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Conformational Studies of Dithymidine Boranomonophosphate Diastereoisomers

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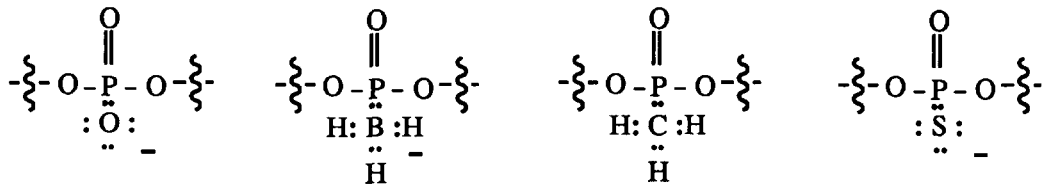
Abstract—The boranophosphate ester nucleotides are a new class of nucleic acid analogues that are isoelectronic and isostructural to normal phosphodiester nucleic acids and that maintain the anionic charge of the nucleic acid backbone. The two P-diastereoisomers of dithymidine boranomonophosphates were separated using reverse phase HPLC; the faster and slower eluting isomers are designated as d(Tp^{B}T)-1 and d(Tp^{B}T)-2, respectively. Conformations of the isomers were studied using circular dichroism (CD) and NMR, and compared to the analogous phosphate diester, d(TpT). This comparison allowed the effects of the borane group and chirality of the boranophosphate linkage on sugar and base conformations to be assessed. The CD spectra of the diastereoisomers are consistent with both having a B-type conformation. Analysis of the ^{1}H–^{1}H and ^{1}H–^{31}P coupling constants showed that these conformations are similar to those of the unmodified parent dimer; specifically, the 2′-deoxyribose rings prefer the *S* (C2′-*endo*) conformation, and the C4′–C5′ and C5′–O5′ rotamers are primarily in the γ^{+} and β^{+} conformations, respectively. Conformational differences between the diastereoisomers and between the modified and unmodified dimers are manifested by differences in the preferences of the 3′-residues to adopt S sugar pucker and β^{+} conformations. There is reduced preference for the S sugar pucker of the 3′-residue in d(Tp^{B}T)-1 relative to d(Tp^{B}T)-2, which is similar to d(TpT). There is less preference for the β^{+} conformation of the 3′-residue in d(Tp^{B}T)-2 relative to d(Tp^{B}T)-1 and d(TpT). Based on the CD results, the temperature dependences of the thymidine H6 chemical shifts, and the derived sugar ring and backbone conformational parameters, we conclude that the borane group exerts a minimal influence on the sugar conformations and base stacking interactions. Preliminary assignment of the absolute configuration of the pair of S_p and R_p diastereoisomers to d(Tp^{B}T)-1 and d(Tp^{B}T)-2, respectively, is made on the basis of enzyme selectivity and NOE difference experiments. © 1997 Elsevier Science Ltd.

Introduction

Recently, our group has synthesized modified nucleotides with a boronated internucleotide backbone.[1] These compounds have potential use in antisense and therapeutic applications, especially as boron neutron capture therapy agents.[2] The boranophosphate internucleotide group **2** is structurally related to the natural phosphodiester **1** (*O*-), phosphorothioate **4** (*S*-) and methylphosphonate **3** (*Me*-) groups. Further, the internucleotide group in boranophosphate is negatively charged like that in *O*- and *S*-oligonucleotides. Replacement of one of the two non-bridging oxygens with a $-BH_3$ group produces a chiral center at the phosphorus atom of an oligonucleotide. The absolute configuration can be defined according to Cahn et al.[3] The R_p and S_p diastereoisomers of boranomonophosphate dimers are shown in Figure 1. The stereochemistry of boron-modified phosphorus has been shown to affect physical properties and biological functions. For example, the retention times on reverse phase high performance



1 **2** **3** **4**

Abbreviations: CD, circular dichroism; EDTA, ethylenediaminetetraacetic acid; d(TpT), dithymidine monophosphate; d(Tp^{B}T)-1 and d(Tp^{B}T)-2, the diastereoisomers of dithymidine boranomonophosphate; RP-HPLC, reverse phase high performance liquid chromatography; MeOH, methanol; NMR, nuclear magnetic resonance spectroscopy; NOE, nuclear Overhauser effect; ppm, parts per million; ROESY, rotating frame nuclear Overhauser effect spectroscopy; SVPD, snake venom phosphodiesterase; TOCSY, total correlation spectroscopy; TSP, 3-trimethylsilyl-propionate-2,3,3,3-d_4 sodium salt.

Key words: boron-modified nucleic acids, boranophosphonate, nucleotides, DNA analogues, CD and NMR conformational analysis.

liquid chromatography (RP-HPLC) columns, rates of phosphodiester hydrolysis, and hydrolytic cleavage by phosphodiesterases are different for the two diastereoisomers.[4,5]

Due to differences in electronegativity, polarity, hydrophobicity and size between the borane group ($-BH_3$) and oxygen, boranophosphate-containing dimers are expected to have different structural properties than the unmodified parent dimer, d(TpT). We have therefore examined the conformational properties of these boranomonophosphate dinucleosides using CD and NMR methods. These results were compared with data obtained with unmodified d(TpT).

