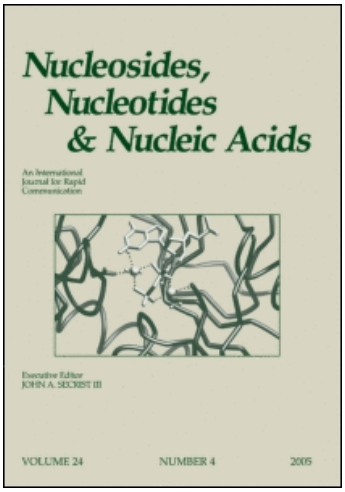

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SYNTHESIS OF NEW CLASSES OF BORON-CONTAINING NUCLEOTIDES

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SYNTHESIS OF NEW CLASSES OF BORON-CONTAINING NUCLEOTIDES

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ABSTRACT

Four different types of boron-modified nucleotides are reported: *P*-boranophosphorothioates, *P*-cyanoboranophosphates, *P*-boranomethylphosphonates, and *P*3′-*N*5′-boranophosphoramidates. Synthesis of dinucleoside borano-phosphorothioates and nucleoside *P*-borano-*P*-thiomonophosphates via a lithium sulfide method is described. The Li_2S method also provides an alternative way to synthesize phosphorothioates through a dinitrophenyl P(V) phosphotriester precursor. The mechanism of Li_2S substitution was investigated.

INTRODUCTION

Novel modified nucleotides are currently attracting attention as probes in biochemistry and molecular biology and as possible therapeutic agents against cancer and viral diseases (1–2). These studies have highlighted certain challenges that need further investigation. For example, there is a need for new analogs having increased resistance towards nucleases, and an ability to be transported into cells via mechanisms leading to biological activity. Previously our laboratory introduced a boranophosphate, the first boronated phosphodiester analogue (3). Here, by introducing borano-, thio-, cyanoborano-, amino-groups or their combinations into phosphate backbones, we present four new classes of boronated phosphodiester analogues (Fig. 1): (a) the *P*-boranophosphorothioate $[S{=}P{-}BH_3]^-$, wherein the two nonbridging oxygen atoms in a phosphodiester group are replaced with sulfur and

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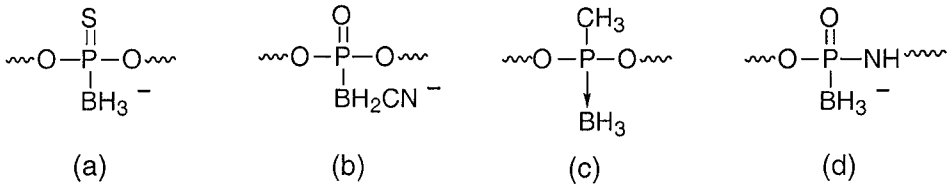


Figure 1. Novel internucleotide linkages: (a) *P*-boranophosphorothioate $[S{=}P{-}BH_3]^-$, (b) *P*-cyanoboranophosphate $[O{=}P{-}BH_2CN]^-$, (c) *P*-boranomethylphosphonate $[CH_3{-}P{-}BH_3]$, (d) *P*3′-*N*5′ boranophosphoramidate $[5'NH{-}P{-}BH_3]^-$.

borane groups respectively; (b) the *P*-cyanoboranophosphate $[O{=}P{-}BH_2CN]^-$, wherein one of the two nonbridging oxygen atoms in a phosphodiester group is replaced with a cyanoborane group; (c) the *P*-boranomethylphosphonate $[CH_3{-}P{-}BH_3]$, wherein the two nonbridging oxygen atoms in a phosphodiester group are replaced with borane and methyl groups; and (d) the *P*3′-N5′-boranophosphoramidates $[5'NH{-}P{-}BH_3]^-$, wherein one of the two nonbridging oxygen atoms in a phosphodiester group is replaced with a borane group and the 5′-bridging oxygen atom is replaced with an NH group.

