Synthesis and Conformational Properties of Oligonucleotides Incorporating 2′-*O*-Phosphorylated Ribonucleotides as Structural Motifs of Pre-tRNA Splicing Intermediates

Hiroyuki Tsuruoka, Koh-ichiroh Shohda, Takeshi Wada, and Mitsuo Sekine*

Department of Life Science, Tokyo Institute of Technology, Nagatsuta, Midoriku, Yokohama 226-8501, Japan

msekine@bio.titech.ac.jp

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To synthesize oligonucleotides containing 2′-*O*-phosphate groups, four kinds of ribonucleoside 3′-phosphoramidite building blocks **6a**−**d** having the bis(2-cyano-1,1-dimethylethoxy)thiophosphoryl (BCMETP) group were prepared according to our previous phosphorylation procedure. These phosphoramidite units **6a**−**d** were not contaminated with 3′-regioisomers and were successfully applied to solid-phase synthesis to give oligodeoxyuridylates **15**, **16** and oligouridylates **21**, **22**. Self-complementary Drew−Dickerson DNA 12mers **24**−**28** replaced by a 2′-*O*-phosphorylated ribonucleotide at various positions were similarly synthesized. In these syntheses, it turned out that KI_3 was the most effective reagent for oxidative desulfurization of the initially generated thiophosphate group to the phosphate group on polymer supports. Without using this conversion step, a tridecadeoxyuridylate **17** incorporating a 2′-*O*-thiophosphorylated uridine derivative was also synthesized. To investigate the effect of the 2′-phosphate group on the thermal stability and 3D-structure of DNA(RNA) duplexes, T_m measurement of the self-complementary oligonucleotides obtained and MD simulation of heptamer duplexes **33**−**36** were carried out. According to these analyses, it was suggested that the nucleoside ribose moiety phosphorylated at the 2′-hydroxyl function predominantly preferred C2′-endo to C3′-endo conformation in DNA duplexes so that it did not significantly affect the stability of the DNA duplex. On the other hand, the 2′-modified ribose moiety was expelled to give a C3′-endo conformation in RNA duplexes so that the RNA duplexes were extremely destabilized.

Introduction

In 1981, Konarska first discovered that when the circular RNA fragments of tobacco mosaic virus RNAΩ73 were treated with wheat germ, an unprecedented species, phosphorylated at the 2′-hydroxyl function, was formed as the ligated product.[1,2] This unique nucleotide has both phosphomonoester and phosphodiester linkages on the 2′,3′-*cis*-diol.

After the discovery of such a species, Abelson and co-workers also reported that pre-tRNA^{Leu}, transcribed from DNA, underwent splicing in tRNA synthesis with elimination of its intron part to generate a spliced tRNA product 2′-*O*-phosphorylated at the ribonucleoside at the junction point near the 3′-side of the anticodon region.[3] Since it is considered that the mature function of tRNA would not be completely acquired unless the 2′-phosphate group was removed,[4] the influence of the 2′-phosphate group on the structure of tRNA was expected to be connected with the recognition of aminoacyl-tRNA synthetases (ARS). To date, a number of pre-tRNAs having an intron have been found in yeast pre-tRNAs.[5] The mechanism of this tRNA splicing is quite different from those of Group I and II self-splicing and eukaryotic mRNA splicing.[3,6] Therefore, this unique processing was considered to be developed from a diverse origin. Moreover, Phizicky et al. reported that an NAD-dependent 2′-phosphotransferase playing a critical role at the final step of tRNA splicing transferred the 2′-phosphoryl function from 2′-*O*-phosphorylated tRNA to NAD to give an ADP-ribose 1′-2′ cyclic phosphate.[7]

With respect to another biochemical property of 2′-phosphorylated RNA species, Szostak and co-workers reported that when the in vitro selection from a random RNA pool and mutagenic PCR was performed to obtain evolved ribozymes with polynucleotide 5′-*O*-thiophosphorylation activity, some of the products selected were eventually phosphorylated at the internal 2′-position.[8] This result strongly suggested the possibility that RNAs involving a 2′-phosphorylated ribonucleoside exist in the process of molecular evolution of nucleic acids. Interestingly, they also disclosed that reverse transcription using an RNA oligomer having a 2′-*O*-thiophosphoryl group as