Results

CD experiments

The boron-modified diastereoisomers were separated by RP-HPLC; d(Tp^{B}T)-1 and d(Tp^{B}T)-2 are the faster and slower eluting diastereoisomers, respectively. CD spectra of unmodified and boron-modified dimers obtained in the 230–340 nm range and at temperatures ranging from 25 to 80 °C are shown in Figure 2. The CD spectrum of unmodified parent d(TpT) is consistent with previously published results.[6,7] Aside from some changes in magnitudes, the differences between the CD spectra of the two modified diastereoisomers and between the boron-modified and the unmodified dimers are minimal. This suggests that boron modification and the configuration about phosphorus do not greatly impact the conformation of the dimers. Signs, positions and magnitudes of the CD bands indicate that both of the modified diastereoisomers adopt B-type conformations, the same as the unmodified d(TpT) dimer.

Increasing the temperature leads to a reduction in the magnitudes of both positive and negative bands, suggesting that at lower temperatures the two aromatic pyrimidine bases interact extensively,[8] and that at higher temperatures these interactions decrease. By comparison, the unmodified d(TpT) shows larger CD intensities (>10%) at a given temperature, and a greater change in CD intensity (>7%) with temperature than either of the two modified diastereoisomers. For the modified diastereoisomers, the spectrum of d(Tp^{B}T)-1 shows ca. 11% larger CD intensity than that of d(Tp^{B}T)-2. These results suggest that boron-modified dimers may have different intramolecular interactions than the unmodified dimer and that the configuration of boronated phosphorus exerts a small but noticeable influence on the base stacking interactions. Following the interpretation of Weinfeld et al.,[9] the order of increasing ability to adopt the stacked states would be d(TpT) > d(Tp^{B}T)-1 > d(Tp^{B}T)-2.

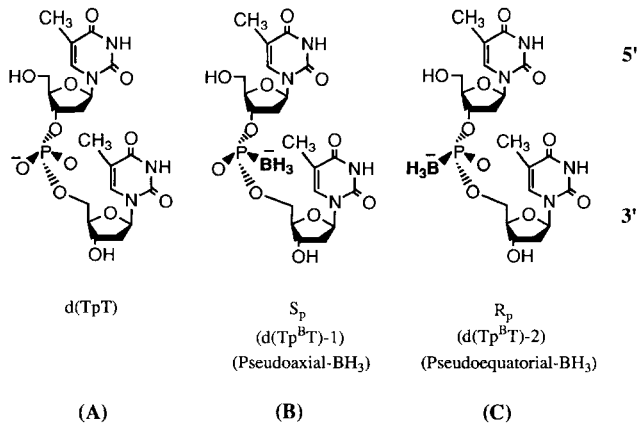


Figure 1. Stereochemical structures of boron-modified and unmodified d(TpT). (**A**) d(TpT), (**B**) d(Tp^{B}T)-S_p, and (**C**) d(Tp^{B}T)-R_p. As shown, the 5′-residues are at the top and the 3′-residues at the bottom.

^{1}H spectral assignments

The 5′- and 3′-thymidine residues in the dimers are not geometrically equivalent (Figure 1). Assignments of proton resonances in the boron-modified dimers were made by analogy to those in d(TpT)[7,8,10–12] and the methylphosphonate analogues.[13,14]

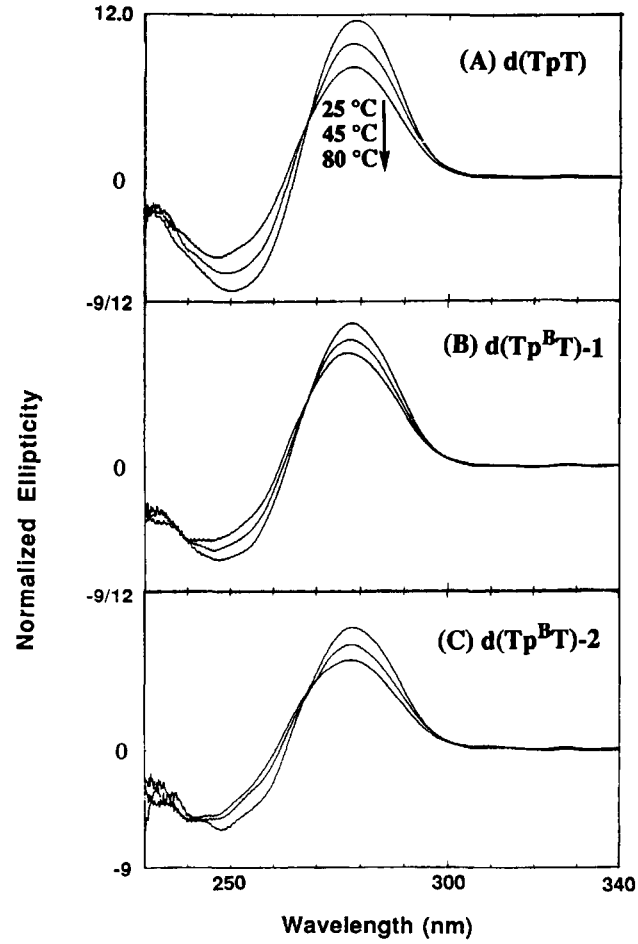


Figure 2. CD spectra of boron-modified diastereoisomers and unmodified d(TpT). Spectra were recorded at 25, 45 and 80 °C in 100 mM NaCl, 1 mM EDTA, 10 mM potassium phosphate, pH 7.4 at 0.1 mM dimer concentrations. (**A**) d(TpT), (**B**) d(Tp^{B}T)-1, and (**C**) d(Tp^{B}T)-2.