RESULTS AND DISCUSSION

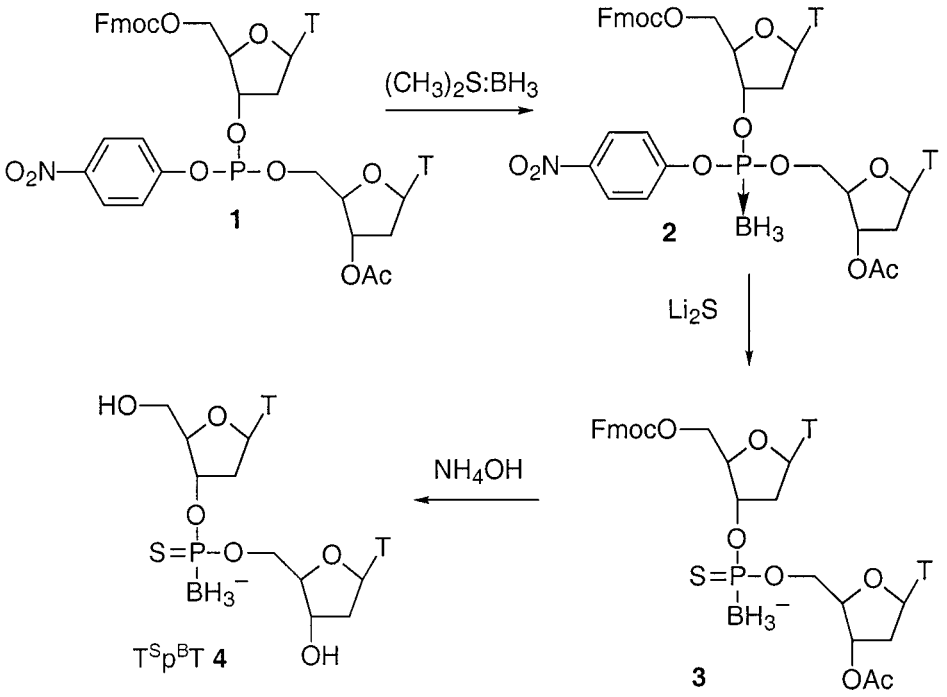
1. *P*-boranophosphorothioate $[S{=}P{-}BH_3]^-$

1a. Synthesis of Dinucleoside Boranophosphorothioate

By structurally combining the phosphorothioate (2) and boranophosphate (3) backbones, we created a new phosphodiester DNa linkage, the boranophosphorothioate (4), $[S{=}P{-}BH_3]^-$.

The modified dinucleoside phosphate **4** is the first example of a boranophosphorothioate compound.

The general procedure for the synthesis of dinucleoside *P*-boranophosphorothioates is outlined in Scheme 1. The phosphite **1** (having ^{31}P NMR signals at 135.9 and 134.8 ppm) (4) was treated with excess borane-methyl sulfide complex to afford phosphite-borane **2** with a ^{31}P NMR signal at 116.6 ppm (br). Dry compound **2** was reacted with lithium sulfide to give **3** which was converted to **4** with NH_4OH/CH_3OH. The overall yield of dithymidine *P*-boranophosphorothioate **4** (T^Sp^BT) was about 28%. Successful separation of two diastereomers (Rp and Sp) of **4** was achieved by reverse-phase HPLC. T^Sp^BT I (the first eluted diastereomer, **4a**): ^{31}P NMR (D_2O, 161.9 MHz) δ (ppm) 161.9 (br); ^1H NMR (D_2O, 400 MHz) δ (ppm) 7.54 (s, 1 H, H6), 7.47 (s, 1 H, H6), 6.13 (t, 1 H, J = 6.8 Hz, H1′), 6.06 (t, 1 H, J = 6.8 Hz, H1′), 4.96–4.91 (m, 1 H, H3′), 4.40–4.37 (m, 1 H, H3′), 4.04–3.89, 3.70–3.59 (2m, 6 H, H4′, H5′), 2.37–2.14 (m, 4 H, H2′), 1.77 (s, 3 H, 5-CH_3), 1.70 (s, 3 H, 5-CH_3), 0.58 (br, 3 H, BH_3). T^Sp^BT II (the second eluted diastereomer, **4b**): ^{31}P NMR (D_2O, 161.9 MHz) δ (ppm) 161.7 (br); ^1H NMR (D_2O, 400 MHz)



Scheme 1.

(ppm) 7.50 (s, 1 H, H6), 7.45 (s, 1 H, H6), 6.15 (t, 1 H, J = 6.8 Hz, H1′), 6.06 (t, 1 H, J = 6.8 Hz, H1′), 4.80 (m, 1 H, H3′), 4.41 (m, 1 H, H3′), 4.17–3.83, 3.68–3.57 (2m, 6 H, H4′, H5′), 2.36–2.29, 2.22–2.13 (m, 4 H, H2′), 1.75 (s, 3 H, 5-CH_3), 1.70 (s, 3 H, 5-CH_3), 0.54 (br, 3 H, BH_3).