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(2) Konarska, M.; Filipowicz; Gross, H. J. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 1474.
(3) Greer, C. L.; Peebles, C. L.; Gegenheimer, P.; Abelson, J. *Cell* **1983**, *32*, 537.
(4) Zillmann, M.; Gorovsky, M. A.; Phizicky, E. M. *J. Biol. Chem.* **1992**, *267*, 10289−10294.
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(6) (a) Cech, T. R. *Cell* **1983**, *34*, 713−716. (b) Filipowicz, W.; Shatkin, A. J. *Cell* **1983**, *32*, 547−557. (c) Mattoccia, E.; Baldi, I. M.; Gandini-Attardi, D.; Ciafre, S.; Tocchini-Valentini, G. M. *Cell* **1988**, *55*, 731−738. (d) Rauhut, R.; Green, P. R.; Abelson, J. *J. Biol. Chem.* **1990**, *30*, 18180−18184.
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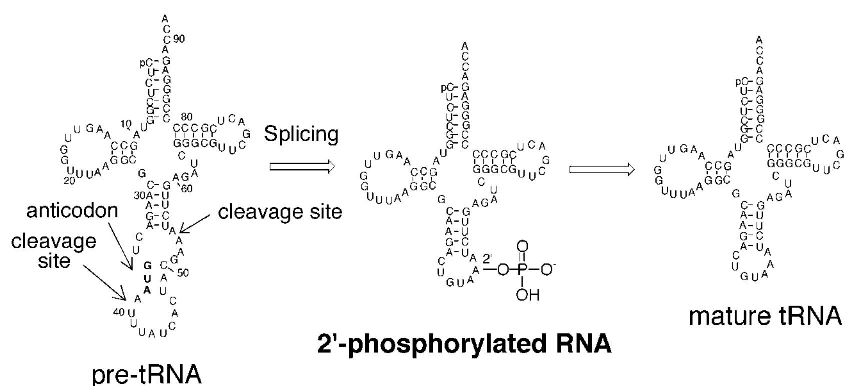
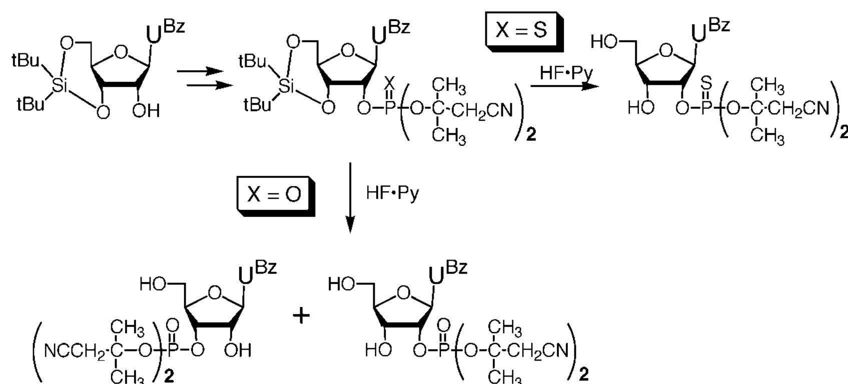
Scheme 1



Scheme 2



the templates caused a pause in AMV and MMLV H⁻ reverse trascriptases, while these ultimately read through this point.[9]

In consideration of these facts found in tRNA splicing and reverse transcription, studies on the 3D-structure of oligonucleotides containing a 2′-phosphorylated ribonucleoside could provide valuable information for molecular recognition of ARSs and HIV-reverse transcriptases and structure−activity relationships. In our recent studies, an effective method for the polymer-supported synthesis of 2′-*O*-phosphorylated RNAs has been established to give oligouridylates (2−6mers) phosphorylated at the 2′-positions of all the uridines except the 3′-terminal one by use of the phosphoramidite approach.[10] However, this polymer support synthesis, which involved removal of the 2-cyano-1,1-dimethylethyl group used as a protecting group for the thiophosphate group and the successive conversion of the unprotected thiophosphate group into a phosphate group, was accompanied with unexpected side reactions and therefore limited to short oligonucleotides. Later, this polymer-supported synthesis was improved by use of KI_3 as the reagent for the S−O conversion to give oligodeoxynucleotides incorporating a 2′-phosphorylated uridine as briefly reported in our previous communication.[11] Our recent NMR and CD studies revealed that the 5′-upstream uridine ribose residue of U(2′-p)pU has a C2′-endo conformation similar to that of B-DNA rather than A-DNA.[11] It was also shown by T_m experiments and MD simulation that, when incorporated into a tridecadeoxyuridylate, the 2′-*O*-phosphorylated uridine behaved as a rigid C2′-endo nucleoside.

