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Chemical synthesis of diastereomeric diadenosine boranophosphates (Ap^{b}A) from 2′-*O*-(2-cyanoethoxymethyl)adenosine by the boranophosphotriester method

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ABSTRACT

We have synthesized diastereomerically pure diadenosine 3′,5′-boranophosphates (Ap^{b}A) by using the boranophosphotriester method from ribonucleosides protected with the 2′-hydroxy protecting group 2-cyanoethoxymethyl (CEM). Melting curves of the triple-helical complex of the dimer Ap^{b}A and 2poly(U) at high ionic strength revealed that presumptive (*S*p)-Ap^{b}A had a much higher affinity and presumptive (*R*p)-Ap^{b}A a much lower affinity for poly(U) than the natural dimer ApA did. In contrast, the affinities of these dimers for poly(dT) were similar. Both the (*R*p)- and the (*S*p)-boranophosphate diastereomers showed much higher resistance to digestion by snake venom phosphodiesterase and nuclease P1 than ApA did. They have potential for use as synthons to be incorporated into boranophosphate oligonucleotides. In particular, because oligonucleotides containing *S*p boranophosphate nucleotides are expected to bind more strongly and specifically to RNA than natural oligoribonucleotides do, they may find application in the isolation and detection of functional RNA in basic research and diagnostics.

1. Introduction

Many types of modified oligonucleotides have been reported over the past couple of decades.[1–5] Among them, oligonucleoside boranophosphates, in which one nonbridging oxygen in the phosphate moiety is replaced by a borane group (BH$_3$–), have attracted much attention because of their high chemical stability, high nuclease resistance, and potential for use in boron neutron-capture therapy.[6] Furthermore, Hall et al.[7] have reported that boranophosphate modification is useful for increasing the activity of siRNA. Since oligodeoxyribonucleoside boranophosphates (BH$_3$-ODNs) were first synthesized in 1990 by Sood et al.,[5] both enzymatic and chemical methods for synthesizing BH$_3$-ODNs have been reported.[8–15] The enzymes which have been used for enzymatic synthesis of BH$_3$-ODNs, such as T7 RNA polymerase, recognize only the *R*p diastereomer of nucleoside 5′-(α-P-borano)triphosphates to incorporate *S*p-boranophosphate internucleotide linkages, so that *R*p linkages cannot be incorporated into an oligonucleotide by the enzymatic methods. For the comparative study of the *S*p and *R*p diastereomers, therefore, chemical synthetic methods are required. However, there are few reports of the chemical synthesis of oligoribonucleoside boranophosphates (BH$_3$–ORNs).[14,16,17] In addition, because of undesirable reductions that occur at the *N*-acyl protecting groups of the base moiety during the boronation step,[18,19] most of the chemical methods reported for the synthesis of BH$_3$-ODNs and BH$_3$-ORNs are limited to thymidine or uridine derivatives. Although we have reported a boranophosphotriester method that can be used to synthesize BH$_3$-ODNs containing all four nucleobases, A, C, G, and T,[12] there are still no reports of the chemical synthesis of BH$_3$-ORNs with nucleobases besides U. In addition, the coupling efficiency of the boranophosphotriester method is lower than that of the phosphoramidite method, and this disadvantage would be even more marked when the boranophosphotriester method is applied to the synthesis of RNA analogues from ribonucleosides protected with the bulky conventional *t*-butyldimethylsilyl (2′-*O*-TBDMS) protecting group.

We have developed an achiral 2′-hydroxy protecting group, 2-cyanoethoxymethyl (CEM), for RNA synthesis.[20] Using this protecting group, we have succeeded in efficiently synthesizing RNA oligomers up to 110 nt.[21] With the CEM method, RNA synthesis can be carried out much more simply and easily than by the conventional TBDMS method. Largely because the CEM protecting group shows less steric hindrance than the TBDMS protecting group, RNA synthesis by the CEM method is comparable in efficiency with DNA

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synthesis. Even though the boranophosphotriester method has a poor coupling yield when used with conventional 2′-hydroxy protecting groups, it can be expected to give a high yield of coupled product when used with the CEM protecting group.

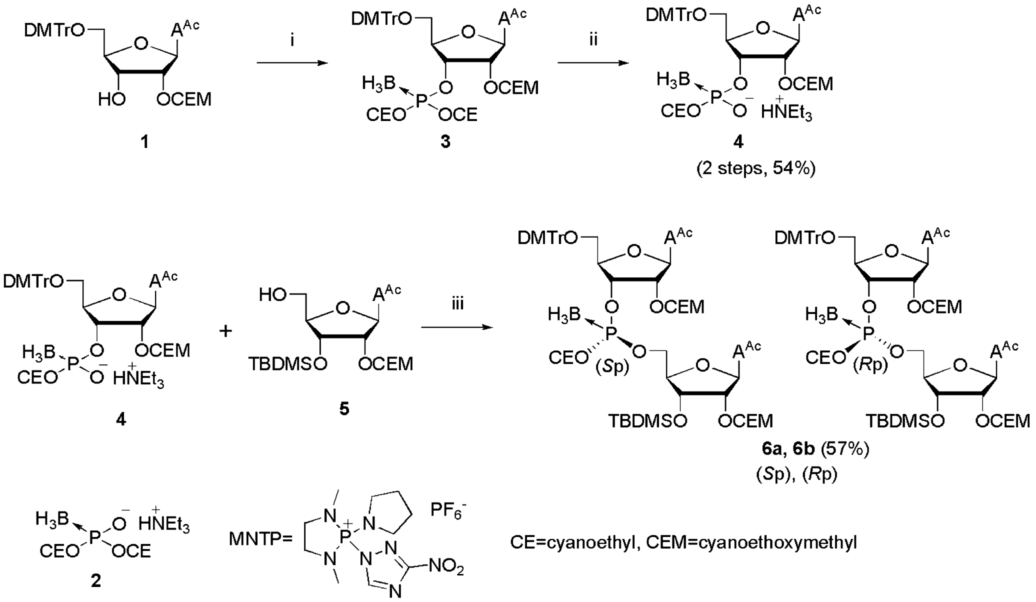
In the present paper, we describe the synthesis of diadenosine 3′,5′-boranophosphate (Ap^{b}A) from CEM-protected ribonucleosides by the boranophosphotriester method. The reason we chose adenosine is that the interaction of ApA analogues with poly(U) has often served as a model system for oligonucleotides with the same modifications because of the strong base-stacking between the adenine bases in ApA analogues.[22–24] We separated the two Ap^{b}A diastereomers by reverse-phase column chromatography and carried out a comparative study of their physicochemical properties. We found that the configuration of BH_3–ORNs at the phosphorus center affected their nuclease resistance, the strength of their base-stacking, and the thermal stability of their complexes with poly(U). As far as we know, this is the first report of the chemical synthesis of a diribonucleoside boranophosphate by using conventional nucleobase protection with an acyl group which is susceptible to reduction by boronation reagents.