Our studies show that the *P*-boranophosphorothioate group is very stable toward acidic or basic hydrolysis at pH 3 or pH 11. The *P*-boranophosphorothioate internucleotide linkage in dimer **4** is also quite stable toward cleavage by both snake venom phosphodiesterase (SVPDE) and bovine spleen phosphodiesterase (BSPDE) (4). As determined by partitioning into octanol/water, the T^SpBT dimer

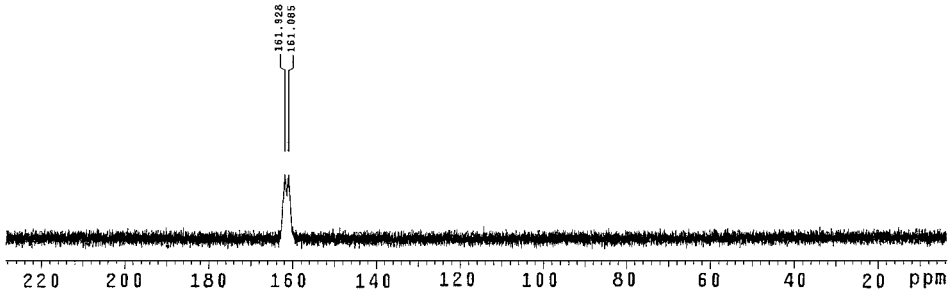


Figure 2. ^{31}P NMR (D_2O) spectrum of T^SpBT **4**.

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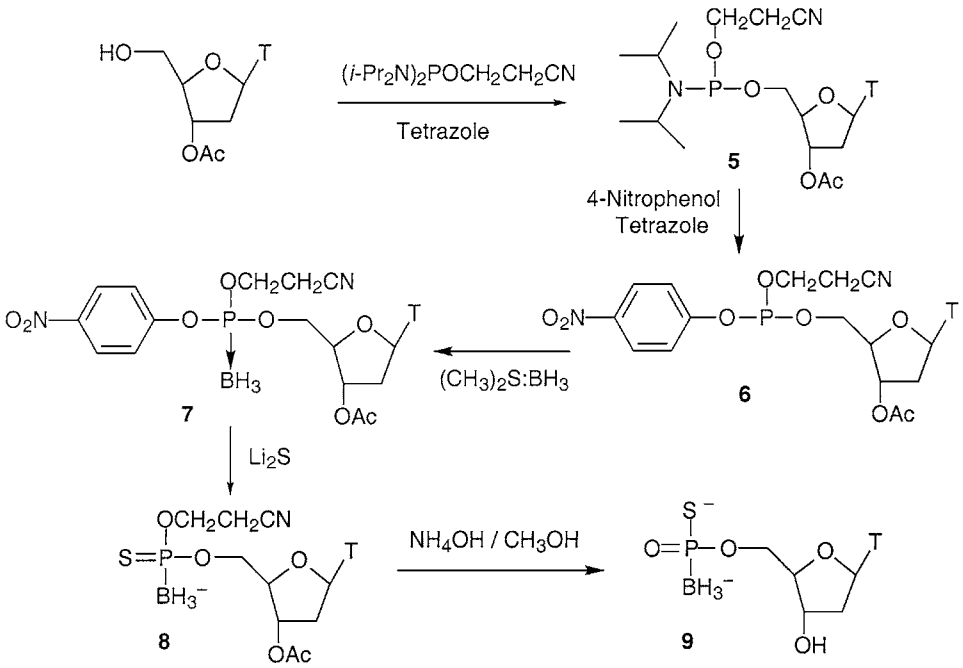
is 320- and 18-fold more lipophilic than normal TpT and Tp^BT) (dithymidime boranophosphate) accordingly.