In this paper, we report a general procedure for the synthesis of four kinds of 2′-*O*-phosphorylated ribonucleoside building blocks and their application to the synthesis of oligonucleotides containing a 2′-*O*-phosphorylated ribonucleotide. Finally, to study the structural effect of only one 2′-*O*-phosphate group on recognition of ARSs, thermodynamic and conformational properties of these modified oligomers were examined.

Results and Discussion

Synthesis of Four Kinds of Ribonucleoside 3′-Phosphoramidites Having the 2′-*O*-BCMETP Group. Previously, we first reported the 2′-*O*-phosphorylation of ribonucleosides by the use of the bis(*tert*-butoxy)phosphoryl (BTBP) group masked by sterically hindered protecting groups.[12]

However, removal of the two *tert*-butyl groups from the BTBP group required rather drastic conditions (20% trifluoroacetic acid in acetic acid at room temperature for 3−5 h) which were apparently unsuitable for the synthesis of longer oligonucleotides. To overcome this problem, we considered the use of a bis(2-cyano-1,1-dimethylethoxy)phosphoryl (BCMEP) group protected by the 2-cyano-1,1-dimethylethyl (CME) groups (Scheme 2), which not only was sterically hindered but also could be simultaneously eliminated by treatment with a combined reagent of DBU and bis(trimethylsilyl)acetamide (BSA).[10,13]

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(10) Sekine, M.; Tsuruoka, H.; Iimura, S.; Kusuoku, H.; Wada, T.; Furusawa, K. *J. Org. Chem.* **1996**, *61*, 4087−4100.
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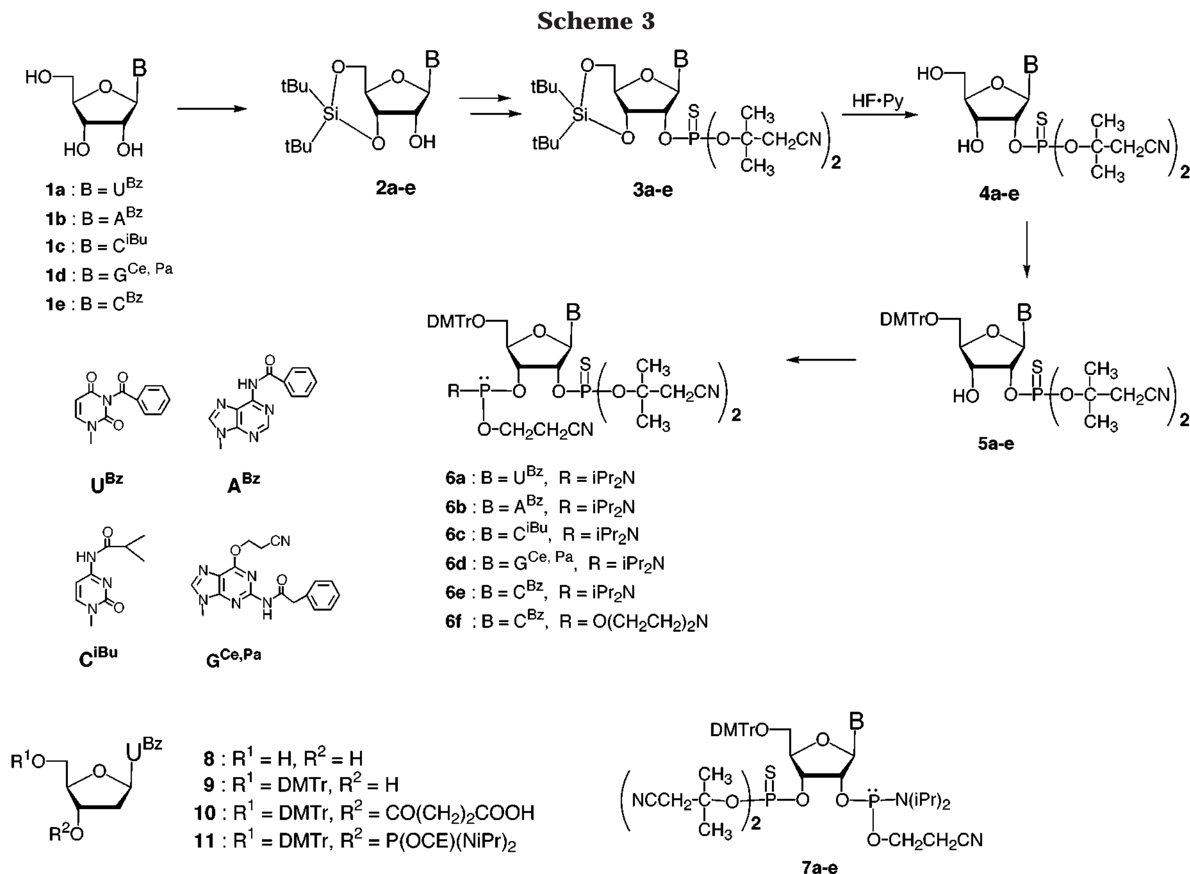
(13) Sekine, M.; Tsuruoka, H.; Iimura, S.; Wada, T. *Nat. Prod. Lett.* **1994**, *5*, 41−46.

