Individual Isomers of Dinucleoside Boranophosphates as Synthons for Incorporation into Oligonucleotides: Synthesis and Configurational Assignment

by **Zinaida A. Sergueeva**[a]), **Dmitri S. Sergueev**[a]), **Anthony A. Ribeiro**[b]), **Jack S. Summers**[a]), and **Barbara Ramsay Shaw***[a])

[a]) Department of Chemistry, Duke University, Durham, NC 27708, USA
[b]) Duke NMR Spectroscopy Center and Department of Radiology, Duke University, Durham, NC 27708, USA

Dedicated to Prof. Dr. *Frank Seela* on the occasion of his 60th birthday

Individual isomers of the protected boranophosphates **5a** and **5b**, *i.e.*, the N^6-benzyl-2′-deoxy-5′-*O*-(4,4′-dimethoxytrityl)adenosin-3′-yl 2′-deoxy-4-*O*-(4-nitrophenyl)uridin-5′-yl boranophosphates, were synthesized *via* stereospecific silylation and boronation of their *H*-phosphonate precursors. 2D-NMR Spectroscopic studies yielded an initial assignment of the isomer configuration, which was further confirmed unambiguously by a parallel chemical synthesis. Deprotection of the 'dimers' **5a** and **5b** yielded the individual [*P*(*R*)]- and [*P*(*S*)]-isomers **7a** and **7b**, respectively, *i.e.*, the 2′-deoxyadenosin-3′-yl 2′-deoxycytidin-5′-yl boranophosphates. Their substrate properties toward phosphodiesterase I were identical to those of the previously characterized isomers of dithymidine boranophosphate. The protected 'dimers' **5a** and **5b** can be used as synthons to incorporate the boranophosphate linkage with a defined configuration to selected positions of an oligonucleotide chain.

Introduction. – In recent years, much attention has been drawn to applications of oligonucleotides as diagnostic tools and therapeutic remedies. Among numerous modified oligonucleotides that were tested [1], several showed considerable promise, *e.g.* phosphorothioates [2], peptide nucleic acids [3], morpholinos [4], and methylphosphonates [5]. Oligonucleoside boranophosphates (BH_3^--ODNs), in which borane (BH_3) is substituted for one of the non-bridging phosphate O-atoms (= *P*-boranophosphate) [6], could be a useful addition to this arsenal of oligonucleotide tools. The boranophosphate modification maintains the negative charge of the normal phosphate and imparts valuable properties to oligonucleotides. The BH_3^--ODNs exhibit high nuclease resistance [6][7], increased hydrophobicity [6][7a,d], altered metal affinities [8], and have potential for use in boron neutron-capture therapy [9]. The usefulness of the boranophosphate modification is demonstrated in a one-step direct PCR sequencing method that exploits the excellent substrate properties of deoxynucleoside P^{α}-boranotriphosphates and the nuclease resistance of the boranophosphate linkage [10]. Further, the ability of a BH_3^--ODN to support RNase H mediated cleavage of the complementary RNA suggests they might find application in antisense technology [11].

The demand for better performing oligonucleotides in therapeutic and diagnostic applications has led to the design of a next generation of oligonucleotides, comprised of mixed or alternating backbones. Such oligonucleotides have demonstrated enhanced nuclease resistance, improved hybridization properties, and fewer immune-stimulatory

side effects [2][12]. In view of this, oligonucleotides containing alternating boranophosphate linkages deserve attention as potentially useful molecules. In this account, we describe the first synthesis of a protected dinucleoside boranophosphate, which can be further used as a synthon for incorporation into an oligonucleotide chain.

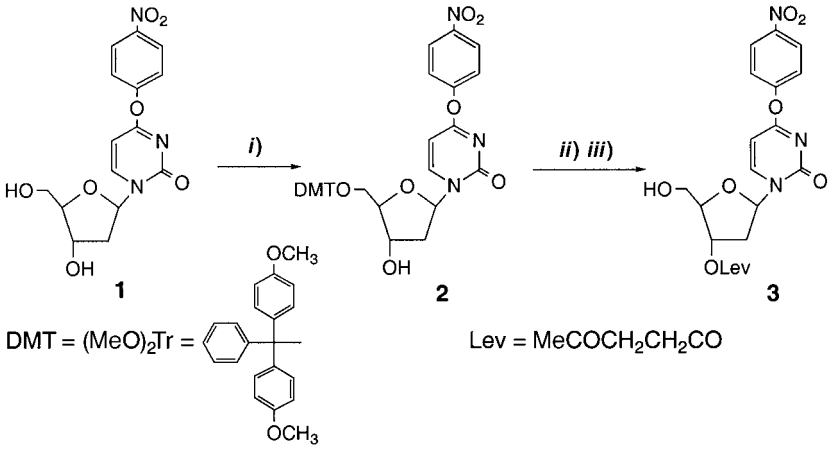
NMR Spectroscopy has been growing as a tool for probing the structure of nucleic acids and their interactions with proteins [13]. The determination of nucleic-acid tertiary structures by NMR can be limited by the small number and redundancy of available protons and their limited exposure to exterior contacts. BH_3^--ODNs may find use as probes in NMR spectroscopy if NOE contacts to the borane H-atoms can be measured. The measurement of NOEs to H-atoms at the phosphonyl positions in boranophosphate-substituted nucleic acids will enhance the reliability and resolution of NMR structures. Since boranophosphates are chiral at the P-atom, comparison of the NOE contacts for the two stereoisomers of a particular boranophosphate linkage could potentially yield important structural information. Introduction of the linkage requires synthesis of diastereoisomerically pure dinucleoside synthons. Here we describe the preparation of the individual isomers of N^6-benzyl-2′-deoxy-5′-*O*-(4,4′-dimethoxytrityl)-adenosin-3′-yl 2′-deoxy-4-*O*-(nitrophenyl)uridin-5′-yl boranophosphates in high stereotopic purity and the assignment of their configuration.

Results and Discussion. – *Synthesis and Separation of Isomers.* We have shown that the synthesis of BH_3^--ODNs *via* their phosphonate precursors proceeds smoothly and in high yield [7d][14]. In addition, the two dinucleoside *H*-phosphonate stereoisomers can be conveniently separated by silica gel chromatography [15]. We recently demonstrated that individual isomers of dithymidine *H*-phosphonate (= *P*-deoxythymidinyl-(3′→5′)-thymidine) could be converted to their boranophosphate analogs stereospecifically with retention of configuration at the P-atom [16]. Based on these results, our strategy for the present synthesis included *H*-phosphonate condensation followed by separation of the isomers, stereospecific silylation and boronation, and finally hydrolysis of trimethylsilyl ester and 3′-OH group deprotection (see below, *Scheme 2*).