1b. Synthesis of Nucleoside *P*-borano-*P*-thiomonophosphate

By introducing both a BH_3 and S into a nucleoside *mono*phosphate (NMP), it is possible to create new NMP analogues, nucleoside *P*-borano-*P*-thiomonophosphates ($NMP^{\alpha}BS$), such as thymidine *P*-borano-*P*-thiomonophosphate ($TMP^{\alpha}BS$ **9**). The borano-thio-disubstitution of nucleoside monophosphate should increase the lipophilicity relative to natural NMP and its stability against enzymatic cleavage, thus facilitating studies of enzymes which utilize NMPs and enabling determination of the nature of bond cleavage, the stereochemical course for a particular NMP-mediated activity, and related metal ion effects. Also, *P*-borano-*P*-thiomonophosphates could be very useful tools as prodrugs to increase the bioavailability.

The general procedure for the synthesis of thymidine *P*-borano-*P*-thiomonophosphate is shown as Scheme 2. 3′-*O*-acetylthymidine (0.5 mmol) was phosphitylated with 2-cyanoethyl tetraisopropylphosphorodiamidite ($(i\text{-}Pr_2N)_2POCH_2CH_2CN$) (0.55 mmol) catalyzed by tetrazole (0.25 mmol) in anhydrous DMF at 0°C for 20 min under argon protection to give **5**. Phosphite **5** was treated *in situ* with 4-nitrophenol (0.6 mmol) and tetrazole (1.5 mmol) in anhydrous DMF at room temperature for 30 minutes to afford **6** (^{31}P NMR, around 134 ppm). Compound **6**



Scheme 2.

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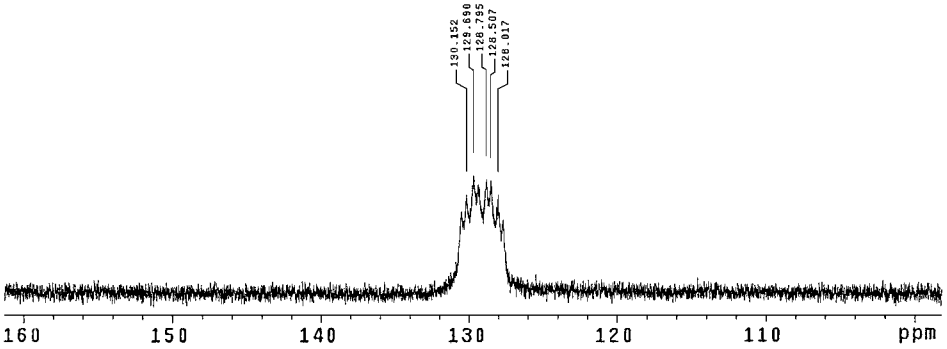


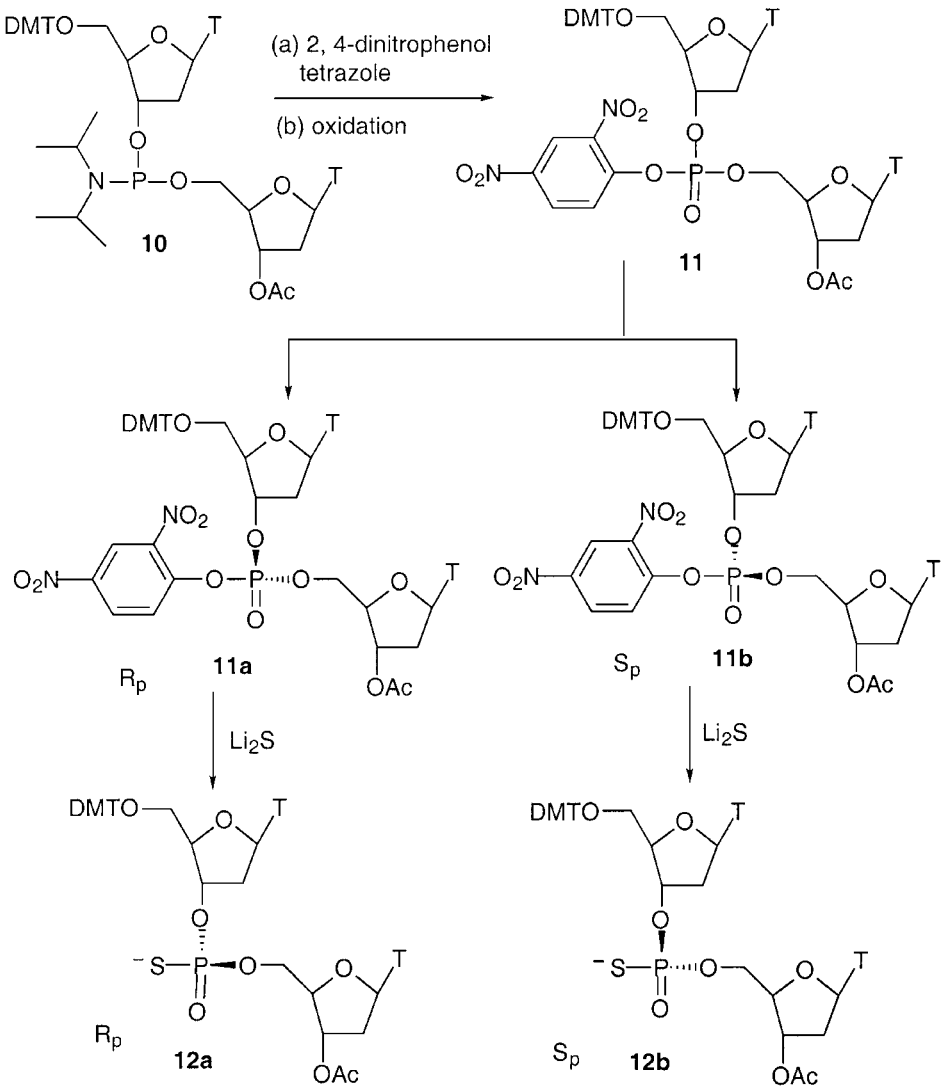
Figure 3. ^{31}P NMR (D_2O) spectrum of **9**, TMP$^{\alpha}$BS.