2. Results and discussion

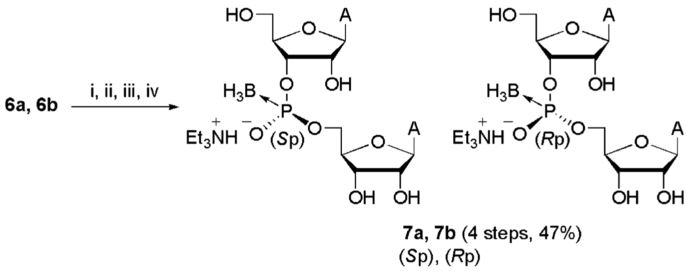
2.1. Synthesis of diadenosine 3′,5′-boranophosphate (Ap^{b}A)

We synthesized the boranophosphorylated monomer **4** from 5′-*O*-dimethoxytrityl-2′-*O*-CEM-*N*^{6}-acetyladenosine **1** by the boranophosphotriester method. Compound **1**, the boranophosphorylating reagent triethylammonium bis(2-cyanoethyl) boranophosphate **2**, and the condensing reagent 1,3-dimethyl-2-(3-nitro-1,2,4-triazol-1-yl)-2-pyrrolidin-1-yl-1,3,2-diazaphospholidinium hexafluorophosphate (MNTP), were synthesized as previously described.[21,25,26] Compound **1** was allowed to react with the boranophosphorylating reagent **2** in the presence of MNTP and 2,6-lutidine. The resulting fully protected intermediate **3** was then treated with Et_3N in CH_2Cl_2 to remove one of the two 2-cyanoethyl groups of **3** to give the corresponding boranophosphate monomer **4**. The monomer **4** was coupled with 2′-*O*-CEM-3′-*O*-TBDMS-*N*^{6}-acetyladenosine **5**, which was prepared from compound **1**, resulting in fully protected diastereomeric dimers Ap^{b}A **6a** and **6b** in 57% yield (Scheme 1). Because the boranophosphate group in **6a** and **6b,** which bears a 2-cyanoethyl protecting group, is not particularly stable during workup and purification, the isolated yield of this reaction is only moderate.

We next set out to remove the protecting groups of the dimers **6a** and **6b**. The dimethoxytrityl cation (DMTr$^+$) is known to react with borane groups, resulting in the cleavage of the internucleotide linkages.[9,19] Therefore we used Et_3SiH as a DMTr$^+$ scavenger for the deprotection of the DMTr group, as previously reported.[12] Finally, the other protecting groups of the dimers **6a** and **6b** were removed by the usual procedure for 2′-*O*-CEM-protected RNA oligomers to yield the diastereomeric diadenosine boranophosphates **7a** and **7b** (Scheme 2). When the diastereomers **7a** and **7b** were subjected to reverse-phase column chromatography, **7a** eluted earlier than



Scheme 1. Synthesis of diadenosine 3′,5′-boranophosphates (**6a**, **6b**). Reagents and conditions: (i) **2** (1.2 equiv), MNTP (2.4 equiv), and 2,6-lutidine (10 equiv) in CH_3CN, rt, 1 h; (ii) Et_3N (10 equiv) in CH_2Cl_2, rt, 1 h; (iii) MNTP (3.0 equiv), and 2,6-lutidine (10 equiv) in CH_3CN, rt, 1 h.



Scheme 2. Deprotection of diadenosine 3′,5′-boranophosphates (**7a**, **7b**). Reagents and conditions: (i) 3% dichloroacetic acid in Et_3SiH/CH_2Cl_2 (1:1, v/v), rt, 1 min; (ii) Et_3N/CH_3CN (1:1, v/v), rt, 1 h; (iii) concd NH_3 aq./EtOH (3:1, v/v), rt, 1 h; (iv) 1 M TBAF in THF containing 1% nitromethane, rt, overnight.

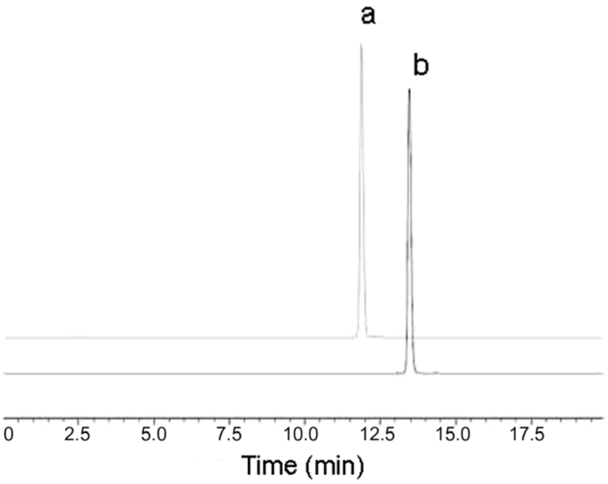
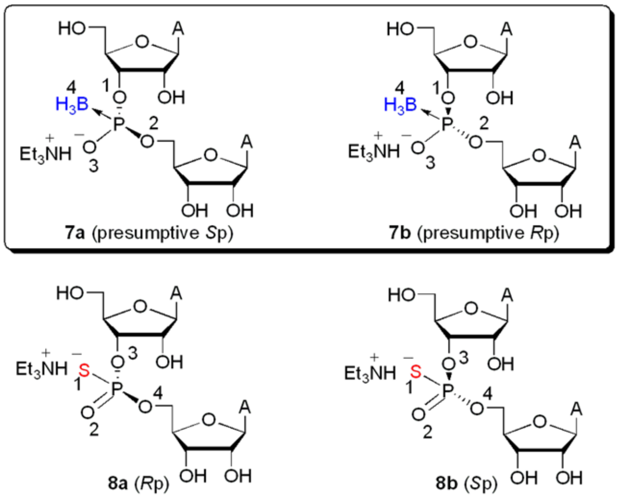


Figure 1. HPLC analysis of diadenosine 3′,5′-boranophosphate. (a) **7a**, (b) **7b**; Cadenza CD-C18 HPLC reverse-phase column (3 mm × 75 mm, 3 μm; Imtakt); buffer A, 5% CH_3CN, 50 mM TEAA, pH 7.0; buffer B, 90% CH_3CN, 50 mM TEAA, pH 7.0; gradient of 0–20% buffer B in 20 min; flow rate, 0.5 mL/min; 40 °C. UV detection was at 260 nm.