Table 1. Chemical shifts and coupling constants for boron-modified and unmodified d(TpT) in 10 mM phosphate, 100 mM NaCl, 0.1 mM EDTA, pH 7.4 at 30 °C

	Chemical shift (ppm)				Coupling constant (Hz)		
	d(Tp^{B}T)-1	d(Tp^{B}T)-2	d(TpT)		d(Tp^{B}T)-1	d(Tp^{B}T)-2	d(TpT)
5′-residue							
H1′	6.246	6.247	6.220	J1′2′	6.75	7.2	7.3
H2′	2.351	2.381	2.352	J1′2″	6.3	6.3	6.2
H2″	2.514	2.522	2.566	J2′2″	−14.0	−14.0	−14.0
H3′	4.836	4.892	4.784	J2′3′	6.2	6.2	6.5
H4′	4.160	4.172	4.192	J2″3′	3.4	3.2	3.6
H5′	3.848	3.841	3.837	J3′4′	3.0	2.8	3.4
H5″	3.777	3.785	3.787	J4′5′	3.4	3.0	3.3
H6	7.649	7.663	7.660	J4′5″	4.8	4.8	4.8
TCH_3	1.892	1.892	1.887	J5′5″	−13.0	−12.5	−12.5
				J6TCH_3	1.5	1.0	1.2
				J3′P	8.0	8.2	6.5
3′-residue							
H1′	6.344	6.314	6.322	J1′2′	7.0	7.0	7.0
H2′	2.376	2.357	2.384	J1′2″	6.5	6.5	6.5
H2″	2.376	2.389	2.370	J2′2″	−14.1	−14.1	−14.1
H3′	4.592	4.571	4.596	J2′3′	5.0	6.5	6.4
H4′	4.152	4.166	4.137	J2″3′	5.0	4.2	4.3
H5′	4.110	4.120	4.157	J3′4′	4.0	3.1	4.4
H5″	4.096	4.080	4.089	J4′5′	2.8	2.4	2.6
H6	7.680	7.722	7.690	J4′5″	2.8	3.4	3.1
TCH_3	1.931	1.927	1.899	J5′5″	−12.5	−11.5	−12.5
				J6TCH_3	1.0	1.5	1.1
				J4′P	3.2	3.0	3.2
				J5′P	4.15	4.9	4.3
				J5″P	4.7	5.4	3.9
$H(BH_3)$	0.379	0.354		JB-P	139.8	138.5	
^{11}B	−37.0	−36.8		JH(B)-P	22.0	22.0	
^{31}P	90.4	90.7	−3.25	JH(B)-B	107.0	104.0	

The ^{1}H signal from the -P-BH_3 moiety is located as a very broad ^{1}H quartet spanning nearly 400 Hz and centered at ca. 0.3 ppm for both diastereoisomers (data not shown). The breadth of this line is due to quadrupolar relaxation by the ^{11}B nucleus (^{11}B, I=3/2). This assignment was confirmed by ^{11}B-decoupling experiments in which a sharper doublet (due to phosphorus coupling) was observed at ca. 0.3–0.4 ppm (Figure 3). The -BH_3 groups in the two diastereoisomers have similar ^{1}H chemical shift values (0.379 and 0.354 ppm for d(Tp^{B}T)-1 and d(Tp^{B}T)-2, respectively, see Table 1). The coupling constant between ^{1}H and ^{31}P determined from the ^{11}B-decoupled ^{1}H spectrum was estimated to be 22 Hz for each diastereoisomer (see Table 1).

Assignment of the thymidine H6 and -CH_3 resonances is straightforward. They have first-order splitting patterns and appear similar to those of unmodified d(TpT). The upfield resonances were assigned to the 5′-residue and the downfield resonances were assigned to the 3′-residue.[7,8,10–12]

Assignment of the H1′, H2′, H2″, H3′, H4′, H5′ and H5″ resonances of the 2′-deoxyriboses was based on comparison with d(TpT). Without site-specific deuteration it was not possible to unambiguously distinguish the resonance of H2′ from H2″ or of H5′ from H5″. H2″ was assumed to be downfield of H2′ based on arguments by Fang et al.[15] The H5′ peak was assumed to be downfield of the H5″ peak following Remin and Shugar.[16] Distinguishing H2′ from H2″ or H5′ from H5″ is not crucial to our purpose, i.e. determining the effects of replacing one of the non-bridged phosphate oxygens with a borane group on the overall conformation of the sugar–base backbone.

Assignments of H3′, H4′, H5′ and H5″ resonances corresponding to the 5′- and 3′-residues were made based on heteronuclear ^{31}P-decoupling experiments. Phosphorus is coupled to the H3′ proton of the 5′-