was boronated by excess $(CH_3)_2S{:}BH_3$ in CH_2Cl_2 to yield phosphite-borane **7** with ^{31}P NMR signal around 116 ppm (br). After removing low-boiling point solvents under reduced presure, the residue was dissolved with anhydrous DMF and treated with lithium sulfide (1 mmol) at room temperature for 1 hour to obtain the crude **8**, with ^{31}P NMR signal around 166.1 ppm (br). Compound **8** was converted to **9** with 4 ml $NH_3/H_2O/CH_3OH$. The resulting crude mixtures were separated by ion-exchange chromatography on QA-Cellulose (HCO_{3^-}) column, eluting with a linear gradient of ammonium bicarbonate buffer. The desired fractions were collected and dried by lyophilization to yield the ammnonium salt of TMP$^{\alpha}$BS **9** (33% overall yield): ^{31}P NMR (D_2O) [Fig. 3] δ 131–128 ppm (br), a key signature of **9**. ^{1}H NMR(D_2O, 400 MHz) δ (ppm) 7.67, 7.48 (2s, 2 isomers, 1 H, H6), 6.23–6.11 (unresolved, 1 H, H1′), 4.48–4.32 (m, 1 H, H3′), 4.13–3.96 (m, 1 H, H4′), 3.91–3.82 (m, 2 H, H5′), 2.29–2.18 (m, 2 H, H2′), 1.81, 1.77 (2s, 3 H, 5-CH_3), −0.20 to 0.67 (br, 3 H, BH_3); MS (FAB$^-$): m/z for M^- 335.

1c. Proposed Mechanism of Li_2S Substitution and New Method for the Preparation of Nucleoside Phosphorothioates from the Dinitrophenyl P(V) Phosphotriester via Li_2S Substitution

For the synthesis of *P*-boranophosphorothioates, the key step is the introduction of a thio-moiety via Li_2S substitution. To better understand and investigate the stereochemical course of Li_2S substitution with nitrophenyl as a leaving group, we synthesized a variety of modified triesters. It was found that the dinitrophenyl containing phosphate triester, compound **11**, is a very good precursor for the synthesis of phosphorothioates.

Previously, phosphorothioate (2) oligonucleotides were prepared using phosphoramidite, H-phosphonate or H-phosphonothioate approaches. All those methods share trivalent P(III) synthetic intermediates and utilize S_8, H_2S, or thiol to introduce the S moiety into target molecules. Here, we report a new method for the preparation of phosphorothioates from the pentavalent P(V) dinitrophenyl phosphotriester via Li_2S substitution and examine the mechanism.



Scheme 3.