The choice of sugar and especially nucleobase protecting groups is very important to the successful synthesis of BH_3^--ODNs with bases other than thymine. We have discovered that acyl base-protecting groups in nucleosides such as benzoyl, isobutyryl, and acetyl are readily reduced by various borane-amine complexes to give *N*-alkylated nucleosides [17] that could not be easily deprotected. While our synthetic strategy was to avoid the use of amides for base-protection, we were able to employ N^6-benzoyl-2′-deoxyadenosine in our synthesis, as the reduced N^6-benzyl-2′-deoxyadenosine can be deprotected by several methods [18]. Thus, commercially available N^6-benzoyl-2′-deoxy-5′-*O*-(4,4′-dimethoxytrityl)adenosine 3′-*H*-phosphonate was chosen here as the 5′-nucleotide component for the condensation reaction. The convertible nucleosides, *i.e.* 4-*O*-substituted 2′-deoxyuridines, are convenient intermediates to various N^4-substituted 2′-deoxycytidine derivatives [19]. Nucleophilic substitution at the 4-position of such 2′-deoxyuridines by ammonia results in 2′-deoxycytidine as the only product [19a,c,d]. We selected for our synthesis 2′-deoxy-4-*O*-(4-nitrophenyl)-uridine (**1**), described by *Miah et al.* [19c], which is quite stable under mild alkaline conditions, yet is completely converted to 2′-deoxycytidine during standard oligonucleotide deprotection by concentrated aqueous ammonia. Conversion of **1** to nucleoside

component **3** followed a standard procedure depicted in *Scheme 1*. Protection of the 5′-OH group with the 4,4′-dimethoxytrityl ($(MeO)_2Tr$ group resulting in 2′-deoxy-5′-(4,4′-dimethoxytrityl)-4-*O*-(4-nitrophenyl)uridine (**2**) proceeded smoothly with 78% yield. Subsequent 3′-OH acylation with levulinic anhydride ($(Lev)_2O$) and *in situ* 5′-OH deprotection provided 2′-deoxy-3′-*O*-levulinoyl-4-*O*-(4-nitrophenyl)uridine (**3**; 80%) as a slightly yellow solid. The levulinoyl group was preferred over the (*tert*-butyl)dimethylsilyl group ($^t BuMe_2Si$) or other acyl groups for 3′-OH protection because of the very mild conditions required for its removal. Treatment with sufficient hydrazine hydrate for 3′-OH deprotection did not cause significant substitution of 4-nitrophenol in **3**, whereas alkaline deprotection of acyl groups or treatment with fluoride ion to remove the $^t BuMe_2Si$ group could cause hydrolysis of the aryl group.

Scheme 1. *Synthesis of the 3′-Protected Convertible 2′-Deoxyuridine* **3**

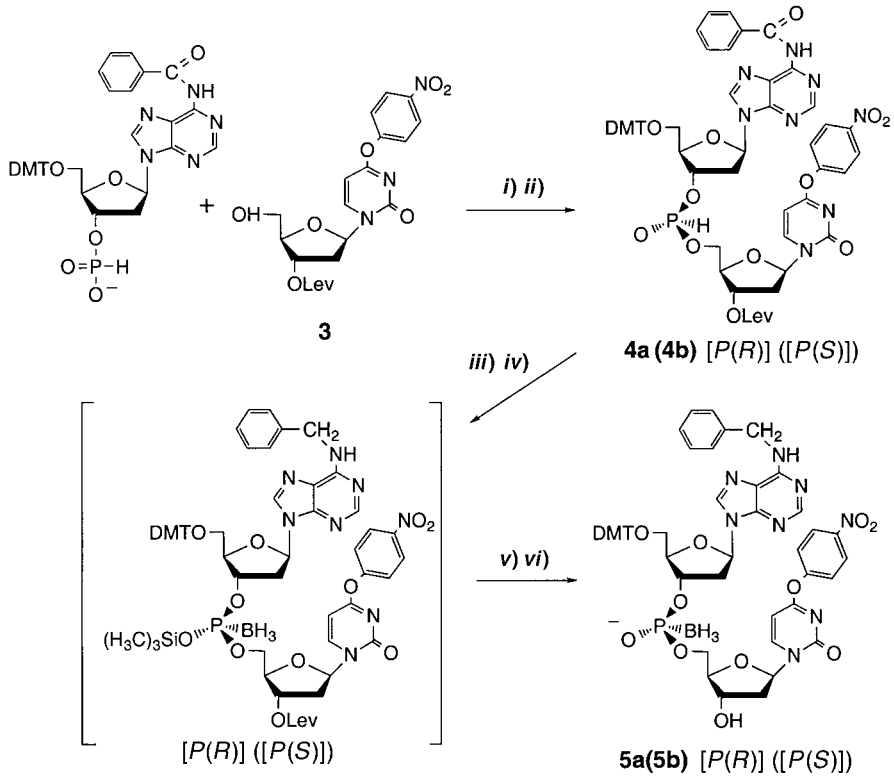


i) $(MeO)_2TrCl$. *ii*) $(Lev)_2O$; *iii*) H^+.

Condensation of N^6-benzoyl-2′-deoxy-5′-*O*-(4,4′-dimethoxytrityl)adenosine 3′-phosphonate and **3** with pivaloyl chloride (=2,2-dimethylpropanoyl chloride; PivCl) resulted in a *ca.* 1:1 diastereoisomer mixture of N^6-benzoyl-2′-deoxy-5′-*O*-(4,4′-dimethoxytrityl)adenosin-3′-yl 2′-deoxy-3′-*O*-levulinoyl-4-*O*-(4-nitrophenyl)uridin-5′-yl *H*-phosphonate (**4**) according to ^{31}P-NMR (*Scheme 2*). After extraction with saturated $NaHCO_3$ solution, the isomer mixture was separated by HPLC (silica gel, isocratic mode). The fast migrating isomer **4a** was isolated in 98% diastereoisomer purity, while the diastereomer purity of the slower migrating isomer **4b** was 84%, as judged by ^{31}P-NMR. The combined yield of **4a** and **4b** was 73%. The enriched *H*-phosphonate isomer preparations **4a**,**b** were converted to boranophosphates **5a**,**b** without isolation of the protected intermediates.

As was shown previously [16], conversion of the *H*-phosphonate diester to the boranophosphate diester proceeds stereospecifically with retention of the configuration. Therefore, we anticipated that diastereoisomeric purity will not change throughout the transformation. Silylation and boronation reactions proceeded near quantitatively, as determined by ^{31}P-NMR analysis. The excess of the silylating and boronating

Scheme 2. *Synthesis of the Individual Isomers* **5a** *and* **5b** *of Protected Dinucleoside Boranophosphate*



i) PivCl. *ii*) Direct-phase HPLC separation of the isomers. *iii*) BSA. *iv*) $BH_3 \cdot {}^{i}Pr_2EtN$. *v*) H_2O. *vi*) $NH_2NH_2 \cdot H_2O$.

agents were removed by precipitation in hexane, and the residue was treated with pyridine/H_2O to hydrolyze the boranophosphate trimethylsilyl ester. Removal of the 3′-*O*-levulinoyl protection group proceeded readily with hydrazine hydrate. During the reaction there was an appearance of a faint yellow color, indicating some release of 4-nitrophenol. The estimated extent of the hydrolysis and/or substitution of 4-nitrophenol (based on the color intensity, TLC, and reversed-phase HPLC analysis) was less than 5%. The resulting N^6-benzyl-2′-deoxy-5′-*O*-(4,4′-dimethoxytrityl)adenosin-3′-yl 2′-deoxy-4-*O*-(4-nitrophenyl)uridin-5′-yl boranophosphates (**5a** and **5b**; enriched isomer preparations) were isolated from the crude reaction mixtures by reversed-phase HPLC (*Fig. 1*). Analytical reversed-phase HPLC assessments of the diastereomer purity of the boranophosphates **5** in the crude reaction mixtures (> 98% for **5a** and 80–85% for **5b**) agree well with the ^{31}P-NMR assessments of the phosphonates **4a** and **4b**[1]), confirming the stereospecific character of the boronation procedure. The

[1]) We could not assess the diastereoisomer purity of the boranophosphates **5** by ^{31}P-NMR due to broad overlapping signals of the boranophosphate isomers.