Scheme 3



However, when the di-*tert*-butylsilanediyl (DTBS) group was removed from the 3′,5′-positions after introduction of the BCMEP group into the 2′-hydroxyl group of a uridine derivative, 2′−3′ phosphoryl migration slightly took place to give a mixture of the desired 2′-*O*-phosphorylated product and its 3′-regioisomer. It seemed that such isomerization occurred because of the electron-withdrawing effect of the two cyano groups involved in the CME groups, although four covalent bonds exist between the phosphorus and cyano groups. This result led us to examine the bis(2-cyano-1,1-dimethylethoxy)-thiophosphoryl (BCMETP) group, having the lesser electronegative sulfur atom instead of oxygen in the BCMEP group. Consequently, we found that introduction of the BCMETP group resulted in complete suppression of the 2′−3′ phosphoryl migration.

In the present study, our preliminary method of 2′-*O*-phosphorylation using uridine derivatives was applied to preparation of three other kinds of nucleoside 3′-phosphoramidites building blocks having the 2′-*O*-BCMETP group.

4-*N*-Benzoylated cytidine (**1e)** was silylated by treatment with di(*tert*-butyl)dichlorosilane in the presence of silver nitrate (Scheme 3). However, silylation of **1e** was slightly complicated due to the remaining reactivity of the 3-nitrogen atom despite introduction of the benzoyl group into the nucleobase. Furusawa reported that when di(*tert*-butyl)silyl bis(trifluoromethanesulfonate) was employed in the presence of silver nitrate that could favorably coordinate to the 3-nitrogen, the desired product was obtained quantitatively.[14] In fact, **2e** was synthesized in 85% yield. A characteristic feature of **2e** was its low solubility in various solvents such as CH_2Cl_2, THF, and CH_3CN. Because of this inherent problem, an excess amount (2.5 equiv) of bis(2-cyano-1,1-dimethylethoxy)-diethylaminophosphine (CMEAP)[10] was required to obtain a satisfactory result in the 2′-*O*-phosphitylation in CH_2Cl_2. The successive in situ sulfurization of the phosphitylated product in pyridine−CS_2 for 30 min gave the 2′-thiophosphorylated derivative **3e** quantitatively according to the TLC analysis. Desilylation of the DTBS group was successively carried out by treatment with pyridinium hydrogenfluoride to produce a 3′,5′ unprotected 2′-*O*-thiophosphorylated cytidine derivative **4e** in 77% yield from **2e**. The detailed analysis of ^1H, ^{13}C, and ^{31}P NMR of this product revealed that it was obtained free from the 3′-regioisomer as reported in the case of the 2′-*O*-thiophosphorylated uridine derivative **4a**. After the usual 5′-dimethoxytritylation of **4e**, the 3′-hydroxyl group of the resulting product **5e** could subsequently be phosphitylated cleanly. However, the corresponding phosphoramidite **6e** was considerably unstable during silica gel column chromatography. Attempts of other purification methods, such as aluminum oxide and gel-filtration column chromatography, and precipitation by use of various solvent systems, failed, and consequently **6e** could not be obtained in sufficiently pure form. Therefore, the phosphoromorpholidite **6f** was prepared as a more stable derivative[15] instead of **6e**. Compound **6f** could be synthesized purely by use of $O(CH_2CH_2)_2NP(OCE)Cl$ in the presence of Et_3N without the 2′−3′ phosphoryl migration. The condensation using **6f** was attempted with the 5′-hydroxyl of a uridine derivative bound to aminopropyl-CPG **12b**. However, the average coupling yield did