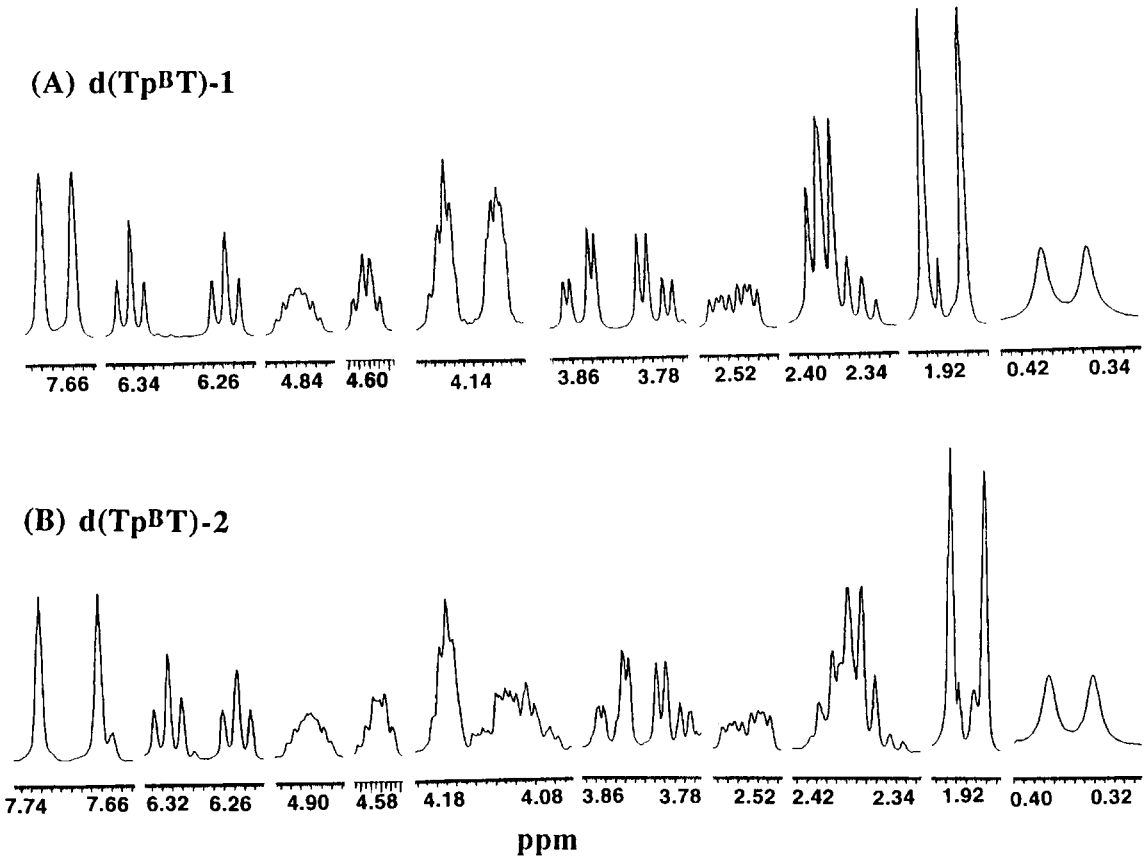


Figure 3. ^{11}B-decoupled ^{1}H NMR spectra of d(Tp^{B}T)-1 and d(Tp^{B}T)-2 at 30 °C. Spectra were recorded in 100 mM NaCl, 0.1 mM EDTA, 10 mM potassium phosphate, pH 7.4 at 2 mM dimer. A total of 725 scans were collected and a 1 Hz line broadening was applied to each spectrum. (**A**) d(Tp^{B}T)-1 and (**B**) d(Tp^{B}T)-2.

residue and to the H5′, H5″ and H4′ protons of the 3′-residue. The frequency difference between the outer components of a J-coupled multiplet peak represents the sum of its coupling interactions. The phosphorus coupling constant was estimated from the difference between the sum of the coupled and decoupled subspectra. Assignments of all the ^{1}H resonances on the bases and sugar rings of the boron-modified diastereoisomers were confirmed by ^{1}H TOCSY and ROESY experiments (data not shown).

Scalar couplings (^{1}H–^{1}H and ^{1}H–^{31}P) and ^{1}H chemical shifts were determined from one-dimensional spectra and were used to generate initial spectral simulations. These parameters were then adjusted to create a simulated spectrum that fit the experimental data. A complete set of ^{1}H, ^{31}P and ^{11}B chemical shifts, and ^{1}H–^{1}H, ^{1}H–^{31}P, ^{11}B–^{31}P and ^{1}H–^{11}B coupling constants for the two modified diastereoisomers and unmodified d(TpT) obtained from experiments and spectral simulation are reported in Table 1. The chemical shifts and coupling constants reported here for the unmodified parent d(TpT) are in agreement with previously reported values obtained under similar experimental conditions.[7,17] While small ^{1}H chemical shift differences exist between boron-modified and unmodified dimers and within the pair of diastereoisomers, these differences are generally less than 0.05 ppm (see Table 1). The largest ^{1}H chemical shift differences (up to 0.11 ppm) were observed for protons in close proximity to the modified site, e.g. H5′/H5″ and H4′ in the 3′-residue, and H3′, H4′ and H2′/H2″ in the 5′-residue of the respective dimers. There is a very small difference (generally within 0.03 ppm) between the ^{1}H chemical shifts of the sugar protons at analogous positions in the diastereoisomers (see Table 1).

Replacing the phosphate oxygen with the -BH_3 group should induce a large alteration in the magnetic environment of the phosphorus atom. Results in Table 1 show that the ^{31}P chemical shift changes from ca. −3 ppm for d(TpT) to ca. +90 ppm for the modified dimer at 30 °C. This large downfield shift is attributed to the decrease in electronegativity and to the attendant change in hybridization of the phosphorus orbital on substitution of oxygen by the borane group.[18]

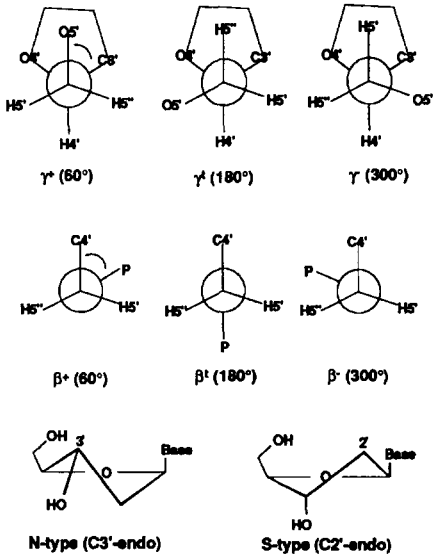
Conformational analysis

Conformational analyses of C4′–C5′ and C5′–O5′ torsion angles and sugar puckering were performed using ^{1}H–^{1}H and ^{1}H–^{31}P coupling constants. We assumed that the presence of the -BH_3 group does not require modification of routine procedures used to characterize the conformation (following studies on methylphosphonate dimer by Kan et al.[14] as well as other modified nucleotide analogues by Glemarec et