Scheme 3. Absolute structures of boranophosphate and phosphorothioate diastereomers and their designations according to Prelog's rule. The substituents at the phosphorus atom are numbered from 1 (highest priority) to 4 (lowest priority) in decreasing order of the atomic number of the atoms attached to the stereocenter. Note that boranophosphate and phosphorothioate diastereomers with the same absolute configuration have opposite Prelog designations.

7b (Fig. 1). The structures of **7a** and **7b** were confirmed by their ^1H, ^{13}C, and ^{31}P NMR spectra, reverse-phase HPLC analysis, and electrospray ionization (ESI) mass spectrometry. With a view to determining the configuration at the chiral phosphorus center in **7a** and **7b**, we carried out NMR measurements and nuclease-resistance tests as described below.

2.2. NMR measurements

The borane groups of the two diastereomers of boron-modified dimers are in different positions relative to the base-stacking region.[27] In the *S*p isomer, the borane group is directed toward the base-stacking region and is close to the H3′ proton of the 5′-residue (pseudoaxial position), while in the *R*p isomer, the borane group is directed away from the base-stacking region and is therefore far from the H3′ proton (pseudoequatorial position). Therefore we carried out a 1D-NOE difference experiment to estimate the distance between the BH_3 protons and the H3′ proton of the 5′-residue of each dimer. Irradiation of the H3′ proton of the 5′-residue of **7a** resulted in a small increase in the intensity of the BH_3 proton resonances (data not shown). Similar experiments with **7b** resulted in no signal change. These results provide strong evidence that **7a** is the *S*p isomer and **7b** is the *R*p isomer. However, the observed NOE in the **7a** 1D-NOE difference experiments was not large enough to allow unambiguous assignment of the absolute configuration of the diastereomers.

2.3. Nuclease resistance of ApA, Ap^{b}A, and Ap^{s}A

It is important to investigate the nuclease resistance of the dimers not only from a biological but also from a chemical point of view, because the results can be used to determine the configuration at the phosphorus center. An enzymatic method has been reported for the determination of the configuration at the phosphorus center of phosphorothioates based on the observation of different rates of hydrolysis of the *R*p and *S*p diastereomers catalyzed by snake venom phosphodiesterase (SVP).[28] According to Prelog's rule, boranophosphates and phosphorothioates with the same absolute configurations have opposite *S*p/*R*p designations (see Scheme 3). The boranophosphate diastereomer that is resistant to SVP is proposed to be the *R*p configuration.[16,18,27] Boranophosphate dimers have higher resistance to SVP than natural dimers do, and the *R*p isomer, which elutes more slowly on reverse-phase HPLC, is more resistant than the *S*p isomer.[16,27,29] In addition, Shaw and coworkers[10] have found that an oligothymidine boranophosphate dodecamer synthesized as a diastereomeric mixture is much more resistant to SVP than are their natural and phosphorothioate counterparts.

To investigate the effect of modifications at the phosphorus center on nuclease resistance, we compared the resistance of five dimers, ApA (**9**), Ap^{b}A (faster-eluting; **7a**), Ap^{b}A (slower-eluting; **7b**), Ap^{s}A (*R*p; **8a**), and Ap^{s}A (*S*p; **8b**) to SVP and nuclease P1 (Figs. 2 and 3).[30] The boranophosphate dimers **7a** and **7b** and the phosphorothioate dimers **8a** and **8b** were significantly more resistant to both enzymes than was the natural dimer ApA. In particular, **7b** and **8b** were more resistant to SVP than were the other dimers. From this diastereospecific resistance to SVP, we infer that **7b** corresponds to the *R*p configuration, a result that is consistent with the NMR measurements and that provides independent support

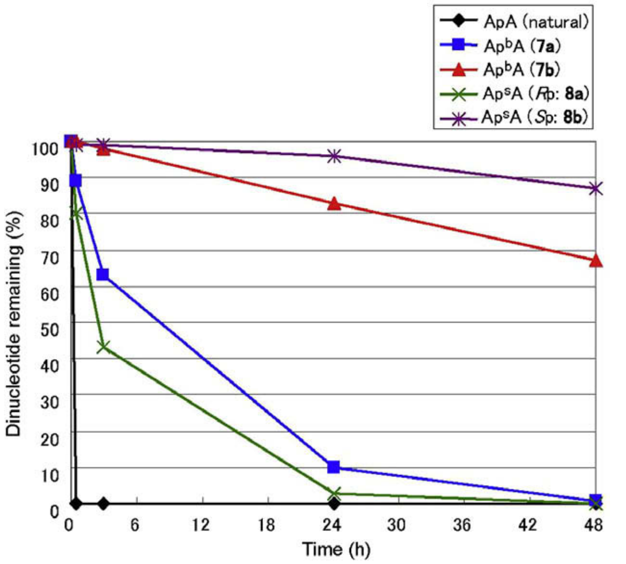


Figure 2. Digestion of dimers with snake venom phosphodiesterase. Residual dimer was measured by reverse-phase HPLC after treatment of ApA, Ap^{b}A (**7a**), Ap^{b}A (**7b**), Ap^{s}A (*R*p; **8a**), or Ap^{s}A (*S*p; **8b**) with SVP.