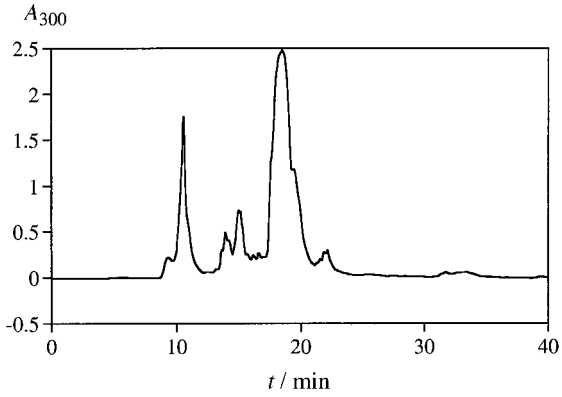


Fig. 1. *Preparative reversed-phase HPLC isolation of* **5b** *from the reaction mixture* (*Scheme 1*). The main-peak shoulder corresponds to **5a** impurity. Conditions: *Microsorb RP-18*. 21.4 × 250 mm column, elution with gradient 40–80% MeCN in 20 mM $(Et_3NH)HCO_3$ (pH 8.0) for 45 min, flow rate 6 ml/min.

elution order of the isomers observed on reversed-phase HPLC was reversed when compared to elution on silica gel. As shown in *Fig. 1*, **5b** elutes first on reversed-phase HPLC, with the isomer impurity **5a** appearing as a shoulder with greater retention time. The diastereomer purity in the final **5a** and **5b** preparations was increased after reversed-phase HPLC separation to 98 and 94%, respectively.

Configuration Assignment of the Isomers **5a** *and* **5b** *by NOE Contacts to the Borane Protons.* The configuration at the P-atom has been found to be important to a number of properties of substituted nucleic acids, such as nuclease resistance, hybridization ability, and hydrophobicity [20]. However, there is not currently available a general method for assigning the configuration at the P-atom of boranophosphate nucleic acids. Two dimensional (2D) NMR has been used to establish the configuration at the P-atom for a number of phosphotriester and methylphosphonate analogs [21], which are structurally similar to BH_3^--ODNs. We hope to develop a reliable physical method for assigning configuration in boranophosphate analogs on measured NOE contacts to the borane protons. Concurrently, we would obtain useful information about the overall conformation of the BH_3^--ODNs.

COSY and NOESY spectra were recorded for each isomer. All ribose and nucleoside proton resonances of isomers **5a** and **5b**, and most of the protecting-group resonances, were unambiguously assigned based on the cross-peaks in the 2D COSY and NOESY plots. The chemical shifts of the base and sugar protons are presented in *Table 1*.

While, in principle, the connection of the ribose rings *via* five single bonds, *i.e.* C(3′)–O–P–O–C(5′)–C(4′), gives rise to a large number of possible states, evidence suggests that the protected isomers **5a** and **5b** reside in a limited number of accessible conformations, which are not unlike *A*- or *B*-form DNA. The program Insight (*Molecular Simulations Inc.*, San Diego, CA) was used to calculate distances between the two non-bridging phosphonyl O-atom positions and the protons of the bases and ribose units by assuming that the 'dimer' adopted either an *A*- or *B*-like conformation. All NOE contacts predicted by the *A*- and *B*-form models that were not obscured by overlapping resonances were observed (including NOE cross-peaks between protons

Table 1. *Resonance-Frequency Assignments for the ^{1}H-NMR Spectra of Isomers* **5a** *and* **5b**

Proton[2])	**5a** δ [ppm]	**5b** δ [ppm]	Proton[2])	**5a** δ [ppm]	**5b** δ [ppm]
2′-Deoxyuridine:			2′-Deoxyadenosine:		
H–C(5)(U)	6.175	6.188	H–C(2)(A)	8.090	8.087
H–C(6)(U)	8.558	8.538	H–C(8)(A)	8.212	8.215
OH–C(3U)(U)	5.396	5.333	H–C(1′)(A)	6.359	6.362
H–C(1′)(U)	5.369	6.080	H–C(2′)(A)	2.976	2.977
H–C(2′)(U) (up)	2.037	1.992	H′–C(2′)(A)	2.464	2.47
H′–C(2′)(U) (down)	2.230	2.223	H–C(3′)(A)	4.936	4.926
H–C(3′)(U)	4.246	4.195	H–C(4′)(A)	4.199	4.182
H–C(4′)(U)	3.919	3.945	H–C(5′)(A)	3.234	3.245
H–C(5′)(U)	3.960	3.93	H′–C(5′)(A)	3.186	3.158
H′–C(5′)(U)	3.754	3.750			

from distant parts of the dinucleotide, such as those of the uracil base and the adenine deoxyribose (H–C(**6**)/H–C(3′)(A) and 2 H–C(2′)(A); H–C(**5**)(**U**)/H–C(3′)(A) and 2 H–C(2′)(A)), those between the adenine base and deoxyribose H–C(**2**)(**A**)/H–C(5′)(A) and H–C(2′)(A); H–C(**8**)/H–C(1′)(A), H–C(3′)(A), H–C(4′)/A), H–C(5′)(A), and 2 H–C(2′)(A)), and contacts between the two deoxyribose units H–C(**5′**)(**U**) and H–C(**4′**)(**U**)/2 H–C(2′)(A); H′–C(**5′**)(**U**)/2 H–C(2′)(A); H–C(**1′**)(**A**)/H–C(4′)(U) and H–C(5′)(U), and H′–C(5′)((U))[2]). Only a few observed NOE contacts could not be explained on the basis of the molecular model. A strong observed NOE that was not predicted by either the *A*- or *B*-form model is the NOE cross-peak H–C(4′)(A)/H–C(3′)(U). The appearance of this cross-peak could be explained if rotation about the backbone allows two riboses to slide across each other. The similarity of the conformations adopted by the dinucleotide and those of the *A*- and *B*-forms of DNA might be due to favorable stacking interactions between the bases and/or protecting groups [22].

The data do not, however, suggest that the dinucleotide has a strong preference for either of the tested two conformations. Plots of observed NOE *vs.* r^{-6} (where r is the calculated distance) showed a great deal of scatter for both the distances calculated for the *A* and *B* conformations (data not shown), indicating that neither model accurately reflects the conformation adopted by the compound. Analysis of ribose-proton splitting patterns (which correlate with the conformation of the ring [23]) indicates that neither the uridine nor adenosine deoxyribose of either isomer has a great preference for adopting an *N*- *vs. S*-conformation, and suggests a rapid equilibrium between the available states. The data agree with a one-dimensional NOE and *J*-coupling analysis previously carried out for dithymidine boranophosphate isomers [24].

The similarity of the one-dimensional ^{1}H-NMR spectra of the two isomers **5a** and **5b** suggests that the configuration at the P-atom has little effect on the conformation. The chemical shifts and splitting patterns of the ribose-proton resonances for equivalent protons were very similar for the two isomers, indicating similar ribose and linkage conformations. Additional support for the contention that the config-

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[2]) For convenience, A, U, and C are used instead of A_d, U_d, and C_d, respectively, for the 2′-deoxynucleoside moieties.

uration at the P-atom does not affect the overall conformation of the dinucleotides is provided by a statistical comparison of the integrated volumes of the cross-peaks in their NOESY plots. If the two isomers adopted substantially different conformations, the differences in the NOEs of protons whose proximities are constrained by their being in the same ribose or nucleoside unit would be expected to display less variation than the NOEs of protons that are not constrained. We found that this was not the case. Of the 46 pairs of data used in the analysis, 8 had a difference in NOE intensity greater than the standard deviation in the differences ($|\Delta i_m| > \sigma$, symbols defined in the *Exper. Part*). The data shows that the rate of occurrence of peaks having $|\Delta i_m| > \sigma$ was greatest when the protons were within a single ribose unit (3 of 14 for contacts between two ribose protons of either A or U[2]), next greatest if between a base proton and a ribose of the same nucleoside (4 of 16), rarer for contacts between protons of the two ribose units (1 of 9), and were not observed for contacts between the base of one and the ribose of the other (0 of 9). Since the integrated volumes change with r^6, we feel this provides good evidence that conformational behaviors of the two isomers were very similar.