The general procedure for the synthesis of protected dinucleoside *P*-phosphorothioates is outlined in Scheme 3. Phosphite **10** (4) was treated with 2,4-dinitrophenol and tetrazole in DMF for 30 min and then left standing, open to air for 30 min to yield compound **11**. Compound **11** was applied to silica gel HPLC column to give two diastereomers of **11**: HPLC conditions: Partisil M9 Whatman 10/50 column; eluants were 3% methanol and 97% ethyl acetate; flow rate, 3.0 ml/min. For the first eluted isomer, **11a**: *Rt* (**11a**) = 14.4 min; ^{31}P NMR (DMSO-d_6, 161.9 MHz) δ (ppm) 9.81; ^{1}H NMR (DMSO-d_6, 400 MHz) δ (ppm) 7.49 (s, 1 H, H6), 7.44 (s, 1 H, H6), 7.33–7.18 (3m, 9 H, Ar-H), 6.84 (d, J = 7.6 Hz, 4 H, Ar-H), 6.18 (t, 1 H, J = 7.0 Hz, H1′), 6.11 (t, 1 H, J = 7.0 Hz, H1′), 5.15 (unresolved m, 2 H),

4.23–4.17 (m, 4 H, H3′, H4′), 4.11–4.09 (m, 4 H, H5′), 3.69 (s, 6 H, $-OCH_3$), 2.34–2.18 (m, 4 H, H2′), 2.01 (s, 3 H, O=C$-CH_3$), 1.94 (s, 3 H, 5-CH_3), 1.70 (s, 3 H, 5-CH_3); For the second eluted isomer, **11b**: *Rt* (**11b**) = 18.0 min, ^{31}P NMR (DMSO-d_6, 161.9 MHz) δ (ppm) 10.63; ^{1}H NMR (DMSO-d_6, 400 MHz) δ (ppm) 7.47 (s, 1 H, H6), 7.43 (s, 1 H, H6), 7.34–7.19 (3m, 9 H, Ar-H), 6.85–6.83 (m, 4 H, Ar-H), 6.19–6.06 (2t, 2 H, H1′), 5.11 (unresolved m, 2 H), 4.21–4.16 (m, 4 H, H3′, H4′), 4.11–4.06 (m, 4 H, H5′), 3.68 (s, 6 H, $-OCH_3$), 2.38–2.16 (m, 4 H, H2′), 2.01 (s, 3 H, O=C$-CH_3$), 1.94 (s, 3 H, 5-CH_3), 1.71 (s, 3 H, 5-CH_3). **11a** and **11b** were reacted with lithium sulfide to give **12a** (^{31}P NMR at 54.2 ppm) and **12b** (^{31}P NMR at 53.4 ppm) respectively. After removing the DMT protecting group by 2.5% $CHCl_2COOH$ in dichloromethane and deprotecting the acetyl group by NH_4OH/CH_3OH, the Rp or Sp dithymidine phosphorothioate was obtained. From empirical rules (2a), which allow the configuration of dinucleoside phosphorothioates to be assigned on the basis of their ^{31}P NMR chemical shift values and relative retention times on reverse phase HPLC, the absolute configuration has been assigned to the diastereomers of **12** by comparison with these criteria. Thus, **12a** which resonates at lower field in the ^{31}P NMR spectra was assigned to the Rp-configuration, and **12b** to the Sp-configuration.

The dinitrophenyl P(V) phosphate intermediates are useful synthons. They are very stable in the dry air while trivalent P(III) intermediates are sensitive to air oxidation. Also the dinitrophenyl is sufficiently stable at acidic conditions to survive the DMT removal step. Lithium sulfide is a solid that can be partly dissolved in DMF. These advantages over the phosphite approach make the pentavalent P(V) method easy to handle for the preparation of phosphorothioates. Further, the two diastereomers of dinucleoside dinitrophenyl phosphate are easily and cleanly separated by silica gel HPLC. For example, the two diastereomers of **11** (5′-DMT dithymidine 3′-acetyl dinitrophenyl phosphate) were obtained in 10 mg scale by silica gel HPLC separation. By appropriately choosing the protecting group, dinitrophenyl phosphate precursors will be very good building blocks for the synthesis of oligonucleoside phosphorothioates with R_p or S_p phosphorothioate linkages inserted at certain positions.

The displacement of the dinitrophenyl group by lithium sulfide successfully introduces an S moiety into a phosphodiester under very mild conditions (room temperature) with high efficiency (almost quantitative conversion). This method with some modifications also has been applied to the syntheisis of the first boranophosphorothioate (4).