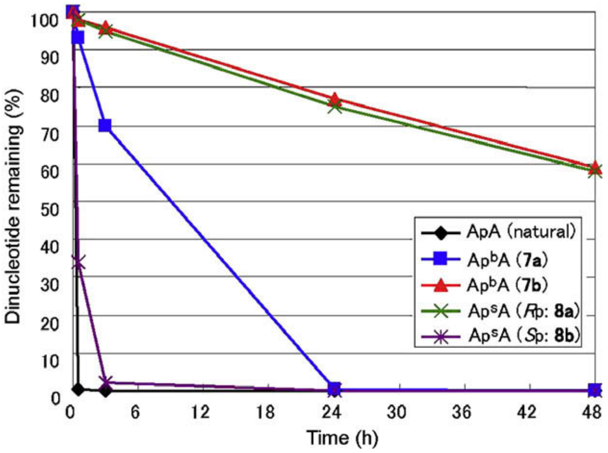


Figure 3. Digestion of dimers with nuclease P1. Residual dimer was measured by reverse-phase HPLC after treatment of ApA, Ap^bA (**7a**), Ap^bA (**7b**), Ap^sA (*R*p; **8a**), or Ap^sA (*S*p; **8b**), with nuclease P1.

for the assignment of configuration. Modification of the phosphorus center by boronation was as effective as thiolation in providing resistance to hydrolysis by SVP. Compounds **7b** and **8a** were equally resistant to nuclease P1. In the context of Prelog's rule, it is worth noting that the rank order of resistance to nuclease P1 observed for the boranophosphate diastereomers, **7a** (presumptive *S*p) < **7b** (presumptive *R*p), was the opposite of that observed for the phosphorothioate diastereomers, **8b** (*S*p) < **8a** (*R*p). Further investigation of these differences between the two types of modification is underway.

2.4. CD spectra of ApA and Ap^bA

We recorded circular dichroism (CD) spectra of natural and boranophosphate dimers (**7a** and **7b**) at 0 °C (Fig. 4). All dimers showed typical CD spectra with a positive band at about 270 nm and a negative band at 252 nm. Compound **7a** showed greater CD intensity than the natural dimer ApA or **7b** did. These results suggest that all dimers have a right-handed stacked conformation and that the two purine bases show stronger stacking interactions in **7a** than in the other dimers.

2.5. Thermal stability of ApA/poly(U) and Ap^bA/poly(U) complexes

The adenosine dimer ApA forms an ApA/2poly(U) triple-helical complex in the presence of 1 M NaCl.[31,32] We performed mixing experiments to investigate the stoichiometry of the interaction between Ap^bA and poly(U). The mixing curves for Ap^bA **7a** or **7b** and poly(U) at 260 nm in the presence of 1.0 M NaCl (not shown) revealed a break at Ap^bA:poly(U) ~ 1:2, indicating the formation of an Ap^bA/2poly(U) triple-helical complex. We then measured the T_m values of the dimer/2poly(U) triple-helical complexes to compare the hybridization properties of ApA, Ap^bA (**7a**), and Ap^bA (**7b**) in the presence of 1.0 M NaCl (Table 1) (the T_m values were too low to be measured at low ionic strength). The poly(A)/2poly(U) triple-helical complex is denatured directly to single strands on heating at high ionic strength, as evidenced by the simple shape of the melting curve.[33] The melting curve for each dimer/2poly(U) triple-helical complex (Fig. 5a) also indicates a single-step transition. The T_m value of the **7a**/2poly(U) complex (16 °C) was substantially higher than that of the natural dimer ApA/2poly(U) complex (9.1 °C). In contrast, the T_m value of the **7b**/2poly(U) complex (4.0 °C) was substantially lower than that of ApA/2poly(U) complex. Compound **7a** is proposed to be the *S*p boranophosphate dimer on the basis of the NMR measurements and hydrolysis by SVP. These results indicate that the *S*p boranophosphate adenosine dimer hybridizes more strongly to poly(U) than the natural dimer does. Meanwhile, the T_m values of the dimer/2poly(dT) complexes were below 0 °C and similar to each other (Fig. 5b and Table 1). Therefore, the increase in the strength of hybridization between the *S*p boranophosphate adenosine dimer and polymer is specific for the RNA polymer. These findings point the way to the development of a functional nucleic acid to recognize RNA with higher specificity than natural oligomers do.

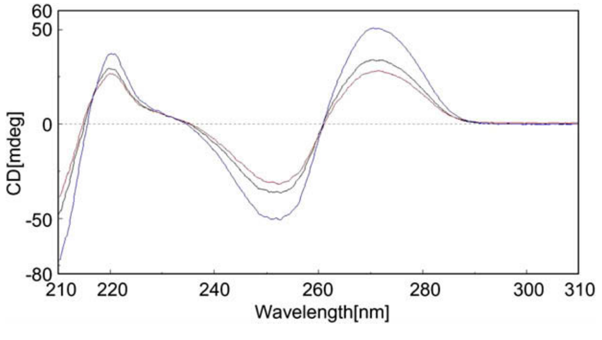


Figure 4. CD spectra of **7a** (blue), **7b** (red), and ApA (black) at 0 °C. The total base concentration was 0.1 mM. The spectra were taken in 10 mM sodium phosphate, pH 7.4, containing 1.0 M NaCl.