Other authors have assigned configuration at the P-atom to substituted nucleotide 'dimers' using either coupling constants between the P-atom and ^{13}C of the ribose [25], or from NOEs to $H-C(4')$ at the 5′-terminus of the 'dimer' [21b,c][26]. Neither of these approaches worked for our case. Lines in the ^{13}C-NMR spectra of the two isomers were too broad for us to make an accurate comparison of coupling constants. Comparison of the NOEs of the two isomers from the adenosine $H-C(4')$ (as done in the case of the methyl phosphate NMR study) was not possible because of overlap of the H–C(4′)(A) resonance of isomer **5b** with the H–C(3′)(U) resonance. We have had more success using the NOE contacts to the borane to distinguish the configuration at the P-atom.

Significantly, the NOESY spectrum showed cross-peaks from a variety of the protons to the borane protons (*Table 2*), with integrated cross-peak volumes dependent on the isomer. The differences in the NOEs observed to the borane ^1H-resonances of the two isomers were much greater than the differences observed for other protons, reflecting the different environments of the borane moiety in the two isomers. Of the 46 pairs of non-borane NOE peaks, 38 differed by less than the standard deviation; in contrast, only 3 of the 10 borane contacts differed by less than 1 σ, and 5 of the 10 differed by more than 2 σ (see *Exper. Part*).

Table 2. *Intensity of the NOESY Contacts to the BH_3 H-Atoms*

Borane to H contact[2])	**5a**	**5b**	Borane to H contact	**5a**	**5b**
2′-Deoxyuridine:			2′-Deoxyadenosine:		
H–C(5)(U)	1.245	1.157	H–C(3′)(A)	3.363	2.775
H–C(6)(U)	1.123	1.292	H–C(4′)(A)	1.693	1.247
H–C(2′)(U)	0.425	0.556	H–C(5′)(A)	0.485	0.000
H–C(3′)(U)	0.546	–[a])	H′–C(5)(A)	0.666	0.000
H–C(4′)(U)	0.329	–[b])			
H–C(5′)(U)	0.451	1.072			
H′–C(5′)(U)	0.752	1.102			

[a]) Overlaps the H–C(4′)(A) resonance. [b]) Overlaps with H–C(5′)(U) resonance.

Both isomers displayed NOE transfer to the borane resonance from the 2′-deoxyadenosine H–C(1′) and H–C(3′) and the 2′-deoxyuridine H–C(6). In the spectrum of isomer **5b**, there was a NOE to a position where the signals from the 2′-deoxyadenosine H–C(4′) and the 2′-deoxyuridine H–C(3′) protons overlapped. In **5a**, the H–C(4′)(A) and H–C(3′)(U) resonances were resolved, and both contributed NOEs to the spectrum. Significant differences in the cross-peaks to the borane in the two spectra included the NOEs from H–C(4′) and H–C(5′)(U) and H′–C(5′)(U) and from 2 H–C(5′)(A). This is clearly demonstrated in the portion of the 2D spectra reproduced in *Fig. 2*. While better information would surely come with a more rigid structure, our NOE data is most consistent with isomer **5a** having the [*P*(*R*)] configuration, and isomer **5b** being [*P*(*S*)].

Assignment of the **5a** *and* **5b** *Configuration by Parallel Synthesis of the Boranophosphate and Phosphorothioate Analogs.* Conversion of a phosphonate diester to a boranophosphate proceeds under retention of the configuration at the P-atom [16]. Thus, based on our 2D-NMR assignments, we expect that fast eluting H-phosphonate **4a** has a [*P*(*R*)] configuration and slow eluting **4b** has [*P*(*S*)]. In principle, the elution order of protected dinucleoside *H*-phosphonates in silica-gel chromatography strongly correlates with their configuration, being [*P*(*R*)] for the fast-eluting and [*P*(*S*)] for the slow-eluting isomer [15]. However, as the *H*-phosphonates **4** are new compounds, we could not rely entirely on the elution order to establish their configuration.

Fortunately, we could take advantage of a study by *Seela* and *Kretschmer* [15a,b], who demonstrated that sulfurization of *H*-phosphonates occurred with retention of the configuration. Both the phosphorothioate and the boranophosphate dinucleosides can be prepared with retention of configuration from a common *H*-phosphonate intermediate. Since assignments of dinucleoside phosphorothioate configuration are well-established [20a][27], we could confirm by parallel synthesis the configurational assignment of the dinucleoside boranophosphates. Thus, the *H*-phosphonates **4a** and **4b** were treated as in *Scheme 1*, but the boronation step was replaced by thioation with elemental sulfur. The phosphorothioate analogs of **5a** and **5b** were then deprotected in analogy to the transformations represented in *Scheme 3*. The resulting isomers of 2′-deoxyadenosin-3′-yl 2′-deoxycytidin-5′-yl phosphorothioate were characterized by their relative mobility on reversed-phase HPLC and their resistance to snake venom phosphodiesterase (not shown). The study revealed that the phosphonate **4a** is converted to phosphorothioate 'dimer' having [*P*(*S*)] configuration, thereby confirming that the faster migrating phosphonate 'dimer' has indeed an [*P*(*R*)] configuration[3]). Therefore, assignment of the boranophosphate isomers made by 2D-NMR fully agrees with the data obtained by the parallel chemical synthesis of the well-characterized phosphorothioate analogs.

Preparation of the 2′-Deoxyadenosin-3′-yl 2′-Deoxyuridin-5′-yl Boranophosphates **7a** *and* **7b** *and Study of Their Resistance toward Snake Venom Phosphodiesterase (SVP).* Deprotection of **5a** and **5b** (*Scheme 3*) required optimization of the standard conditions. A number of reports have indicated that the acidic removal of a 5′-$(MeO)_2$Tr

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[3]) The change in the assignment [*P*(*S*)] to [*P*(*R*)] is due to the *CIP* rules, which consider the H-atom in the phosphonates as the least preferred, and the S-atom in the phosphorothioates as the most preferred substituent.

