^{-6}	60
Deuterium Substitution of $O_3P-BH_3^{3-}$			
$-P-BH_3^- \rightarrow -P-BH_2D^-$		1×10^{-4} [b]	50
		2×10^{-4} [b]	60
$-P-BH_2D^- \rightarrow -P-BHD_2^-$		4×10^{-5} [b]	50
		9×10^{-5} [b]	60
$-P-BHD_2^- \rightarrow -P-BD_3^-$		2×10^{-5} [b]	50
		4×10^{-5} [b]	60

[a] d(p^BT) is as shown in Scheme 1. [b] Upper limit value (see the Discussion).

We assume that hydrolysis is slower than deuterium substitution of boranophosphates. The fact that the hydrolysis rate for the same species (e.g., I in Scheme 3) is 10-fold smaller than the calculated upper limit deuterium substitution rate constant (see Table 1) validates this assumption. When the boranophosphate is completely deuterated, the decrease in species IV intensity can be attributed exclusively to hydrolysis, and the hydrolysis rate constant is estimated to be 8×10^{-6} s^{-1} at 60 °C. Estimated rate constants for hydrolysis and deuterium substitution are given in Table 1. Hydrolysis rate constants for intermediates $O_3P-BH_3^{3-}$, $O_3P-BDH_2^{3-}$, and $O_3P-BD_2H^{3-}$ in D_2O could not be assessed at this time. Although ^{31}P spectral data offered only upper limit rates for stepwise deuterium substitution of $O_3P-BH_3^{3-}$, Figures 5 and 6 clearly show that the borane hydrogens in boranophosphate can exchange with D_2O in a stepwise manner.

The rates of boranophosphate hydrolysis and deuteration are temperature-dependent. For $O_3P-BH_3^{3-}$ hydrolysis in H_2O, k_1 values are 2×10^{-5} and 7×10^{-5} s^{-1} at 50 and 60 °C, respectively (Table 1).

The rates of boranophosphate hydrolysis and deuteration are solvent- and isotope-dependent. Each stage of deuterium substitution of borane hydrogens in $O_3P-BH_3^{3-}$ in D_2O takes place at a progressively slower rate. Thus, the presence of more deuterium substituents in the $-BH_3$ group slows further substitution. For example, the upper limit deuterium substitution rate constant k_1 for the transformation of $-P-BH_3$ into $-P-BH_2D$ is *ca.* 2×10^{-4} s^{-1}, the rate of $-P-BH_2D$ deuteration is *ca.* 9×10^{-5} s^{-1}, and the rate of $-P-BHD_2$ deuteration is *ca.* 4×10^{-5} s^{-1} at 60 °C (Table 1). In addition, hydrolysis rates for $O_3P-BH_3^{3-}$ in H_2O and $O_3P-BD_3^{3-}$ in D_2O are 7×10^{-5} and 8×10^{-6} s^{-1} at 60 °C, respectively. Thus, $O_3P-BH_3^{3-}$ hydrolyzes *ca.* 10-fold faster in H_2O than $O_3P-BD_3^{3-}$ in D_2O. These observed kinetic isotope effects are probably the result of a combination of isotope solvent (H_2O *vs* D_2O) and composition ($O_3P-BH_3^{3-}$ *vs* $O_3P-BD_3^{3-}$) effects. Since

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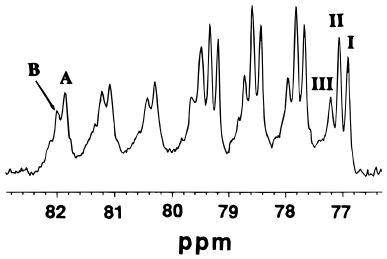


Figure 8. ^{1}H-decoupled ^{31}P spectrum of d(p^{B}T) in D_2O buffer at 25 °C. The sample was incubated at *ca.* 25 °C for at least 30 h before collecting the spectrum. The downfield multiple quartets centered at *ca.* 80.9 ppm correspond to d(p^{B}T) resonances, and the upfield multiple quartets centered at *ca.* 78.2 ppm are from boranophosphate resonances (the right-most peaks of the downfield quartets overlap with the left-most peaks of the upfield quartets). In the downfield multiple quartets, subquartet B is due to the resonance of partially deuterated borane (P−BH_2D) in d(p^{B}T), and subquartet A is the resonance of the non-deuterated d(p^{B}T). In the upfield multiple quartets, subquartets I−IV are the same as assigned in Figure 5. A total of 1024 scans were collected, and a line-broadening of 5 Hz was applied.

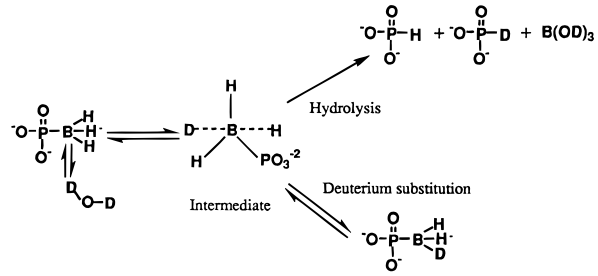
we were not able to measure the hydrolysis rates of $O_3P-BH_3^{3-}$ in D_2O or fully deuterated boranophosphate, $O_3P-BD_3^{3-}$, in H_2O without interfering with the solvent exchange process, these isotope effect contributions could not be analyzed.