Table 2. Conformer populations (%) of sugar ring and C4′–C5′ and C5′–O5′ torsions for boron-modified and unmodified d(TpT) in 10 mM phosphate, 100 mM NaCl, 0.1 mM EDTA, pH 7.4 at 30 °C

	d(Tp^{B}T)-1	d(Tp^{B}T)-2	d(TpT)
5′-residue			
S (C2′-*endo*)[1]	74	76	73
N (C3′-*endo*)[2]	26	34	27
γ^{+} [3]	57	61	58
γ^{t} + γ^{-} [4]	43	39	42
3′-residue			
S (C2′-*endo*)[1]	57	65	64
N (C3′-*endo*) [2]	43	35	36
γ^{+} [3]	84	81	83
γ^{t} + γ^{-} [4]	16	19	17
β^{+} [5]	78	71	81
β^{t} + β^{-} [6]	22	29	19

(1) C2′-*endo* = [17.8–(J1′2″ + J2″3′)]/10.9. (2) C3′-*endo* = [(J1′2″ + J2″3′) – 6.9]/10.9. (3) γ^{+} = [13.7 – (J4′5′ + J4′5″)]/9.7. (4) (γ^{t} + γ^{-}) = 1 – γ^{t}. (5) β^{+} = [25 – (J5′P + J5″P)]/20.8. (6) (β^{t} + β^{-})=1–β^{+}.



al.[7]). The C4′–C5′ conformations are described in terms of a time-averaged distribution over the staggered rotamers: gauche(+) (γ^{+}), gauche-trans (γ^{t}), and gauche(–) (γ^{-}) proposed by Klyne and Prelog.[19] The results of our analysis are presented in Table 2. The C4′–C5′ rotamer populations were calculated from the experimental ^{1}H–^{1}H coupling constants, J4′5′ and J4′5″, using the empirical generalized Karplus equation.[20–22] Analogously, the C5′–O5′ conformation can be described as a rapid equilibrium between β^{+}, β^{t} and β^{-} torsion angles. The population distribution of C5′–O5′ conformers was obtained using the methods of Altona[20] and Lee et al.[22] Because unambiguous assignment of the two C(5′) protons was not possible, we could not distinguish between γ^{t} and γ^{-} conformations or between β^{t} and β^{-} conformations. Hence, only the contributions from γ^{+} versus the γ^{t} + γ^{-} mixture or from β^{+} versus the β^{t} + β^{-} mixture could be evaluated.

The five-membered furanose ring is non-planar and its conformation is generally treated as a two-state equilibrium between C2′-*endo* (S) and C3′-*endo* (N) type puckered ring forms based on the pseudorotational concept[23,24] and generalized Karplus equation.[21] The sugar conformation was calculated using empirical expressions developed by Altona,[20] which should yield values close to those obtained from full pseudorotation analysis.[20] Although several Karplus relations are available to calculate the conformer fractions, data calculated using the same equation should be reliable for comparing conformational preference among a series of analogous compounds.[22,25]

The calculated conformer fractions for sugar ring and backbone are summarized in Table 2. Both of the boron-modified diastereoisomers as well as the unmodified dimer adopt very similar conformations in solution. In general, the 2′-deoxyribose rings are preferentially in the S form (C2′-*endo*), the C4′–C5′ torsion is predominantly in the γ^{+} conformation, and C5′–O5′ is predominantly in the β^{+} conformation. Thus, a standard right-handed conformation[10,12] is found for all three dimers, which is consistent with our CD results. Some conformational differences, however, are seen due to the boron modification and/or configuration of boronated phosphorus. The parameters in Table 2 indicate that conformational differences due to -BH_3 modification occur somewhat more in the 3′- than the 5′-residue. The conformations of the 5′-residues for the boron-modified diastereoisomers are almost identical to that of the unmodified parent d(TpT). Specifically, the sugar ring in the 3′-residue of d(Tp^{B}T)-1 has ca. 10% less S conformer population compared to that in d(TpT), while the conformation in the 3′-residue sugar ring of d(Tp^{B}T)-2 does not change relative to d(TpT); the 3′-residue in d(Tp^{B}T)-1 has a similar population of β^{+} C5′–O5′ rotamer relative to d(TpT), while d(Tp^{B}T)-2 has a ca. 12% smaller β^{+} population. Boron modification did not noticeably change the C4′–C5′ γ torsion angle distributions.

Temperature-dependence studies

Variable temperature ^{1}H NMR experiments focused particularly on the chemical shift changes of the non-exchangeable TH6 protons. This signal is known to be sensitive to changes in base stacking due to ring-current magnetic anisotropy of neighboring thymidine base.[26,27] As temperature increased (Figure 4), the TH6 resonances of all three dimers underwent upfield shifts, indicating destacking of the bases with increasing temperature.[28–30] This result is consistent with the results of our temperature-dependent CD studies. For the 5′-residues (Figure 4A), the TH6 chemical shifts in the three dimers exhibited slightly different slopes; d(Tp^{B}T)-2 and d(TpT) had almost the same chemical shifts; whereas those of d(Tp^{B}T)-1 were found upfield from those of d(Tp^{B}T)-2 and d(TpT) by ca. 0.02 ppm at 20 °C.