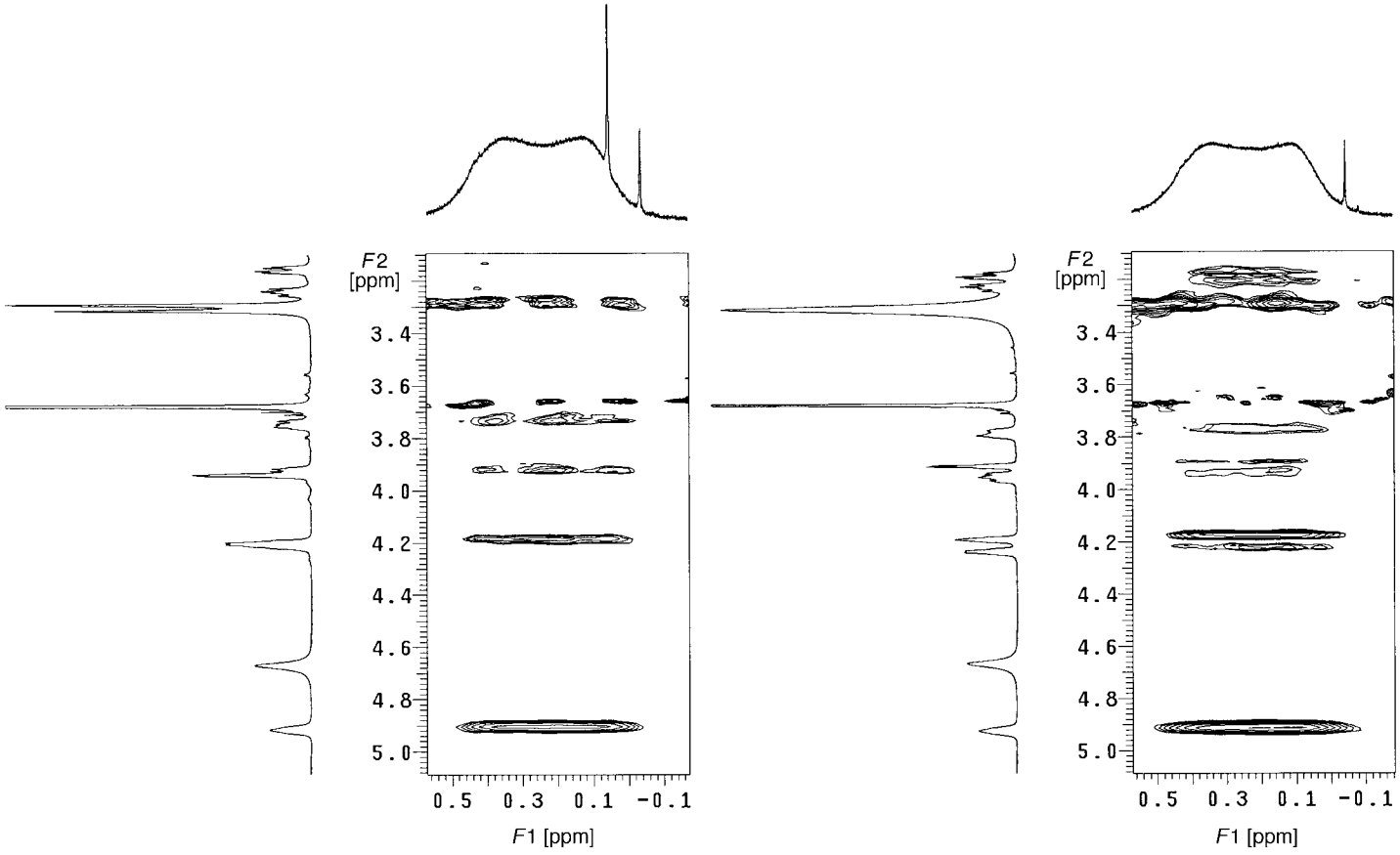
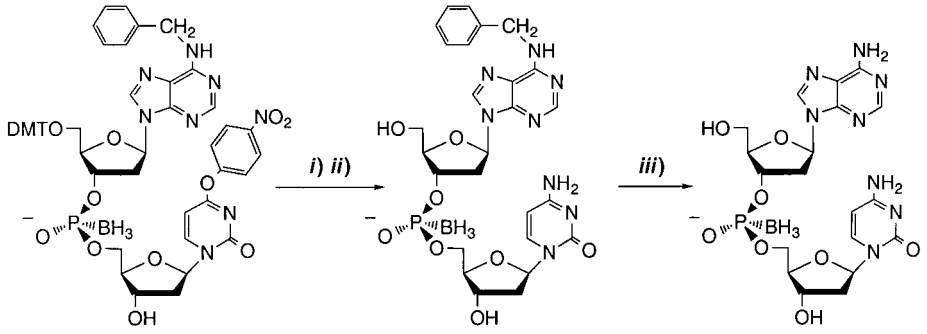


Fig. 2. *Fragments of 2D-NOESY plots of* **5a** (right) *and* **5b** (left), *illustrating interactions of* BH_3 *and deoxyribose protons*

group caused partial degradation of the boranophosphate bond [28] or loss of a cyanoborane group in base-boronated nucleotides [29]. A detailed study of this phenomena will be published elsewhere. Briefly, we found that the addition of $(MeO)_2$Tr-cation scavengers efficiently suppressed the loss of the boranophosphate group. Borane-pyridine complex was used as a scavenger at 5-fold molar excess over **5a** (**5b**). Without intermediate isolation of the 5′-OH derivative, the reaction mixture was treated with concentrated ammonia for 4 h at room temperature to convert the 2′-deoxy-4-*O*-(4-nitrophenyl)uridine residue to 2′-deoxycytidine. The resulting N^6-benzyl-2′-deoxyadenosin-3′-yl 2′-deoxycytidin-5′-yl boranophosphates **6a** and **6b** were isolated by reversed-phase HPLC with 85% yield.

Scheme 3. *Synthesis of the Deprotected 2′-Deoxyadenosin-3′-yl 2′-Deoxycytidin-5′-yl Boranophosphates* **7a** *and* **7b**



i) H^+, $BH_3 \cdot$ pyridine. *ii*) NH_4OH. *iii*) $(NH_4)_2S_2O_8$.

We tried a number of methods for N^6-benzyladenine deprotection. Oxidative deprotection of **6a** or **6b** with $NaIO_4$ in the presence of catalytic amounts of RuO_2 [18b,c] resulted in a large amount of by-products and a low yield. The reductive debenzylation with $(NH_4)HCOO$ in the presence of Pd/C [18d] was very inefficient. Reduction through hydrogenolysis as exampled in [18e] was not tried. The most satisfactory results so far were obtained by $(NH_4)_2S_2O_8$ deprotection as described in [18a]. While the original report called for addition of the oxidant in one batch and reaction at 80° for 2 h, we achieved a better yield when the oxidant was added in small portions at 2 h intervals at 60°. Although the yields of the completely deprotected boranophosphate dinucleotide did not exceed 35%, sufficient amounts of the final compounds **7a** and **7b** of 2′-deoxyadenosin-3′-yl 2′-deoxycytidin-5′-yl boranophosphates were isolated by reversed-phase HPLC.

The elution order of the deprotected isomers **7a** and **7b** on the reversed-phase HPLC was the same as for the protected **6a** and **6b**, *i.e.* the [*P*(*S*)] isomer was eluted faster than the [*P*(*R*)] isomer. It coincided with the elution pattern of the previously synthesized dithymidine boranophosphate isomers [7a][16][24]. The reversed-phase HPLC analysis showed no evidence of boranophosphate-bond racemization during the deprotection procedure.

It was shown previously that oligothymidine boranophosphates are hydrolyzed by snake venom phosphodiesterase (SVP) in a stereospecific manner [7a,b,d]. While the

[$P(S)$] isomer of dithymidine boranophosphate is much more stable (330 times) than its natural counterpart, it can be degraded at high enzyme concentration [7a,b]. In contrast, the [$P(R)$] isomer is completely resistant to the enzyme.

To confirm that the SVP differentiation of boranophosphate isomers is a general phenomenon, we studied the stability of the two isomeric boranophosphates **7a** and **7b** toward SVP hydrolysis. As expected, the [$P(S)$] isomer **7b** was hydrolyzed slowly, giving 2′-deoxyadenosine and 2′-deoxycytidine[4]), and the [$P(R)$] isomer **7a** was completely inert after even prolonged incubation (*Fig. 3*). Even after 65 h of incubation with SVP at 37°, no sign of **7a** hydrolysis was observed. The enzyme in this mixture maintained at least 20% of its original activity at the end of the experiment. The normal d(ApC) was hydrolyzed by SVP at the studied conditions very quickly (*Fig. 3*, insert). Comparison of the initial rates of hydrolysis suggests that the [$P(S)$] boranophosphate isomer is *ca.* $5 \cdot 10^2$ times more resistant to SVP than the natural counterpart. We conclude that the substrate properties of **7a**,**b** towards SVP are very similar to those of the dithymidine boranophosphates [7a,b] and are in good agreement with those for boranophosphate RNA dimers [30].