3. Conclusions

We have synthesized diadenosine 3′,5′-boranophosphate (Ap^bA) from CEM-protected ribonucleosides by the boranophosphotriester method and isolated the pure Ap^bA diastereomers. As far as we know, this is the first report of the chemical synthesis of a diribonucleoside boranophosphate by using conventional nucleobase protection with an acyl group which is susceptible to reduction by boronation reagents. It is inferred from the analysis of 1D-NOE difference spectra and nuclease resistance to SVP of the dimers that **7a** is the *S*p isomer. The T_m values of the dimer/2poly(U) complexes revealed that **7a** had a much higher and **7b** a much lower affinity for poly(U) than did the natural dimer, ApA. In contrast, the T_m values of the dimer/2poly(dT) complexes were all similar. These results indicate that the diastereopure boranophosphate RNA, which is inferred to be the *S*p isomer, hybridizes better to RNA than natural RNA does. The diastereomeric boranophosphate dimers **6a** and **6b** synthesized by this method can be subjected to specific removal of the 3′-protecting group, TBDMS, and transformed by the phosphitylating reagent bis(*N*,*N*-diisopropylamino)cyanoethylphosphite into the fully protected dimer block CEM-amidite suitable for use in the solid-phase synthesis of oligoribonucleotides having 3′,5′-boranophosphate internucleotide linkages. Therefore these dimers have the potential to be synthons that can be incorporated into oligonucleotides. Although it is known that dimers may differ from oligomers in their hybridization properties and that longer oligomers interacting with mixed-base RNA tend to form double-helical rather than triple-helical complexes, studies of dimers are sometimes carried out to provide an initial insight into the hybridization properties of oligomers.[22–24] Accordingly, we propose to extend the chemistry described in this paper to oligomers containing 3′,5′-boranophosphate linkages and to assess their interaction with complementary RNA. Meanwhile, our preliminary studies on the physicochemical properties of the diastereopure oligoribonucleotide boranophosphate dimers suggest that BH_3-ORNs synthesized with the *S*p diastereomers can be developed as a promising new class of

Table 1
T_m values of dimer/2poly(U) complexes and dimer/2poly(dT) complexes

dimer	T_m (°C) with poly(U)	T_m (°C) with poly(dT)
ApA	9.1	−1.9
Ap^{b}A **7a**	16.0	−2.8
Ap^{b}A **7b**	4.0	−0.5

functional RNA that will form stable complexes with complementary RNA.

4. Experimental

4.1. General methods

UV spectra, mixing curves, and melting curves were recorded with a Hitachi U-2810 double-beam spectrophotometer. CD spectra were taken with a Jasco J-720 spectropolarimeter fitted with a Jasco temperature controller. ^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded on a Varian VNMRS600, Bruker DRX 500 or Bruker DPX 300 spectrometer with 3-trimethylsilylpropionate-2,2,3,3-d^4 sodium salt or H_3PO_4 as the standard. High resolution mass spectra (HRMS) were recorded on a JEOL JMS-T100LC mass spectrometer. Analytical high performance liquid chromatography (HPLC) was performed on a Shimadzu HPLC system equipped with a Cadenza CD-C18 (75 × 3 mm, Imtakt) or Mightysil RP-18 GP (150 × 4.6 mm, Kanto Kagaku) column. Thin-layer chromatography (TLC) was performed on Merck silica gel 60 F254 precoated plates and column chromatography on Wako silica gel C-200 (Wako) or LiChroprep RP-18 GP (Merck). Dowex® 50WX8-200 hydrogen-form ion-exchange resin (Sigma–Aldrich) was used after conversion to the pyridinium or sodium form by washing with pyridine/water (1:1, v/v) or 1.0 M sodium hydroxide solution, respectively. We used published molar extinction coefficients for ApA (ε = 13,600 M^{-1} cm^{-1} at 258 nm)[31] and poly(U) (ε = 9200 M^{-1} cm^{-1} at 260 nm).[34] The amount of the final products **7a** and **7b** was determined by measuring the absorbance at 258 nm and converting to micromoles by using the published extinction coefficient for ApA.

4.2. Materials

Sodium polyuridylate (poly(U)) and nuclease P1 were purchased from Yamasa Corp. (Chiba, Japan) and snake venom phosphodiesterase from Worthington Biochemical Corp. (Lakewood, NJ). Reagents and solvents were from commercial suppliers and were used without further purification. Anhydrous solvents were from Wako Pure Chemical Industries, Ltd (Osaka, Japan) and tetrabutylammonium fluoride (TBAF) was from Nacalai Tesque (Kyoto, Japan).

4.3. Chemical synthesis

4.3.1. Triethylammonium 5′-*O*-dimethoxytrityl-2′-*O*-CEM-*N*^{6}-acetyladenosine cyanoethyl boranophosphate (4)

5′-*O*-Dimethoxytrityl-2′-*O*-CEM-*N*^{6}-acetyladenosine **1** (500 mg, 0.72 mmol) and triethylammonium bis(2-cyanoethyl) boranophosphate **2** (262 mg, 0.86 mmol) were dried by repeated coevaporation with dry toluene followed by dry pyridine and finally dissolved in dry acetonitrile (7.2 mL). To the solution were successively added 2,6-lutidine (0.84 mL, 7.2 mmol) and MNTP (769 mg, 1.73 mmol). After having been stirred at rt for 1 h, the mixture was diluted with CH_2Cl_2 (10 mL). The solution was washed with saturated $NaHCO_3$ aq. (3 × 10 mL) and the aqueous layer was back-extracted with CH_2Cl_2 (10 mL). The organic layer and washings were combined, dried over Na_2SO_4, filtered, and concentrated to dryness under reduced pressure. The residue was chromatographed on silica gel (10 g) with a gradient of 10–50% acetone in acetonitrile with 0.5% Et_3N as the eluent. Fractions containing 5′-*O*-dimethoxytrityl-*O*-CEM-*N*^{6}-acetyladenosine cyanoethyl boranophosphate (**3**), unseparated by-products, and reagents, were combined and concentrated to dryness under reduced pressure. To a solution of **3** in CH_2Cl_2 (1 mL) was added Et_3N (1 mL, 7.2 mmol). After having been stirred at rt for 1 h, the mixture was concentrated to dryness under reduced pressure. The residue was chromatographed on silica gel (10 g) with a gradient of 0–50% acetone in acetonitrile with 0.5% Et_3N as the eluent. Fractions containing triethylammonium 5′-*O*-dimethoxytrityl-2′-*O*-CEM-*N*^{6}-acetyladenosine cyanoethyl boranophosphate (**4**) were combined and concentrated to dryness under reduced pressure. Excess Et_3N was removed by repeated coevaporation with toluene to yield **4** (358 mg; 54% from **1**) as a colorless foam. ^{1}H NMR (300 MHz, $CDCl_3$): δ 0–1 (bq, 3H); 1.27 (t, 9H, J = 7.3 Hz); 2.43–2.71 (m, 4H); 2.59 (s, 3H); 3.01 (q, 6H, J = 7.3 Hz); 3.43–4.04 (m, 6H); 3.79 (s, 6H); 4.47–4.57 (m, 1H); 4.79 (dd, 1H, J = 7.2, 10.2 Hz); 4.98–5.07 (m, 3H); 6.26–6.29 (m, 1H); 6.78–6.85 (m, 4H); 7.20–7.45 (m, 9H); 8.20 (d, 1H, J = 5.9 Hz); 8.61 (s, 1H); 8.68 (bs, 1H); ^{13}C NMR (300 MHz, $CDCl_3$): δ 8.6, 14.2, 18.7, 18.8, 20.0, 20.1, 25.6, 29.3, 29.7, 45.7, 55.3, 58.6, 62.9, 63.0, 63.2, 71.6, 86.9, 87.1, 94.9, 95.0, 113.2, 117.7, 117.8, 117.9, 127.1, 127.1, 127.9, 128.4, 130.3, 135.4, 135.4, 135.5, 141.3, 144.3, 149.2, 151.1, 151.2, 152.4, 152.5, 158.6, 158.6, 170.2; ^{31}P NMR (500 MHz, $CDCl_3$): δ 95.0–99.5 (m). HRMS (ESI$^-$) calcd for $C_{40}H_{45}BN_7O_{10}P$ [M−H]$^-$, 824.29748; found, 824.29600.