(14) (a) Furusawa, K.; Ueno, K.; Katsura, T.; *Chem. Lett.* **1990**, 97. (b) Furusawa, K.; Katsura, T. *Tetradedron Lett.* **1985**, *26*, 887. (c) Furusawa, K. *Chem. Lett.* **1989**, 509 (d) Frusawa, K. *Chem. Express* **1991**, *6*, 763.

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not exceed 85% even when a slightly higher concentration (0.15 M) of **6f** was employed. It is desirable that condensation in the solid-phase synthesis is performed in more than 95% yield. Since the 2′-thiophosphorylated uridine derivative (**6a)** reported previously was stable enough to isolate and difference between the 3′-phosphoramidites **6a** and **6e** was only in the structure of the base moiety, the benzoyl group as a 4-*N*-protecting group of cytidine was replaced by the isobutyryl group with the hope that this more sterically hindered aliphatic protecting group might stabilize the 3′-phosphoramidite function. Silylation of 4-*N*-isobutyrylcytidine (**1c**) followed by the subsequent 2′-*O*-thiophosphorylation, desilylation, and 5′-*O*-dimethoxytritylation gave the 5′-masked product **5c** in good yield. Introduction of the phosphoramidite function into the 3′-hydroxyl of **5c** using $i\text{Pr}_2\text{NP(OCE)Cl}$ and Et_3N gave the building unit **6c**. It turned out that **6c** did not decompose during silica gel column chromatography. It was, however, disclosed that this phosphitylation accompanied the 2′−3′ migration of the 2′-thiophosphoryl group so that a desired product was contaminated with the 3′-thiophosphorylated cytidine 2′-*O*-phosphoramidite **7c**, which could not be separated by any purification methods as shown in Figure 1-A_1. It was likely that this migration was ascribed to the basicity of Et_3N (pK_a; 10.7). Therefore, 2,4,6-collidine (pK_a; 7.4) as a weaker base, which Usman et al. used in the synthesis of 2′-*O*-silylated ribonucleoside 3′-phosphoramidites,[16] was used to avoid such a base-induced phosphoryl migration. Consequently, migration was completely suppressed to give a sole product **6c** in 72% yield as evidenced by Figure 1-A_2.

Since the nucleobase structure of ribonucleosides considerably affected the stability of the P−N bond at the 3′-position as well as 2′−3′ phosphoryl migration, these major effects might be explained in terms of the electron-withdrawing effect of the base moiety. To investigate the inductive effect of cytosine derivatives, partial charges of 1-methyl-carbon in 4-*N*-benzoyl-N^1-methylcytosine and 4-*N*-isobutyryl-N^1-methylcytosine as model compounds were estimated by ab initio calculation using the HF/6-31G* basis set. However, there was almost no difference between them (data not shown). Accordingly, the inductive effect would not be an essential factor.