Tentative Evidence for Solvent Exchange of Borane Hydrogens in the d(p^{B}T) Mononucleotide. Whereas we observed stepwise deuterium substitution of borane hydrogens in $O_3P-BH_3^{3-}$ in D_2O, we did not observe deuterium-induced isotope shifts in ^{1}H-decoupled ^{31}P spectra of d(p^{B}T) during hydrolysis to form dT and $O_3P-BH_3^{3-}$ in D_2O at 50 °C (Figure 2). However, we observed deuterium substitution of the borane hydrogens in one d(p^{B}T) sample in D_2O buffer which contained 100 mM NaCl, 1 mM EDTA, and 10 mM phosphate, pH 7.4, upon incubation at or below 25 °C for over 30 h. The ^{1}H-decoupled ^{31}P spectrum (Figure 8) showed that there are at least two resolved subquartets (labeled A and B) from the original d(p^{B}T), whose chemical shifts differed by 0.14 ppm, the same value as observed for each of the deuterium-substituted boranophosphate forms I−IV (Figure 5). We believe that subquartet A in Figure 8 corresponds to non-deuterated d(p^{B}T) and that subquartet B corresponds to a population of partially deuterated d(p^{B}T) (containing the −P−BH_2D linkage). Thus, under these conditions d(p^{B}T) is observed to undergo stepwise solvent exchange, but deuterium substitution is slow relative to hydrolysis of the −P−O-sugar linkage and is thus overtaken by it.

Exchange between borane hydrogens in the −P−BH_3 linkage and solvent protons is significant because it demonstrates that the hydrogens in the borane group (−BH_3) in $O_3P-BH_3^{3-}$ and d(p^{B}T) mononucleotide are in dynamic equilibrium with the solvent. This ready ability of borane hydrogens to exchange with D_2O distinguishes the −BH_3 functionality in $O_3P-BH_3^{3-}$ and d(p^{B}T) mononucleotide from the −CH_3 in methylphosphonate-modified nucleotides.

In contrast to the *mono*nucleotide, the dithymidine boranomonophosphate *di*nucleotide d(Tp^{B}T) is very stable to hydrolysis; hydrolysis rates for the latter (diester) are about 5 orders of magnitude slower than for d(p^{B}T) (monoester), i.e., on the order of 10^{-9} s^{-1} at 50 °C in 0.2 M phosphate (pH 7) buffer.[8,9a] If we assume that the deuterium substitution rate in D_2O obtained for $O_3P-BH_3^{3-}$ (*ca.* $10^{-4}-10^{-5}$ s^{-1} at 60 °C) is applicable to the dinucleotide, it is possible that deuterium replacement of the borane hydrogens in the d(Tp^{B}T) dinucleotide occurs more easily than its hydrolysis in D_2O. However, incubating a d(Tp^{B}T) sample in 100 mM NaCl, 10 mM phosphate (pH 7.4), and 1 mM EDTA at 30 °C for at least 30 h showed no experimental evidence for isotope shifts in the ^{1}H, ^{31}P, or ^{11}B spectra (data not shown). There are two reasonable explanations for why isotope shifts have been detected in the NMR spectra of the mononucleotide, but not those of the dinucleotide. First, the NMR studies utilized 40−85-fold higher concentrations of mononucleotide than the dinucleotide (85 mM *vs* 1−2 mM), so a small fraction of dinucleotide showing the isotope shift may be difficult to observe. Second, the ^{11}B atom in the dinucleotide gives broadened ^{1}H, ^{31}P, and ^{11}B peaks, reducing the "effective" signal/noise ratio. Thus, observation of deuterium substitution by an isotope shift on the ^{31}P or ^{11}B spectra of d(Tp^{B}T) dinucleotides may be obscured.

Scheme 4. One Possible Mechanism for Hydrolysis and Solvent Exchange Reactions of $O_3P-BH_3^{3-}$ in D_2O



Possible Mechanisms for the Hydrolysis and Deuteration of $O_3P-BH_3^{3-}$. The overall hydrolysis and deuteration reactions of boranophosphate are shown in Scheme 3, and one possible mechanism involving a five-coordinate activated complex is shown in Scheme 4. Pentavalent intermediates, e.g., BH_5 and BH_4CN, were previously proposed for the hydrolyses of hydroborate (BH_4^-) and cyanoborate (BH_3CN^-), respectively.[22,30,31] If both hydrolysis and deuterium exchange reactions share the same intermediate, an analogous intermediate containing a −P−BH_4 linkage may operate here for the hydrolysis and deuteration of $O_3P-BH_3^{3-}$ (Scheme 4).