The most striking change occurred for the 3′-residues; the TH6 chemical shifts of the three dimers exhibited

quite different values and responses with changing temperature (Figure 4B). Specifically, the d(Tp^{B}T)-1 resonance was located 0.07 ppm upfield relative to the d(Tp^{B}T)-2 at 10 °C with d(TpT) in between; d(Tp^{B}T)-2 had a similar slope to d(TpT) in the 7 to 60 °C range; d(Tp^{B}T)-1 changed very little with temperature; and at temperatures above 50 °C chemical shifts of d(Tp^{B}T)-1 had the same value as d(TpT). The relatively small change in the TH6 chemical shift of the 3′-residue in d(Tp^{B}T)-1 with increasing temperature cannot be explained as resistance to destacking because a corresponding decrease in the CD intensity with increasing temperature (Figure 3) would suggest that destacking did occur in d(Tp^{B}T)-1.[6,9,31] Rather, the insensitivity of the TH6 chemical shift to temperature indicates that the TH6 of the 3′-residue in d(Tp^{B}T)-1 is in a different magnetic environment relative to that of both d(Tp^{B}T)-2 and d(TpT), which display a temperature response typical of a fully and partially stacked conformational transition.[27]

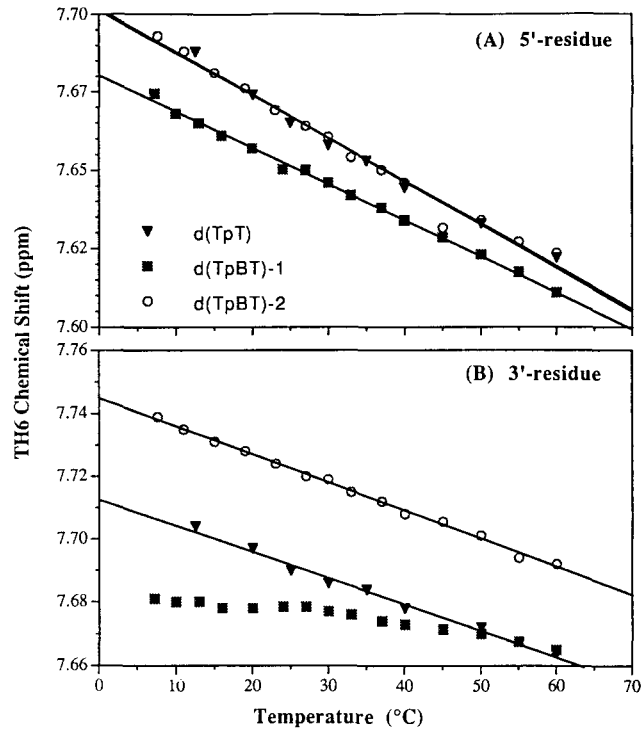


Figure 4. Temperature dependence of TH6 chemical shifts of boron-modified diastereoisomers and unmodified dimer d(TpT). (**A**) 5′-dT and (**B**) 3′-dT residues. Experimental conditions were the same as in Figure 3.

Configuration at phosphorus

When the boron-modified dimers are in a stacked conformation, the borane groups of the two diastereoisomers are in different positions relative to the base-stacking region (as shown in Figure 1). In the S_p form, the borane group is directed toward the base stacking region and is close to the H3′ of the 5′-residue in the dimer (a pseudoaxial position), while in the R_p form, the borane group is directed away from the stacked bases and from other protons of the dimer (a pseudoequatorial position).[32] Therefore, a large NOE effect between the -BH_3 protons and H3′ of the 5′-residue is expected in the S_p configuration. This feature has been successfully used to assign the absolute configurations of methylphosphonate dinucleosides using 1D NOE difference experiments[14] and 2D ROESY experiments.[32–34] Attempts were made to find conditions for ROESY in our study, but a cross-peak between the -BH_3 protons and H3′ of 5′-residue was not observed. Presumably this was due to the quadrupolar effect of boron-11 in the -P-BH_3 moiety. When 1D ^{1}H NOE difference experiments were carried out with the modified diastereoisomers in the ^{11}B-decoupled mode, however, irradiation of the H3′ of the 5′-residue in d(Tp^{B}T)-1 resulted in a 6% increase in the intensity of the -BH_3 proton resonances (data not shown). Likewise, irradiation of the -BH_3 protons resulted in a similar intensity increase of the H3′ resonance of the 5′-residue in d(Tp^{B}T)-1, indicative of an NOE. Similar experiments with d(Tp^{B}T)-2 resulted in no apparent signal change in either the -BH_3 or H3′ protons of the 5′-dT residue (data not shown). Therefore, the -BH_3 group in d(Tp^{B}T)-1 was assigned to the pseudoaxial position in the S_p configuration, while the -BH_3 group in d(Tp^{B}T)-2 was assigned to a pseudoequatorial position or R_p configuration. Unfortunately, the observed NOEs in the d(Tp^{B}T)-1 1D NOE difference experiments were not large enough to allow unambiguous assignment of the absolute configurations of the boronated phosphorus in the diastereoisomers. Absolute assignment must wait for X-ray analysis.