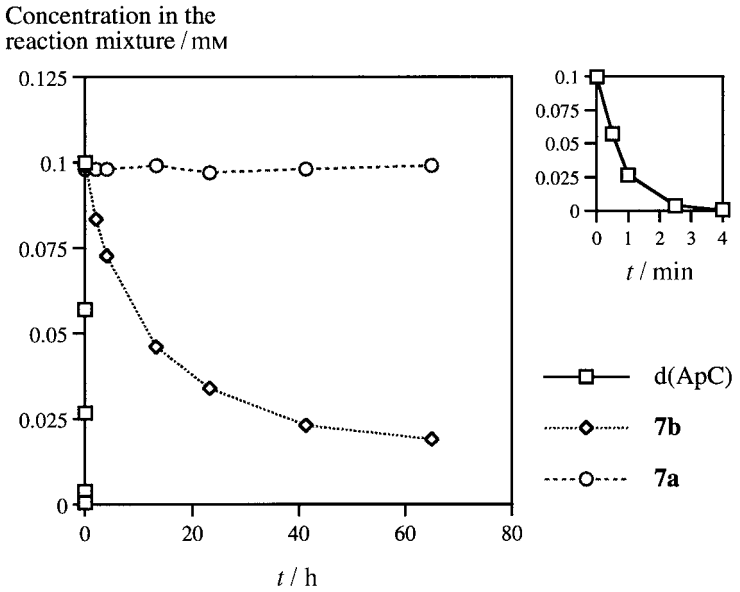


Fig. 3. *Hydrolysis of d(ApC) (insert) and of their boranophosphate analogs* **7a** *and* **7b** *by SVP.* Conditions: 0.1 mM dinucleotide concentration, 0.03 U/ml of SVP activity in 1.25 ml of 0.1M NaCl, 0.1M *Tris*·HCl (9.0), 15 mM $MgCl_2$ buffer, and 0.2 mg/ml of bovine serum albumin at 37°. Hydrolysis of d(ApC) occurred in an appropriate (fast) time scale (insert).

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[4]) Expected 2′-deoxycytidine 5′-boranophosphate is not stable enough under the buffer conditions and slowly hydrolyzed to 2′-deoxycytidine and boranophosphate [32]. An analogous observation was made for the dithymidine boranophosphate hydrolysis [7a].

Experimental Part

General. 2′-Deoxyuridine, pyrrolidine, phosphorus oxychloride, 4-nitrophenol, 4-(dimethylamino)pyridine (DMAP), Et_3N, 4,4′-dimethoxytrityl chloride ($(MeO)_2TrCl$), hydrazine hydrate, *N*,*O*-bis(trimethylsilyl)acetamide (BSA), borane-*N*,*N*-diisopropylethylamine ($^iPr_2EtN \cdot BH_3$), borane-pyridine, levulinic acid, and sulfur were purchased from *Aldrich Chemical Co*. Pivaloyl chloride was purchased from *Sigma*. Levulinic anhydride was prepared according to [31]. 3% $CHCl_2COOH$ in CH_2Cl_2 and N^6-benzoyl-2′-deoxy-5′-*O*-(dimethoxytrityl)-adenosine 3′-*H*-phosphonate were purchased from *Glen Research* (USA). High-performance liquid chromatography (HPLC): *Waters-600E* controller system equipped with a *991* photodiode array detector; direct-phase HPLC on a 19×300 mm μ-*Porasil* column (*Waters*) using the isocratic mixture AcOEt/MeOH/CH_2Cl_2 40:2:58; anal. reversed-phase HPLC on a 3.9×300 mm *DeltaPakC18* (15 μm) column, linear gradient 0–30% MeCN in 0.02M $(Et_3NH)HCO_3$ (pH 7.5) (system *A*) or isocratic 8% MeCN in 0.02M KH_2PO_4 (pH 6.5) (system *B*), 1 ml/min flow rate; prep. reversed-phase HPLC on a 21.4×250 mm *Microsorb RP-18* column or 25×100 mm *RadialDeltaPak* cartridge, linear gradient 0–25% MeCN in 0.02M $(Et_3NH)HCO_3$ (pH 7.5) (system *C*) or linear gradient 40–80% MeCN in 0.02M $(Et_3NH)HCO_3$ (pH 8.0) (system *D*), 6 ml/min flow rate. 1D-^{1}H- and ^{31}P-NMR Spectra: *Varian Inova 400* spectrometer at 399.9 and 161.9 MHz, resp., chemical shifts δ referenced for ^{1}H to internal deuterated solvent signals ($CDCl_3$ or D_2O) and for ^{31}P to external 85% H_3PO_4 in H_2O as a standard. 1D- and 2D-COSY and -NOESY of **5a** and **5b**: at 16 mM concentration in (D_6)DMSO; *Varian-Unity-600-MHz* NMR spectrometer in the Duke NMR Spectroscopy Center; 2D-NOESY experiments with 1.0 s delay time, 0.5 s mixing time, and 6598.5 Hz sweep width. Fast-atom-bombardment mass spectrometry (FAB-MS): *Jeol-300* mass spectrometer, pos. and neg. ion modes; high-resolution FAB MS in neg. ion mode with polyethylene glycol *600* as a matrix.

Statistical Comparison of NOESY Cross-peak Volumes. To determine whether the two isomers adopt similar conformations in solution, cross-peak volumes from the NOESY experiment of the first isomer were compared to those from the NOESY of the second isomer. The volumes were measured using VNMR *Varian* software version 6.1A. Only the negative NOE peaks were considered, and peaks from scalar coupled protons, and those involving contacts to the borane protons were omitted from the analysis. To account for small diferences in the concentrations of the two isomers, the integrated volumes of the cross-peaks from the second isomer were scaled so that the summed intensities of all spots of the two isomers were the same. The variation in the measured peak volumes between the first isomer and the scaled values of the second isomer was investigated for the non-borane peaks. The differences between the volumes of a given cross-peak of the two isomers is Δi_m ($\Delta i_m = i_{1,m} - i_{2,m}$, where i represents the integrated volume of the peak indicated by its subscript; the subscript m refers to a specific pair of protons, such as H–C(1′)(U)/H–C(3′)(U), and the subscripts 1 and 2 refer to the spectra of isomer 1 and isomer 2, resp.). Since the intensities of the isomer-2 peaks were scaled to resemble those of isomer 1, the average of the differences in the volumes of the peaks of the two isomers, Δi_{avg} (calculated as $\Delta i_{avg} = (1/n)\Sigma\Delta i_m$ where n is the number of peaks considered) is, by definition, zero. The distribution of Δi values about the mean value (zero) was characterized by its standard deviation σ (calculated as $\sigma^2 = (1/n-1))\Sigma(\Delta i_m)^2)$,and the differences in each pair of peaks was compared to the standard deviation: $|\Delta i_m|/\sigma$. The 46 peaks included in the analysis were[2]): H–C(**6**)(**U**)/H–C(3′)(U), 2 H–C(5′)(U), 2 H–C(2′)(U), H–C(3′)(A), 2 H–C(2′)(A); H–C(**5**)(**U**)/H–C(3′)(U), H–C(4′)(U), H–C(5′)(A), H′–C(2′)(U), H–C(3′)(A), 2 H–C(2′)(A); H–C(**1′**)(**U**)/H–C(3′)(U), H–C(5′)(U), H–C(4′)(U); H–C(**5′**)(**U**) and H–C(**4′**)(**U**)/2 H–C(2′)(U), 2 H–C(2′)(A); H′–C(**5′**)(**U**)/2 H–C(2′)(U), 2 H–C(2′)(A); H–C(**2**)(**A**)/H–C(5′)(A), H–C(2′)(A); H–C(**8**)(**A**)/H–C(1′)(A), H–C(3′)(A), H–C(4′)(A), H–C(5′)(A), 2 H–C(2′)(A); H–C(**1′**)(**A**)/H–C(3′)(A), H–C(4′)(A), 2 H–C(5′)(A), H–C(4′)(U) and H–C(5′)(U), H–C(5′)(U); 2 H–C(**2′**)(**A**)/2 H–C(5′)(A); H–C(**4′**)(**A**)/2 H–C(2′)(A). The value of σ was 24% of the average value of the volumes of the 92 peaks.

2′-Deoxy-4-O-*(4-nitrophenyl)uridine* (**1**) was synthesized according to [19c].