Next, to prepare 2′-thiophosphorylated adenosine and guanosine 3′-phosphoramidite **6b** and **6d**, 6-*N*-benzoyladenosine **1b** and 6-*O*-cyanoethyl-2-*N*-phenylacetylguanosine **1d** were treated with tBu_2SiCl_2 and $AgNO_3$ to give the 3′,5′-*O*-protected derivatives **2b** and **2d**, respectively, in good yields. Moreover, **2b** and **2d** were converted to the corresponding 5′-*O*-dimethoxytritylated compounds **5b** and **5d** in 78 and 83% yields, respectively, via a four-step reaction. No 2′−3′ phosphoryl migration could be observed during these reactions. However, when the phosphoramidite function was introduced into the 3′-hydroxyl of **5b** and **5d** using $i\text{Pr}_2\text{NP(OCE)Cl}$ and Et_3N, 2′−3′ phosphoryl migration of 2′-thiophosphoryl group was slightly caused like that of the *N*-isobutyrylated cytidine derivative to simultaneously give the phosphitylated regioisomers **7b** and **7d**. The use of 2,4,6-collidine as the acid-scavenger allowed the complete suppression of the phosphoryl migration during the conversion to the 3′-phosphoramidite. Therefore, this base is the most appropriate reagent in the present approach.

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Stability of the 2′-*O*-Phosphorylated Ribonucleoside Derivatives 4a−e in Various Solvents. To evaluate the utility of the 2′-BCMETP group, the stability of four nucleotides **4a−e** were systematically examined.

Compounds **4a−e** were kept in pyridine, 80% acetic acid, and methanol at 25 °C for 50 days and distribution of the unchanged starting material, and the decomposition products in these solutions were estimated by ^{31}P NMR to monitor the phosphoryl migration and degradation. These results are shown in Figure 2. Only when the 3′-resioisomers could be spectroscopically assigned, their amount was measured

As shown in Figure 2-A, it was found that all of the nucleoside derivatives **4a−e** in pyridine underwent degradation of the 2′-BCMETP group rather than the 2′−3′ phosphoryl migration. The guanosine derivative **4d** was exceptionally the most stable compared with the other derivatives **4a−c** and **4e**. The usual conditions (rt, 4 h in pyridine) prescribed for the 5′-*O*-dimethoxytritylation gave the corresponding 3′-regioisomers to a degree of less than 0.1% in all cases. Interestingly, the 4-*N*-isobutyrylcytidine derivative **4c** was apparently more stable and more resistant to migration than the 4-*N*-benzoylcytidine derivative **4e**. This result was in agreement with those observed in conversion of **5c** and **5e** to **6c** and **6e**, respectively. In methanol as a protic solvent, most of **4a−e** remained unchanged even after a few days as shown in Figure 2-B. In contrast to the above result, however, the guanosine derivative **4d** was less stable than **4a−c** and **4e** upon standing for prolonged periods. In 80% acetic acid (Figure 2-C), the uridine and adenosine derivatives **4a** and **4b** were more resistant to migration and degradation than the other nucleoside derivatives. In **4a** and **4b**, 2′−3′ thiophosphoryl migration mainly occurred so that the 2′- and 3′-*O*-thiophosphorylated isomers remained to a degree of 90% even after 50 days. The guanosine derivative **4d** and the cytidine derivative **4c** and **4e** were relatively unstable, and only less than 20% of these compounds remained after 50 days.

In consideration of these stabilities and actual reaction conditions such as above, the present results suggested that **4a−d** were useful intermediates to meet the stability requirements during the synthesis of the building blocks **6a−d** and that protecting groups of the base moieties considerably affect the stability of the 2′-*O*-thiophosphoryl group.

Improved Polymer-Supported Synthesis of Oligonucleotides Incorporating 2′-*O*-Phosphorylated and 2′-*O*-Thiophosphorylated Ribonucleosides. It was previously reported by us that oligouridylates (2−6mers), phosphorylated at all internal 2′-positions, were prepared on polymer supports.[10] In the present study, we first attempted to apply this previous original method to incorporation of a 2′-*O*-phosphorylated uridine into oligodeoxyuridylates 10mer and 13mer only at one position (Scheme 4).