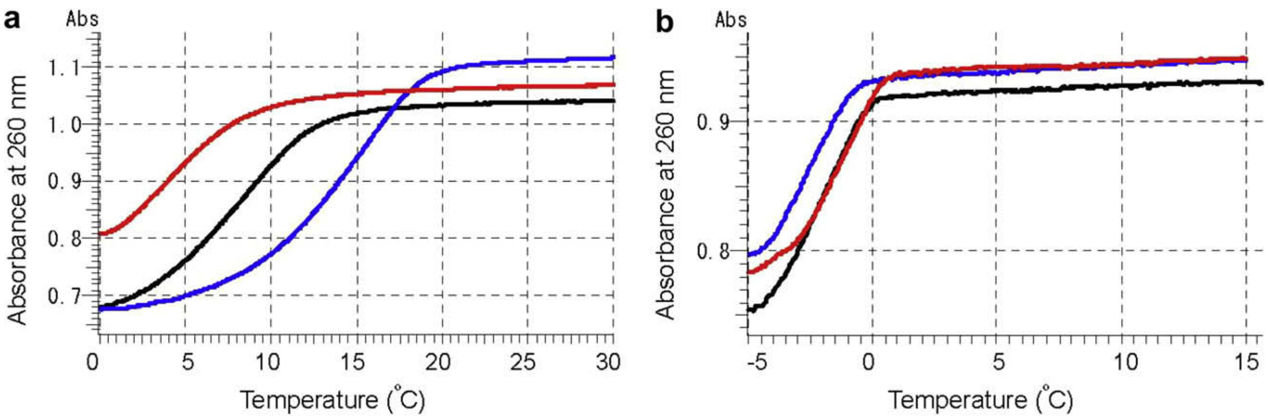


Figure 5. Melting curves of **7a** (blue), **7b** (red), or ApA (black) complexed with (a) poly(U) or (b) poly(dT). The spectra were taken in 10 mM sodium phosphate, pH 7.4, containing 1.0 M NaCl. Each dimer and poly(U) or poly(dT) were mixed at a ratio of 1:2 to give a total base concentration of 0.1 mM.

4.3.2. 2′-*O*-CEM-3′-*O*-TBDMS-N^6-acetyladenosine (5)

5′-*O*-Dimethoxytrityl-2′-*O*-CEM-N^6-acetyladenosine **1** (2.0 g, 2.87 mmol) was dried by repeated coevaporation with dry toluene followed by dry pyridine and finally dissolved in dry dimethylformamide (30 mL). To the solution were successively added imidazole (0.43 g, 6.31 mmol) and *tert*-butyldimethylsilyl chloride (0.87 mg, 5.74 mmol). After having been stirred at rt for 20 h, the mixture was diluted with AcOEt (50 mL). The solution was then washed successively with water (2 × 50 mL) and brine (50 mL). The organic layers were combined and dried over Na_2SO_4, filtered, and concentrated to dryness under reduced pressure. The residue was chromatographed on silica gel (40 g) with a mixture of AcOEt, hexane, and acetone as the eluent. Fractions were combined and concentrated to dryness under reduced pressure to yield 5′-*O*-dimethoxytrityl-2′-*O*-CEM-3′-*O*-TBDMS-N^6-acetyladenosine (1.72 g, 74%) as a colorless foam. The resulting intermediate (1.72 g, 2.12 mmol) was dissolved in CH_2Cl_2 (60 mL). To the solution were successively added dichloroacetic acid (1.75 mL, 21.2 mmol). After having been stirred at rt for 15 min, the mixture was diluted with CH_2Cl_2. The solution was then washed with saturated $NaHCO_3$ aq. (2 × 50 mL) and brine (50 mL). The organic layers were dried over Na_2SO_4, filtered, and concentrated to dryness under reduced pressure. The residue was chromatographed on silica gel with a mixture of CH_2Cl_2 and MeOH as the eluent. Fractions were combined and concentrated to dryness under reduced pressure to yield 2′-*O*-CEM-3′-*O*-TBDMS-N^6-acetyladenosine **5** (730 mg, 68%) as a colorless foam. ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.13 (s, 6H); 0.95 (s, 9H); 2.22–2.27 (m, 2H); 2.65 (s, 3H); 3.27–3.46 (m, 2H); 3.69–3.81 (m, 1H); 3.97 (d, 1H, J = 12.3 Hz); 4.23 (s, 1H); 4.57 (d, 1H, J = 4.6 Hz); 4.59 (s, 2H); 5.02 (dd, 1H, J = 4.6, 7.7 Hz); 5.92 (d, 1H, J = 12.3 Hz); 6.02 (d, 1H, J = 7.7 Hz); 8.14 (s, 1H); 8.67 (s, 1H); 8.83 (s, 1H); ^{13}C NMR (300 MHz, $CDCl_3$): δ −4.7, −4.6, 18.2, 18.6, 25.8, 25.9, 62.9, 63.1, 72.2, 78.8, 89.5, 95.0, 117.2, 123.2, 143.3, 150.0, 152.0, 170.6. HRMS (ESI$^-$) calcd for $C_{22}H_{34}N_6O_6Si$ $[M-H]^-$, 505.22254; found, 505.22072.