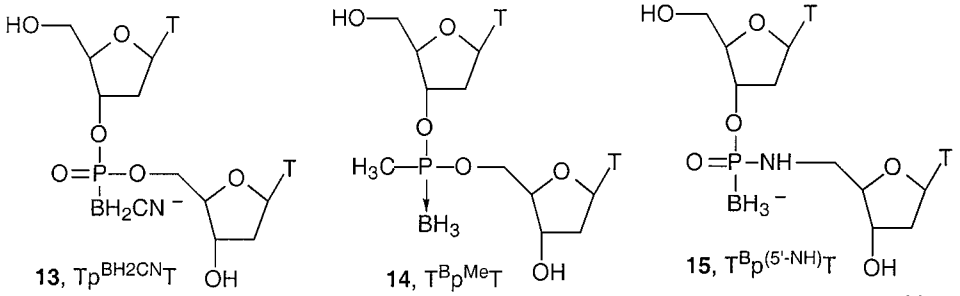
It is important to know whether the Li_2S substitution step is strereospecific or not. After HPLC separation of the individual two diastereomers **11a** and **11b**, which bear a dinitrophenyl leaving group at the P atom, the two diastereomers are allowed to react with nucleophile, sulfide S^{2-}. From the analysis of the ^{31}P NMR chemical shift data before and after the reaction, information about the configuration around the P atom can be obtained and the mechanism of lithium sulfide substitution can be inferred. Typically, at a given set of conditions (solvent, pH, temperature etc.), the ^{31}P NMR chemical shift depends soley on the substituents and the configuration around P. For the two diastereomers **11a** and **11b**, the four

groups at P are the same and the only difference is their relative steric order, the configuration. After lithium sulfide substitution only one group, dinitrophenyl, was replaced by S^{2-}. If the other three groups remain in the same relative order as prior to the reaction, then the configuration would be retained, and the ^{31}P chemical shift change ($\Delta\delta_a = \delta_{12a} - \delta_{11a}$ and $\Delta\delta_b = \delta_{12b} - \delta_{11b}$) for the two diastereomers after the reaction should be the same: $\Delta\delta_a - \Delta\delta_b = 0$. If the reaction occurs with inversion of configuration at the P atom that bears the leaving group, the ^{31}P chemical shift change ($\Delta\delta_a = \delta_{12a} - \delta_{11a}$ and $\Delta\delta_b = \delta_{12b} - \delta_{11b}$) for the two diastereomers after the reaction should differ: $\Delta\delta_a - \Delta\delta_b \neq 0$. If the reaction proceeds through a single S_N1 mechanism, there would be a mixture of two new diastereomers and thus two ^{31}P NMR peaks after the reaction. We observed only one ^{31}P NMR signal after Li_2S substitution (from **11** to **12**). From **11a** to **12a**, the ^{31}P chemical shift difference $\Delta\delta_a$ was 44.35 ppm, while from **11b** to **12b**, ^{31}P chemical shift difference $\Delta\delta_b$ was 42.77 ppm. The observed difference between $\Delta\delta_a$ and $\Delta\delta_b$ is 1.58 ppm, which is definitely greater than experimental error. This difference and the observation that there is only one diastereomer from Li_2S substitution indicate that S^{2-}substitution of the dinitrophenyl group underwent total configuration inversion, i.e. via an S_N2 mechanism.

In this section, the first example of synthesizing dinucleoside phosphorothioates via a pentavalent intermediate, dinucleoside dinitrophenylphosphate, following Li_2S treatment has been described, and the related sterochemical course has been investigated. It was found that the replacement of a nitrophenyl group by S^{2-} likely occurs through an S_N2 mechanism with total configuration inversion at the P atom. Since the phosphorothioate precursor (pentavalent dinitrophenylphosphate) is stable to oxygen and acidic conditions, and can be easily and stereospecifically converted to phosphorothioates with Li_2S treatment, this method should be perceived as a convenient general approach for the synthesis of phosphorothioates, especially for the introduction of a stereochemically pure R_p or S_p phosphorothioate linkage into oligonucleotides.