2′-Deoxy-5′-O-*(dimethoxytrityl)-4*-O-*(4-nitrophenyl)uridine* (**2**). Nucleoside **1** (0.7 g, 2 mmol) was dried by repeated co-evaporation with dry pyridine and finally dissolved in dry pyridine (10 ml). DMAP (12 mg, 0.1 mmol) and Et_3N (0.35 ml, 2.4 mmol) were added to the soln., followed by $(MeO)_2TrCl$ (0.68 g, 2 mmol). After 2 h stirring, the mixture was diluted with MeOH (3 ml), evaporated, and co-evaporated with toluene (to remove pyridine), and the residue was applied to column chromatography (100 ml of silica gel, 0–10% MeOH/CH_2Cl_2). The product fractions (TLC (10% MeOH/CH_2Cl_2): R_f 0.52) gave **2** (1.0 g, 78%). Yellowish foam. ^{1}H-NMR ($CDCl_3$): 2.26 (*m*, 1 H–C(2′)); 2.59 (*m*, 1 H–C(2′)); 3.42–3.56 (*m*, 2 H–C(5′)); 3.78 (*s*, 2 MeO); 4.03 (*m*, H–C(4′)); 4.49 (*m*, H–C(3′)); 5.81 (*d*, $J = 7.2$, H–C(5)); 6.19 (*t*, $J = 6.0$, H–C(1′)); 6.83 (*m*, 2 arom. H);

7.26–7.38 (*m*, 13 H, $(MeO)_2Tr$); 8.25 (*m*, 2 arom. H); 8.33 (*d*, *J* = 7.6, H–C(6)). HR-FAB-MS (pos.): 652.2 ($C_{36}H_{34}N_3O_9^+$, $[M+H^+]$; calc. 651.66).

2′-Deoxy-3′-O-*levulinoyl-4*-O-*(4-nitrophenyl)uridine* (**3**). Starting **2** (0.42 g, 0.6 mmol) was dried over P_2O_5 *in vacuo* overnight and dissolved in dry pyridine (10 ml). A cat. amount of DMAP (12 mg, 0.1 mmol) and levulinic anhydride (0.3 ml, 1.2 mmol) was added and the resulting soln. stirred at r.t. The reaction was completed within 2 h (TLC (10% MeOH/CH_2Cl_2)) and quenched by adding H_2O (2 ml). The mixture was evaporated and the residue treated with 3% $CHCl_2COOH$ in CH_2Cl_2 (20 ml) for 4 min and washed with aq. $NaHCO_3$ soln. Evaporation of the org. layer gave an orange gum, which was purified by chromatography (silica gel, 0–10% MeOH/CH_2Cl_2): **3** (0.21 g, 80%). Slightly yellow solid. ^{1}H-NMR ($CDCl_3$): 2.17 (*s*, Me); 2.34 (*m*, 1 H–C(2′)); 2.56 (*m*, CH_2); 2.64 (*m*, 1 H–C(2′)); 2.75 (*m*, CH_2); 3.97–3.85 (*m*, 2 H–C(5′)); 4.14 (*m*, H–C(4′)); 5.30 (*m*, H–C(3′)); 6.16 (*d*, *J* = 7.6, H–C(5)); 6.19 (*t*, *J* = 6.4, H–C(1′)); 7.32 (*m*, 2 arom. H); 8.11–8.35 (*m*, H–C(6), 2 arom. H). HR-FAB-MS (pos.): 448.11 ($C_{20}H_{21}N_3O_9^+$, MH^+; calc. 448.40).

N^6-*Benzoyl-2′-deoxy-5′*-O-*(4,4′-dimethoxytrityl)adenosin-3′-yl 2′-Deoxy-3′*-O-*levulinoyl-4*-O-*(4-nitrophenyl)-uridin-5′-yl* H-*Phosphonates* **4a** *and* **4b**. A mixture of *N*^{6}-benzoyl-2′-deoxy-5′-*O*-(4,4′-dimethoxytrityl)adenosine 3′-phosphonate (400 mg, 0.48 mmol) and **3** (250 mg, 0.250 mmol) was dried over P_2O_5 under vacuum overnight and then dissolved in 10 ml of dry pyridine. Pivaloyl chloride (0.2 ml, 1.44 mmol) was added at r.t., and the mixture was stirred for 10 min. CH_2Cl_2 (10 ml) was added and the soln. was extracted with aq. sat. $NaHCO_3$ soln. (10 ml). The combined org. layer and washings were dried and evaporated. The resulting mixture contained a 1:1 mixture **4a**/**4b** (^{31}P-NMR: δ 9.18 and 10.32 ppm). Separation of diastereoisomers **4a**/**4b** by HPLC (μ-*Porasil* (19 × 300 mm column), isocratic (AcOEt/MeOH/CH_2Cl_2 40:2:58) gave faster migrating **4a** ([*P*(*R*)]; 190 mg, 34%; 98% diastereoisomer purity) and slower migrating **4b** ([*P*(*S*)]; 220 mg, 39%; 84% diastereoisomer purity).

Sulfurization of Diastereoisomers **4a** *and* **4b**. Starting **4a** or **4b** (1 mg, *ca.* 1 μmol of each) was dried over P_2O_5 under vacuum overnight and then dissolved in dry pyridine (0.1 ml). BSA (5 μl, 0.02 mmol) and Et_3N (3 μl, 0.02 mmol) were added, and the soln. was stirred at r.t. for 1 h. Sulfur (2 mg, 0.5 mmol) was added and the mixture stirred for another 16 h. After filtration, the soln. was evaporated and the residue treated with 0.2 ml of 3% $CHCl_2COOH$ in CH_2Cl_2. After 5 min, the acid was neutralized with 20 μl of Et_3N, and the mixture was evaporated to oil. The oil was dissolved in NH_4OH/MeOH 1:1 (1 ml), the soln. incubated for 48 h at r.t. and then evaporated, and the residue redissolved in H_2O and applied to reversed-phase HPLC (*DeltaPakC18* column, 3.9 × 300 mm, linear gradient *A*). The resulting *2′-deoxyadenosin-3′-yl 2′-deoxycytidin-5′-yl phosphorothioate* obtained upon sulfurization of **4a** had a t_R of 22.5 min ([*P*(*S*)] isomer), and that obtained upon sulfurization of **4b** a t_R of 20.9 min ([*P*(*R*)] isomer).

N^6-*Benzyl–2′-deoxy-5′*-O-*(4,4′-dimethoxytrityl)adenosin-3′-yl 2′-Deoxy-4*-O-*(4-nitrophenyl)uridin-5′-yl Boranosphosphate* **5a** *and* **5b**. Starting **4a** or **4b** (200 mg, 0.17 mmol of each) was dried over P_2O_5 under vacuum overnight and dissolved in THF (5 ml). BSA (212 μl, 0.85 mmol) was added, and the soln. was stirred at r.t. for 1 h. Then boronating agent $BH_3 \cdot {}^iPr_2EtN$ (220 μl, 1.7 mmol) was added, and the mixture was stirred for another 30 min. The mixture was cooled in a freezer and added dropwise to cold hexane. The precipitate formed was washed with hexane, dried, and dissolved in pyridine (2 ml), and H_2O (18 μl, 1 mmol) was added. The mixture was immediately evaporated to an oil, redissolved in pyridine/AcOH 4:1 (4 ml) and treated with hydrazine hydrate (0.25 ml). After 5 min, pentane-2,4-dione (2 ml) was added to stop the reaction. The mixtures were evaporated and co-evaporated with toluene to remove traces of pyridine, and the residue dissolved in MeCN/aq. 0.2M KH_2PO_4 (pH 7.5) 1:1 and purified by reversed-phase HPLC (*Microsorb RP-18* column, gradient *D*).

Data of **5a**: 54 mg (27%). ^{1}H-NMR: *Table 1*. ^{31}P-NMR (CD_3CN): 93.84. HR-FAB-MS (neg.): 1051.30 ($C_{53}H_{53}BN_8O_{13}P_1^-$, M^-; calc. 1051.82).