4.3.3. 5′-*O*-Dimethoxytrityl-2′-*O*-CEM-N^6-acetyladenosine-3′-yl 3′-*O*-TBDMS-N^6-acetyladenosine-5′-yl cyanoethyl boranophosphate (6a and 6b)

Compounds **5** (85.8 mg, 92.5 μmol) and **4** (46.9 mg, 92.5 μmol) were dried by repeated coevaporation with dry toluene followed by dry pyridine and finally dissolved in dry acetonitrile (1.00 mL). To the solution were successively added 2,6-lutidine (0.11 mL, 0.925 mmol) and MNTP (123.5 mg, 0.278 mmol). After having been stirred at rt for 1 h, the mixture was diluted with CH_2Cl_2 (10 mL). The solution was washed with saturated $NaHCO_3$ aq. (3 × 10 mL), and the aqueous layer was back-extracted with CH_2Cl_2 (10 mL). The organic layer and washings were combined, dried over Na_2SO_4, filtered, and concentrated to dryness under reduced pressure. The residue was chromatographed on silica gel (5 g) with a gradient of 0–50% acetone in acetonitrile containing 0.5% pyridine as the eluent. Fractions containing 5′-*O*-dimethoxytrityl-2′-*O*-CEM-N^6-acetyladenosine-3′-yl 3′-*O*-TBDMS-N^6-acetyladenosine-5′-yl cyanoethyl boranophosphate were combined to yield a diastereomeric mixture of **6a** and **6b**. ^{1}H NMR (300 MHz, $CDCl_3$): δ 0–1 (bq, 3H); 0.15 (s, 6H); 0.94 (s, 9H); 2.25–2.33 (m, 2H); 2.48–2.70 (m, 10H); 3.24–3.77 (m, 6H); 3.78 (s, 6H); 4.02–4.52 (m, 6H); 4.60–4.87 (m, 6H); 5.16–5.35 (m, 2H); 6.17–6.23 (m, 2H); 6.79–6.83 (m, 4H); 7.22–7.31 (m, 7H); 7.38–7.41 (m, 2H); 8.16–8.25 (m, 2H); 8.56–8.57 (m, 1H); 8.68 (s, 1H); 8.81(bs, 1H); 8.87 (bs, 1H); ^{13}C NMR (300 MHz, $CDCl_3$): δ −4.8, −4.8, −4.6, 8.7, 14.1, 18.1, 18.1, 18.5, 18.5, 18.7, 18.8, 18.8, 19.6, 19.7, 22.7, 24.3, 25.6, 25.7, 25.7, 25.8, 26.5, 26.6, 29.3, 29.6, 29.7, 31.5, 31.5, 31.8, 46.3, 46.6, 46.6, 47.3, 47.5, 53.5, 55.3, 55.3, 61.5, 62.3, 62.7, 63.1, 63.2, 63.3, 70.5, 70.7, 76.3, 76.8, 78.7, 78.8, 82.6, 83.3, 86.0, 87.2, 87.7, 87.8, 89.4, 95.4, 95.4, 113.3, 116.3, 117.5, 117.5, 117.7, 117.7, 122.2, 122.4, 124.0, 127.1, 127.2, 128.0, 128.1, 130.1, 130.2, 135.2, 135.2, 142.0, 142.2, 144.2, 149.5, 150.9, 151.2, 151.2, 152.5, 152.5, 158.7, 158.7, 170.4, 170.5, 170.5; ^{31}P NMR (500 MHz, $CDCl_3$): δ 120.3–121.2 (m). HRMS (ESI$^-$) calcd for $C_{62}H_{77}BN_{13}O_{15}PSi$ $[M-H]^-$, 1312.51783; found, 1312.51717.

4.3.4. Triethylammonium diadenosine 3′,5′-boranophosphate (Ap^{b}A) (7a and 7b)

The diastereomeric mixture of **6a** and **6b** (69.8 mg, 53.1 μmol) was added to a solution of 3% dichloroacetic acid (DCA) in Et_3SiH/CH_2Cl_2 (1:1 v/v, 5.3 mL), and the mixture was stirred at rt for 1 min. The mixture was then diluted with CH_2Cl_2 and washed with saturated $NaHCO_3$ aq. (2 × 10 mL), and the aqueous layer was back-extracted with CH_2Cl_2 (1 × 10 mL). The organic layer and washings were combined, dried over Na_2SO_4, filtered, and concentrated to dryness under reduced pressure. To a solution of the residue in acetonitrile (1 mL) was added Et_3N (1 mL). After having been stirred at rt for 1 h, the mixture was concentrated to dryness under reduced pressure. The residue was then dissolved in NH_3 aq./EtOH (2 mL) and stirred at rt for 1 h, after which the reaction mixture was concentrated to dryness under reduced pressure. A solution of 1.0 M TBAF in tetrahydrofuran (THF) solution containing 1% nitromethane (2 mL) was added to the residue, and the solution was stirred at rt overnight. The reaction mixture was subjected to reverse-phase column chromatography (LiChroprep RP-18 GP (Merck)) with a linear gradient of 0–20% acetonitrile in 50 mM triethylammonium acetate (TEAA) buffer, pH 7.0. The flow rate was 10 mL/min and the effluent was monitored at 260 nm. Fractions containing triethylammonium diadenosine 3′,5′-boranophosphate were concentrated to dryness under reduced pressure. This purification procedure afforded diastereopure **7a** (394 A_{258} units, equivalent to 14.5 μmol) and **7b** (347 A_{258} units, equivalent to 12.8 μmol) in 51% yield over 4 steps from **6a + 6b**). NMR of **7a** (Na salt): ^{1}H NMR (D_2O) δ 0–1 (bq, 3H); 3.85–3.90 (m, 1H); 3.99–4.03 (m, 1H); 4.14–4.18 (m, 1H); 4.35–4.40 (m, 3H); 4.41–4.44 (m, 1H); 4.47–4.50 (m, 1H); 4.64–4.70 (m, 2H); 5.89–5.90 (m, 1H); 5.91–5.92 (m, 1H); 7.90–7.92 (m, 1H); 8.10–8.12 (m, 1H); 8.19–8.21 (m, 1H); 8.23–8.24 (m, 1H); ^{13}C NMR (D_2O) δ 63.4, 64.1, 71.9, 73.5, 74.3, 75.9, 76.6, 77.6, 85.4, 86.4, 88.8, 90.8, 91.2, 92.7, 141.5, 142.4, 143.4, 154.7, 155.5, 157.6; ^{31}P NMR (D_2O) δ 97.88 (d, $J_{P,B}$ = 413 Hz). HRMS (ESI$^-$) calcd for $C_{20}H_{28}BN_{10}O_9P$ $[M-H]^-$, 593.17877; found, 593.17938. NMR of **7b** (Na salt): ^{1}H NMR (D_2O) δ 0–1 (bq, 3H); 3.81–3.89 (m, 1H); 4.16–4.32 (m, 1H); 4.34–4.38 (m, 2H); 4.47–4.50 (m, 1H); 4.55–4.59 (m, 1H); 4.69–4.72 (m, 1H); 4.80–4.86 (m, 1H); 5.85–5.88 (m, 1H); 5.96–5.99 (m, 1H); 7.97–8.01 (m, 1H); 8.10–8.13 (m, 1H); 8.20–8.23 (m, 1H); 8.32 (s, 1H); ^{13}C NMR (D_2O) δ 63.5, 64.4, 65.6, 72.4, 73.5, 76.1, 76.5, 77.2, 85.8, 86.7, 88.7, 90.6, 91.2, 92.1, 141.9, 142.8, 151.1, 155.4, 155.5, 158.0; ^{31}P NMR (D_2O) δ 97.38 (d, $J_{P,B}$ = 411 Hz). HRMS (ESI$^-$) calcd for $C_{20}H_{28}BN_{10}O_9P$ $[M-H]^-$, 593.17877; found, 593.17813. The two diastereoisomers were designated **7a** and **7b** according to their order of elution on reverse-phase HPLC, and they showed no evidence of diastereomeric cross-contamination on analytical reverse-phase HPLC.