Addition of a D^+ from the D_2O to $O_3P-BH_3^{3-}$ would form the −P−BH_3D linkage. This intermediate could lead down either of two paths: proton−deuterium exchange or hydrolysis. Thus, the intermediate may transfer either an existing H or the incoming D to phosphorus and P−B bond breakage can occur to produce a −P−H or −P−D bond in the phosphonate, resulting in hydrolysis. Alternatively, loss of H^+ occurs, resulting in deuterium substitution. A third possibility is loss of D^+, resulting in no observed solvent exchange.

In this mechanism, we expect to see both −P−H and −P−D bond formation in the phosphonates if P−H and P−D bonds are formed at similar rates under a given condition. Under these circumstances, the ^{1}H-decoupled ^{31}P spectrum should then contain a singlet and a triplet, and the ^{31}P spectrum should contain a doublet and a triplet, corresponding to the ^{1}H- and D-forms of phosphonate, respectively. Qualitatively, at the beginning of boranophosphate hydrolysis, more H-form phosphonate should be present, while at later stages most of the boranophosphate should be in the $O_3P-BD_3^{3-}$ form and only the deuterated phosphonate should be produced as a hydrolysis product. Results from our $O_3P-BH_3^{3-}$ hydrolysis experiments in D_2O demonstrate the presence of a triplet in ^{31}P spectra both with (Figure 5) and without ^{1}H decoupling (data not shown), indicating that D-form phosphonate forms throughout the course of the reactions.

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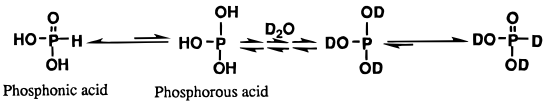
Scheme 5. Tautomeric Equilibrium of Phosphonic Acid and Hypothetical Deuterium Exchange Mechanism



One possible reason for not observing the ^{1}H-form of phosphonate by ^{31}P NMR is that, in D_2O, the ^{1}H-form of phosphonate is readily converted into the D-form. It is known that phosphonic acid is in tautomeric equilibrium with its trivalent phosphorous acid isomers[32,33] (Scheme 5), which can then be converted into deuterated phosphonate (product V in Scheme 3). If the conversion reaction from the ^{1}H-form to the D-form of phosphonate in Scheme 5 were to occur faster (e.g., >500-fold) than the rate of P−B bond hydrolysis, the D-form of phosphonate would be the major observed product in this mechanism. Another possible reason for not observing the ^{1}H-form phosphonate by ^{31}P NMR is that existing H and incoming D in the intermediate (Scheme 4) are not in equivalent positions so that the incoming deuterium is transferred more efficiently to phosphorus during the hydrolysis process than the existing H. In this inequivalent transfer rate case, the D-form of phosphonate would be the dominant product of $O_3P-BH_3^{3-}$ hydrolysis. The present data cannot discriminate between the two possibilities or other possible mechanisms for hydrolysis and solvent exchange of boranophosphate.

Conclusion

Reactions of thymidine 5′-boranomonophosphate, d(pBT), in aqueous solution were studied with the hope that the results would offer important insights regarding the behavior of these compounds in potential pharmaceutical applications. Unlike many borane-containing compounds, phosphate borane is quite stable in aqueous solution due to its unique structure. Thus, ^{1}H, ^{31}P, and ^{11}B NMR could be used to follow hydrolysis of the P−B bond and deuterium substitution of the borane hydrogens by providing a set of interdependent and internally consistent information. Of special utility were ^{31}P and ^{11}B isotope shifts due to ^{1}H/D substitution.

In aqueous solution, d(p^{B}T) slowly decomposes, first into thymidine dT and boranophosphate $O_3P-BH_3^{3-}$ ($k_1 = 4 \times 10^{-4}$ s^{-1} at 50 °C in H_2O), followed by an even slower hydrolysis of $O_3P-BH_3^{3-}$ to produce phosphonate and boric acid ($k_1 = 2 \times 10^{-5}$ s^{-1} at 50 °C in H_2O). Therefore, the P−B bond in the d(p^{B}T) mononucleotide is less susceptible to hydrolysis in aqueous solution than the P−O−sugar linkage. In addition to hydrolysis, the hydrogens in the $-P-BH_3$ linkage of both d(p^{B}T) and $O_3P-BH_3^{3-}$ exchange with solvent. The exchange process was inferred from the presence of ^{31}P and ^{11}B isotope shifts and changes in coupling patterns to involve three-step deuterium substitution of borane hydrogens in D_2O. Each deuterium substitution causes a 0.14 ppm downfield shift of the ^{31}P resonance of the $-P-BH_3^{3-}$ linkage. Deuterium substitution of borane hydrogens in $O_3P-BH_3^{3-}$ occurs in a three-step manner to form intermediates $O_3P-BDH_2^{3-}$, $O_3P-BD_2H^{3-}$, and $O_3P-BD_3^{3-}$ and is superimposed upon hydrolysis of partially and fully deuterated boranophosphate intermediates, producing deuterated phosphonate and boric acid in D_2O. At 50 °C deuterium substitution of $O_3P-BH_3^{3-}$ occurs an order of magnitude faster than its hydrolysis.

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