Data of **5b**: 32 mg (18%). ^{1}H-NMR: *Table 1*. ^{31}P-NMR (CD_3CN): 93.28. HR-FAB-MS (neg.): 1051.40 ($C_{53}H_{53}BN_8O_{13}P_1^-$, M^-, calc. 1051.82).

N^6-*Benzyl-2′-deoxyadenosin-3′-yl 2′-Deoxycytidin-5′-yl Boranophosphates* **6a** *and* **6b**. Protected **5a** or **5b** (10 mg, 10 μmole of each) was dissolved in MeOH/H_2O 1:1 (0.2 ml) and treated with AcOH (0.8 ml) for 30 min. Pyridine-borane (5 μl, 0.05 mmol) was added during treatment to scavenge the $(MeO)_2Tr$ cation. Then the soln. was diluted with NH_4OH soln. (20 ml), stirred for 4 h at r.t., and evaporated and the residue purified by reversed-phase HPLC (*Microsorb RP-18* column, gradient *C*). Product fractions were evaporated and co-evaporated several times with H_2O to remove $(Et_3NH)HCO_3$.

Data of **6a**: 5.1 mg (85%). ^{1}H-NMR (D_2O)[2]): 8.08 (*s*, H–C(2)(A)); 7.99 (*s*, H–C(8)(A)); 7.66 (*d*, *J* = 7.6, H–C(6)(C)); 7.15–7.26 (*m*, 5 H, Bn); 6.24 (*t*, *J* = 6.4, 1 H, H–C(1′)); 6.04 (*t*, *J* = 6.4, 1 H, H–C(1′)); 5.67 (*d*, *J* = 7.6, H–C(5)(C)); 4.72 (*s*, 2 H, CH_2); 4.32–4.36 (*m*, 1 H); 4.12 (*q*, 1 H); 3.95–4.01 (*m*, 2 H); 3.94–3.89 (*m*, 1 H); 3.61–3.71 (*m*, 2 H); 2.74–2.66 (*m*, 1 H); 2.56–2.48 (*m*, 1 H); 2.26–2.19 (*m*, 1 H); 2.10–2.03 (*m*, 1 H); −0.1–0.6 (br. *m*, BH_3). HR-FAB-MS (neg.): 627.30 ($C_{26}H_{33}N_8BO_8P_1^-$; M^-; calc. 627.38).

Data of **6b**: 5.0 mg (84%). ^{1}H-NMR (D_2O)^2): 8.09 (*s*, H–C(2)(A)); 7.97 (*s*, H–C(8)(A)); 7.64 (*d*, *J* = 7.2, H–C(6)(C)); 7.27–7.15 (*m*, 5 H, Bn); 6.22 (*t*, *J* = 6.4, 1 H, H–C(1′)); 6.01 (*t*, *J* = 6.4, 1 H, H–C(1′)); 5.60 (*d*, *J* = 7.2, H–C(5)(C)); 4.82 (*s*, 2 H, CH_2); 4.36–4.31 (*m*, 1 H); 4.12–4.09 (*m*, 1 H); 4.00–3.89 (*m*, 2 H); 3.76–3.63 (*m*, 2 H); 2.71–2.66 (*m*, 1 H); 2.53–2.48 (*m*, 1 H); 2.22–2.17 (*m*, 1 H); 2.10–2.06 (*m*, 1 H); –0.1 to 0.6 (br. *m*, BH_3). HR-FAB-MS (neg.): 627.21 ($C_{26}H_{33}N_8BO_8P_1^-$, M^-; calc. 627.38).

2′-Deoxyadenosin-3′-yl 2′-Deoxycytidin-5′-yl Boranophosphates **7a** *and* **7b**. The benzyl-protected **6a** or **6b** (5.0 mg, 8 μmoles) was dissolved in 10 ml of 1M KH_2PO_4/MeCN 3 : 2 (10 ml), heated to 60°, and treated with $(NH_4)_2S_2O_8$ (2 mg) every 2 h during an 8 h period. Isolation was performed by reversed-phase HPLC (*DeltaPak C18 Radial* 25 × 100 mm cartridge, 8% MeCN in 0.02M KH_2PO_4 (pH 6.5)).

Data of **7a**: 1.5 mg (35%). HPLC: t_R 14.4 min. ^{1}H-NMR (D_2O)^2): 8.08 (*s*, H–C(2)(A)); 7.96 (*s*, H–C(8)(A)); 7.65 (*d*, *J* = 7.6, H–C(6)); 6.20 (*t*, *J* = 6.8, 1 H, H–C(1′)); 6.02 (*t*, *J* = 6.4, 1 H, H–C(1′)); 5.67 (*d*, *J* = 6.9, H–C(5)); 4.82 (*m*, 1 H); 4.34 (*m*, 1 H); 3.85–4.00 (*m*, 2 H); 3.61–3.71 (*m*, 2 H); 2.88 (*m*, 1 H); 2.66 (*m*, 1 H); 2.22 (*m*, 1 H); 2.06 (*m*, 1 H); –0.25–0.65 (br. *m*, BH_3). ^{31}P-NMR (D_2O): 93.80. HR-FAB-MS: 537.1779 ($C_{19}H_{27}N_8BO_8P_1^-$, M^-; calc. 537.2559).

Data of **7a**: 1.4 mg (33%). HPLC: t_R 11.4 min. ^{1}H-NMR (D_2O)^2): 8.11 (*s*, H–C(2)(A)); 7.97 (*s*, H–C(8)(A)); 7.64 (*d*, *J* = 7.2, H–C(6)(C)); 6.20 (*t*, *J* = 6.4, 1 H, H–C(1′)); 6.00 (*t*, *J* = 6.0, 1 H, H–C(1′)); 5.59 (*d*, *J* = 7.2, 1 H, H–C(5)(C)); 4.34 (*m*, 1 H); 4.11 (*m*, 1 H); 3.88–4.00 (*m*, 2 H); 3.64–3.77 (*m*, 2 H); 2.68 (*m*, 1 H); 2.51 (*m*, 1 H); 2.19 (*m*, 1 H); 2.07 (*m*, 1 H); –0.3–0.65 (br. *m*, BH_3). ^{31}P-NMR (D_2O): 93.90. HR-FAB-MS: 537.1811 ($C_{19}H_{27}BN_8O_8P_1^-$, M^-; calc. 537.2559).

Enzymatic Hydrolysis of **7a**, **7b**, *and d(ApC)* was carried out at 0.1 mM concentration of dinucleotide and 1 mg/ml (0.03 U/ml) of SVP (*Sigma*) in 1.25 ml of 0.1M NaCl, 0.1M *Tris* · HCl (9.0), 15 mM $MgCl_2$ buffer, 0.2 mg/ml of bovine serum albumin (*Sigma*) at 37°. At appropriate times, 0.25 ml aliquots were withdrawn, the hydrolysis was stopped by addition of AcOH (50 μl), and the mixture analyzed by anal. reversed-phase HPLC (gradient *A*). Control samples were incubated at identical conditions without SVP. For the reaction mixture containing **7a**, the enzyme activity was checked after 65 h of the hydrolysis at 37° by addition of d(ApC) (0.1 mM). The rate of d(ApC) hydrolysis was determined by reversed-phase HPLC.

This work was supported by *NIH* grant *R01-GM-57693* and *DOE* grant *DE-FG 02-97ER62376* to *B.R.S.* The Duke NMR Spectroscopy Center is partially supported by *NIH* grant *P30-CA-14236*. NMR Instrumentation in the Duke NMR Spectroscopy Center was funded by the *NSF*, the *NIH*, the *NC Biotechnology Center*, and Duke University. We thank Prof. *Terry Oas* for help in preparing the molecular models of *A*- and *B*-form DNA.

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Received February 29, 2000