4.4. Preparation of diadenosine (ApA)

ApA was synthesized on a 15-μmol scale by the phosphoramidite method modified for CEM chemistry on an ÄKTA Oligopilot 10 (GE Healthcare Bio-Science Corp., NJ). Commercially available controlled-pore glass (CPG) derivatized with *N*-benzoyl-2′-*O*-acetylriboadenosine was used as the solid support. 2′-*O*-CEM-phosphoramidite adenosine was synthesized as previously described.[15] Cleavage from the resin and deprotection of the phosphate and base moiety were carried out by treatment with concentrated ammonia in EtOH at 35 °C for 15 h. The other protecting groups were removed by 1 M TBAF in THF and the ApA was purified as described above.

4.5. Mixing curves

Poly(U) and dimer were mixed in various proportions to maintain a constant total base concentration of 0.1 mM. Each nucleotide mixture contained, in a total volume of 4 mL, nucleotide dimer and/or polymer, 1.0 M NaCl, and 10 mM sodium phosphate, pH 7.4. The mixture was scanned at 0 °C from 320 to 205 nm, and from each spectrum A_{260} was determined and plotted against the proportion of poly(U).

4.6. Melting curves

Melting curves (absorbance–temperature profiles) were recorded in the presence of 10 mM sodium phosphate, pH 7.4, containing 1.0 M NaCl. Each dimer and poly(U) were mixed at a ratio of 1:2 to give a total base concentration of 0.1 mM. The absorbance was monitored at 260 nm as the temperature was increased from 0 °C to 30 °C at a rate of 0.5 °C/min. T_m values were determined as the temperature corresponding to the peak of the first-derivative plot of the melting profile.

4.7. CD spectroscopy

The spectropolarimeter was calibrated with (+)-10-camphorsulfonic acid. Spectra were recorded in a 1-cm-path-length quartz cell at a scan speed of 50 nm/min, and the recorded spectra were digitized at 0.1-nm resolution and stored on a floppy disk. The bandwidth was 1 nm and the response time was 1.0 s. At least seven spectra were accumulated for each determination.

4.8. Preparation of NMR samples

Compounds **7a** and **7b** synthesized as described above were subjected to reverse-phase column chromatography (LiChroprep RP-18 GP (Merck)) with a linear gradient of 0–20% acetonitrile in 50 mM triethylammonium hydrogen carbonate (TEAB) buffer, pH 7.4. The flow rate was 10 mL/min and the effluent was monitored at 260 nm. Fractions containing **7a** or **7b** were concentrated to dryness under reduced pressure, coevaporated twice with H_2O to remove TEAB buffer, and passed through a pyridinium-form Dowex® 50WX8–200 ion-exchange resin followed by a sodium-form resin. The dimers were eluted with water at each ion-exchange step, and so were obtained in the sodium form. The dimers were coevaporated twice with 99.96% D_2O (Merck) and lyophilized twice from 99.96% D_2O for NMR analysis.

4.9. HPLC

The HPLC buffer stock solution was 1 M TEAA, pH 7.0 (1 M in triethylamine, titrated to pH 7.0 with acetic acid), and the HPLC buffers were prepared by mixing the appropriate amounts of 1 M TEAA, pH 7.0, and acetonitrile with distilled deionized water. The column was operated at a flow rate of 1 mL/min and the effluent was monitored at 260 nm.

4.10. Nuclease-digestion experiments

4.10.1. Digestion by snake venom phosphodiesterase

Snake venom phosphodiesterase (0.3 U) and buffer (7 μL; 50 mM Tris–HCl, pH 9.3, containing 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$ and 1 mM spermidine; final concentrations) were added to each dimer (0.1 A_{260} units) to give a total volume of 70 μL and the reaction mixture was incubated at 37 °C. Samples of the reaction mixture were taken at 0.5, 3, 24, and 48 h and analyzed directly by reverse-phase HPLC.

4.10.2. Digestion by nuclease P1

Each dimer (0.1 A_{260} units) was incubated with nuclease P1 (2 U) at 37 °C. Samples of the reaction mixture were taken at 0.5, 3, 24, and 48 h and analyzed directly by reverse-phase HPLC.

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