2. *P*-cyanoboranophosphates $[O{=}P{-}BH_2CN]^-$

The first dinucleoside cyanoboranophosphate compound, dithymidine cyanoboranophosphate **13**, $Tp^{BH_2CN}T$, was synthesized and its chemical structure was



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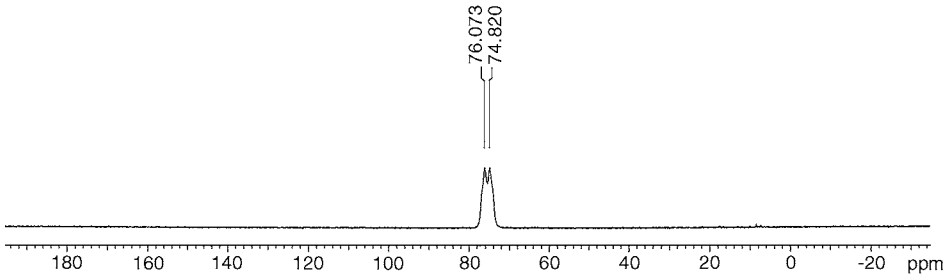

Figure 4. ^{31}P NMR (D_2O) spectrum of **13**, $Tp^{BH_2CN}T$.

established via spectroscopic methods with a typical ^{31}P NMR (Fig. 4) signal at 75 ppm (br).

3. *P*-boranomethylphosphonates [CH_3–P–BH_3]

The first dinucleoside boranomethylphosphonate compound, dithymidine boranomethylphosphonate **14**, $T^Bp^{Me}T$, was synthesized and its chemical structure was established via spectroscopic methods with a typical ^{31}P NMR (Fig. 5) signal at 147.6 ppm.

4. *P*3′-*N*5′-boranophosphoramidates [5′NH–*P*–BH_3]$^-$

The first dinucleoside P3′-N5′ boranophosphoramidate compound, dithymidine P3′-N5′ boranophosphoramidate **15**, $T^Bp^{5'-NH}T$, was synthesized and its chemical structure was established via ^{31}P NMR (Fig. 6), ^{1}H NMR, and FAB$^+$HRMS.

To summarize, we have synthesized four totally new types of boron-containing phosphodiester compounds as model nucleic acid mimics. Their similarity to natural nucleic acids and anticipated unique properties such as high lipophilicity and resistance to enzymatic cleavage, in conjuction with their potential utility as carriers of ^{10}B in boron neutron capture therapy (BNCT) (5) for the treatment of cancer,

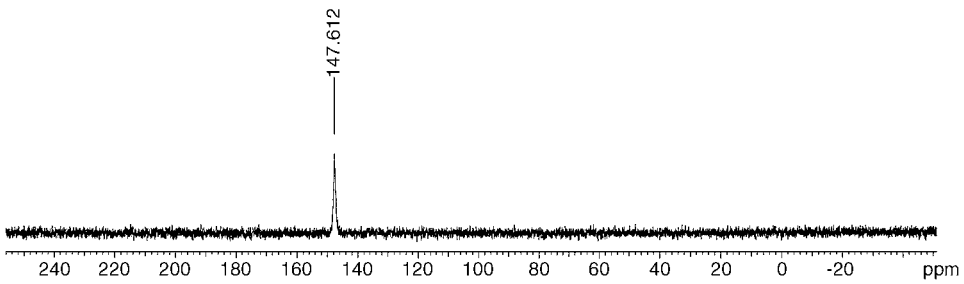

Figure 5. ^{31}P NMR (DMSO-d_6) spectrum of **14**, $T^Bp^{Me}T$.

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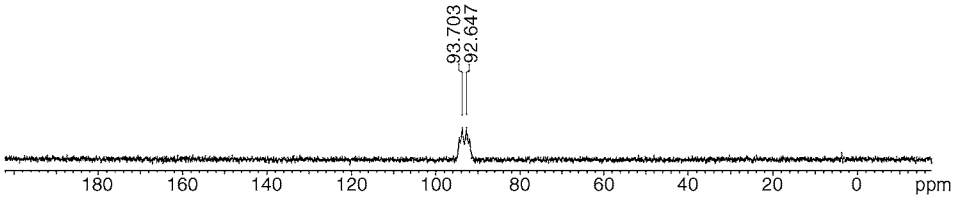

Figure 6. ^{31}P NMR (D_2O) spectrum of **15**, $T^{B}p^{5'NH}T$.

make the new boron analogues promising candidates for mechanistic, diagnostic and therapeutic applications.

ACKNOWLEDGMENT

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