Discussion

The effect of boron modification on dimer conformation

We expected to find small differences in chemical and physical properties of unmodified and modified dimers and within the pair of boranophosphate diastereoisomers. Ab initio quantum mechanical calculations indicate that the electronegativity (X) of the -BH_3 group is similar to that of highly electropositive ligands such as Li (X = 1) and BeH (X = 1.5). For comparison, the electronegativity of oxygen is over 3.[35] The calculated geometries for boranophosphate[35] show considerable deviation from the parent phosphate in that the P–B bond is longer (ca. 1.9 Å) than the P–O bond (ca. 1.5 Å) by 25%. Boron modification results in altered physical, chemical and biological properties, as observed in RP-HPLC retention times, hydrophobicities and hydrolysis by phosphodiesterases.[4,5] Specifically, on an RP-HPLC column, the two modified diastereoisomers eluted after the unmodified parent dimer d(TpT), indicating that they are more hydrophobic. Also, the modified diastereoisomers are considerably more resistant to hydrolysis by the phosphodiesterases from snake venom and bovine spleen.[4,5] The ability to separate the individual diastereoisomers allowed us to examine the effect of the configuration of the phosphoryl borane group on the overall conformation within the pair of diastereo-

isomers and between borane-modified and unmodified dimers.

Circular dichroism spectra of dinucleoside monophosphates are dependent on both the extent of base stacking and the conformation.[36] The CD data reported here (Figure 2) suggest that the boranophosphonate internucleoside linkages do not noticeably perturb the stacking interactions between the bases in each of the boron-modified diastereoisomers. The only differences are in the magnitudes of the respective molar ellipticities. The observation of smaller CD intensities as well as smaller intensity changes with changing temperature for both of the boron-modified diastereoisomers relative to unmodified d(TpT) (Figure 2) indicate that boron modification may somewhat reduce or alter the base stacking. The results also indicate that d(Tp^{B}T)-1 (S_p or pseudoaxial configuration) may have better base stacking interactions than does d(Tp^{B}T)-2 (R_p or pseudoequatorial configuration) since d(Tp^{B}T)-1 has greater CD intensity relative to d(Tp^{B}T)-2 (see Figure 2). The conformation and base-stacking interactions of the boron-modified dimers are undoubtedly affected by -BH_3 substitution.

Configurations of boron-modified diastereoisomers at the phosphorus center

The absolute configuration at the chiral phosphorus center of the diastereoisomeric boranomonophosphate dinucleosides was tentatively assigned by 1D NOE difference experiments with d(Tp^{B}T)-1 as the S_p and d(Tp^{B}T)-2 as the R_p stereoisomer. This assignment was supported by enzymatic experiments[4,5] similar to the work of Burgers and Eckstein.[37] The enzymatic reactivities of phosphorothioate nucleosides were correlated with their absolute configurations.[37] Enzymes like snake venom phosphodiesterase (SVPD) catalyze the hydrolysis of 5′-nucleotide esters to produce a free 3′-hydroxyl group.[38] They differentiate between two diastereoisomers of phosphorothioate internucleotide linkages by hydrolyzing one isomer more efficiently than the other.[39] The hydrolysis reaction of phosphorothioate nucleotides by SVPD is highly specific for the R_p-diastereoisomer.[37,40] Similarly only one of the two boranophosphate diastereoisomers, d(Tp^{B}T)-1, was found to act as a viable substrate for SVPD.[4,5] By presuming that the enzyme utilizes only substrates in the absolute R_p-configuration of the phosphorothioate nucleosides at phosphorus, the absolute stereochemistry for boranophosphate dinucleoside diastereoisomers may be assigned by analogy. Noting also that sulfur is the largest atom around the phosphorus center in a nucleotide phosphorothioate while boron is the smallest atom around the phosphorus center in a nucleotide boranophosphate, *an* R_p *configuration in phosphorothioate corresponds to an* S_p *configuration in boranophosphate*. Thus, d(Tp^{B}T)-1 was assigned as the S_p-configuration and d(Tp^{B}T)-2 was assigned to the R_p-configuration. This assignment based on nuclease reactivity is in agreement with the assigned configurations based on our 1D NOE analysis.

Conclusion

The results of the present ^{1}H NMR and CD investigation lead one to conclude that substitution of a phosphodiester by a boranophosphate in thymidylylthymidine, d(TpT), induces minimal structural changes. A similar conclusion was obtained with methylphosphonate analogues.[14] The conformational perturbations were small, and were found mostly in the 3′-residue. The chirality of the boranophosphate moiety in d(Tp^{B}T)-1 and d(Tp^{B}T)-2 had a minimal effect on the sugar–phosphate backbone conformation and on base-stacking interactions. It seems reasonable that the perturbations by the borane group in a boranophosphate oligonucleotide would be localized within the modified site, and that the DNA backbone would readily accommodate these changes. The near equivalence of melting temperatures in 14-mers containing either a normal or BH_3-modified phosphorus linkage supports this prediction.[41] Given that P-BH_3 modified nucleotides are isoelectronic and isostructural analogues of normal P-O nucleotides and exhibit small changes in conformations, yet have increased resistance to nuclease, we conclude that the BH_3-containing nucleotides are good analogues for the naturally occurring O-nucleotides.

Material and Methods

Sample preparations

Synthesis of dithymidine boranomonophosphate, as described previously,[1,42] resulted in a mixture of two diastereoisomers. The boronated diastereoisomers were separated by reverse phase HPLC using a Waters Delta Pak C18-300 Å, 3.9 × 300 mm column, with a mixture of 80% 20 mM KH_2PO_4, pH 4.6 and 20% MeOH solvents at a flow rate of 2 mL min^{-1}.[5] The two diastereoisomers were eluted at 16.6 and 19.7 min, respectively, and were designated d(Tp^{B}T)-1 and d(Tp^{B}T)-2 according to their order of elution from the HPLC column. Separation of sufficient amounts of resolved diastereoisomers for NMR required multiple injections of 0.1 mg mixture. The baselines of the two peaks slightly overlapped.[5] Separations were achieved by separately pooling the left fraction of the first eluting peak for d(Tp^{B}T)-1 and the right fraction of the second peak for d(Tp^{B}T)-2; these procedures were repeated twice to ensure good separation of the two diastereoisomers. The purities of d(Tp^{B}T)-1 and d(Tp^{B}T)-2 were confirmed by individually injecting the pool of separated solutions onto an RP-HPLC column; d(Tp^{B}T)-1 had almost no detectable d(Tp^{B}T)-2 contaminant and d(Tp^{B}T)-2 contained < 1% of d(Tp^{B}T)-1.[5] A small amount of material lacking absorbance at 260 nm was present (data not shown) but did not interfere with assignments or simulation of ^{1}H spectra. Unmodified dimer (Sigma)

had no ^{1}H resonances other than d(TpT), and thus was used without further purification.

Dimers were dissolved in 100 mM NaCl, 10 mM potassium phosphate, pH 7.4, and 0.1 or 1 mM EDTA (for NMR and CD samples, respectively). To prepare NMR samples in D_2O, the above samples were lyophilized twice from 99.9% D_2O (Cambridge Isotope Laboratories), and then dissolved in the original volume of 99.96% D_2O. The dimer concentration of each boron-modified diastereoisomer was ca. 2 and 0.1 mM in the NMR and CD experiments, respectively. Concentrations were based on the UV absorbance at 260 nm, assuming an extinction coefficient (ε_{260}) of 8.4×10^3 M^{-1} cm^{-1} per dimer[43] for both the unmodified and the boron-modified dimers.

CD spectroscopy

CD spectra were recorded on a JASCO J600 spectrophotometer equipped with a water-jacketed cell (1 cm in path length) and sealed with a Teflon stopper, interfaced to and controlled by an IBM PC computer. The temperature was increased in 5 °C intervals from 25 to 80 °C using a recirculating bath. CD spectra were obtained in the 220–340 nm range at 0.2 nm intervals in one scan. Buffer baselines were subtracted from raw dimer ellipticity data. The final spectral data were smoothed using the JASCO J600 software. CD data were obtained in millidegree units and are reported in terms of a dissymmetry factor (g-factor) derived from the expression[9,31]

$$g_\lambda = \Delta A_\lambda / A_{max} \quad (1)$$

where g_λ is the g-factor, ΔA_λ is the CD ellipticity, and A_{max} is the UV absorbance of the same solution and in the same path length used in the CD experiments. The CD ellipticity is determined at the quoted temperature and wavelength, and the UV absorbance is obtained at 25 °C at the maximum wavelength (ca. 260 nm). This parameter is a dimensionless quantity (which has a meaning of normalized ellipticity) and provides a precise way of comparing a closely related group of molecules in a similar concentration range without the need for accurate concentration determination.[9,31]

NMR experiments

NMR spectra were acquired on either a Varian Unity-500 MHz NMR spectrometer or a GE GN-500 MHz NMR spectrometer (for temperature-dependence experiments). All ^{1}H, ^{31}P and ^{11}B spectra were collected using a reverse-detect probe. All NMR samples were prepared in D_2O and 5-mm NMR tubes (Wilmad). One-dimensional ^{1}H NMR spectra were typically collected with a sweep width of 5498.3 Hz, 16k data points, resulting in a resolution of 0.34 Hz/point, and enough scans (256–1024) to obtain good signal-to-noise. The ^{31}P- or ^{11}B-decoupled ^{1}H NMR spectra were obtained with the probe tuned to the ^{31}P or ^{11}B frequency in the X-nucleus channel. Spectra were measured at 30 ± 0.2 °C except for temperature-dependence experiments which were done in the 7–65 °C range (at least 14 temperatures). All ^{1}H chemical shifts were measured relative to TSP (3-trimethylsilyl-propionate-2,3,3,3-d_4 sodium salt) as internal reference. D_2O was used as lock signal. No bulk susceptibility corrections have been made for any of the NMR data.

One-dimensional NOE difference data were obtained with identical on- and off-irradiation experiments. Resonance intensities were determined relative to the internal standard signal (TSP). NOE data were obtained by determining the intensity changes for the on- and off-irradiation spectra.

All ^{1}H 2D spectra were recorded using the Varian Unity-500 MHz spectrometer. TOCSY[44] and ROESY[45,46] spectra were obtained in phase-sensitive mode and collected in a 512×1024 data matrix with a sweep width of 5498.3 Hz, and by positioning the carrier frequency at the HDO signal. Thirty-two scans were acquired per t_1 value with a delay time of 0.8–1.5 s between scans. TOCSY spectra were obtained using a MLEV-17 spin-lock time of 80 ms. ROESY experiments were conducted with a 350-ms mixing time. Samples were collected in the non-spin mode in all two-dimensional experiments. Data were processed off-line on a SUN SPARC station using FELIX version 2.0 software (Hare Research Inc., Woodinville, WA, USA). Spectra were weighted with a shifted skewed sinebell window function in both dimensions. Prior to Fourier transformation the spectra were obtained as a $2k \times 2k$ matrix.

^{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 diethyletherboron trifluoride $Et_2O{\cdot}BF_3$.

^{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.

Conformational analyses were performed on the basis of *J*-coupling constants derived from ^{1}H spectra. First, approximate *J*-couplings were deduced from the experimental spectra which were subsequently, together with the ^{1}H chemical shifts, used as input for the Varian spin-simulation software package (Version 3.2). Scalar couplings and chemical shifts were adjusted to obtain optimal agreement between the experimental and simulated spectra. Sugar ring resonances were treated as a seven-spin system.[22,47,48] The conformational parameters of the sugar–base backbone were deduced from ^{1}H–^{1}H and ^{1}H–^{31}P coupling constants.

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