With respect to polymer-supported synthesis of the desired oligomers, the classical conditions used in the phosphoramidite method were basically used, but in the case of condensation using **6a** the reaction should be performed at a slightly high concentration (0.15 M) of the phosphoramidite reagent. If not, the average coupling yield was insufficient (ca. 85%). To synthesize deoxyuridylate 10mer $dU_4U(2'\text{-p})dU_5$ **13**, after the chain elongation, the 2-cyano-1,1-dimethylethyl (CME) and 2-cyanoethyl (CE) groups were removed from the fully protected

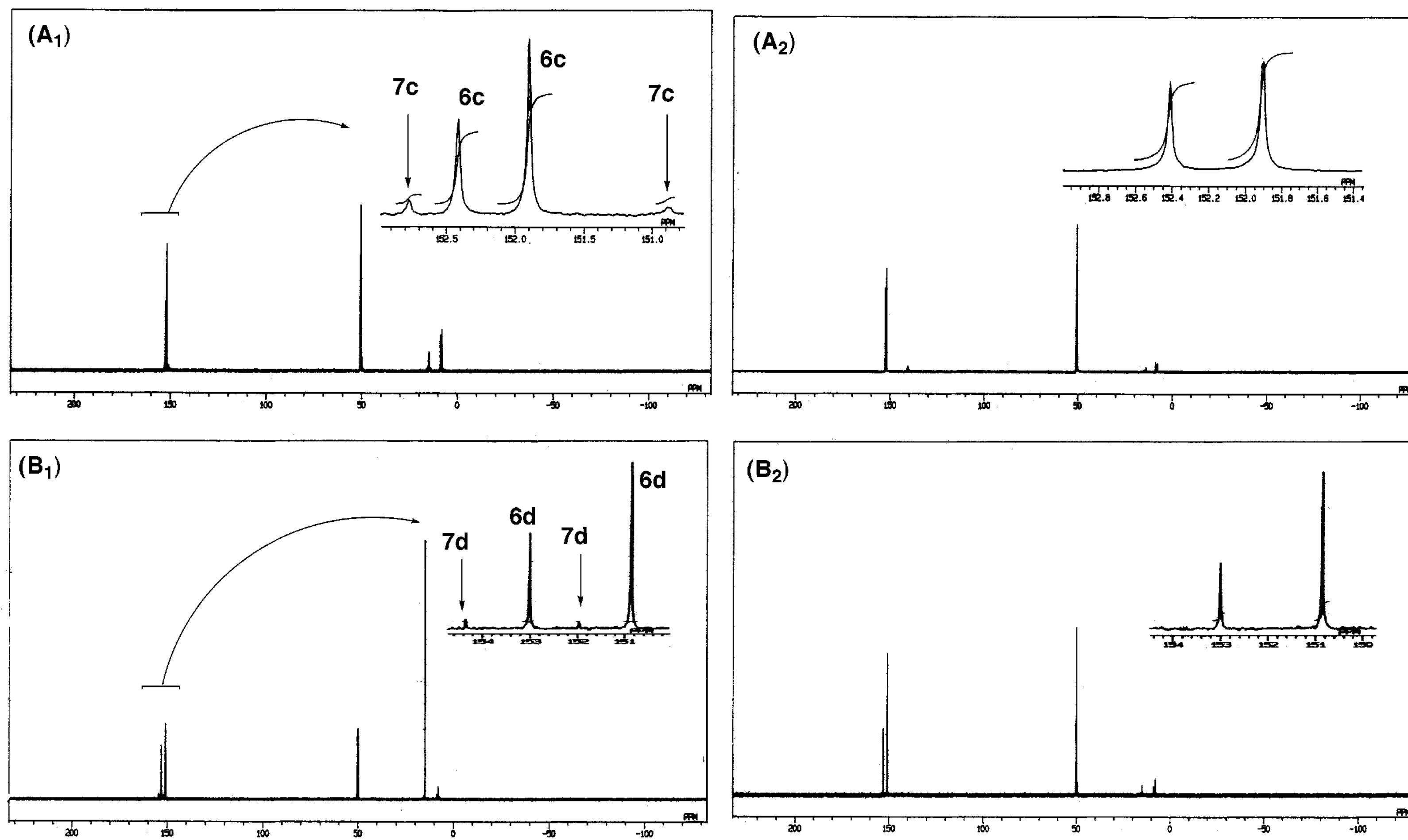


Figure 1. 2′−3′ Thiophosphoryl migration of the BCMETP group of **5c** and **5d** upon 3′-phosphitylation. A_1: The ^{31}P NMR spectrum of the product obtained by phosphitylation of **5c** in the presence of triethylamine. A_2: The ^{31}P NMR spectrum of the product obtained in phosphitylation of **5c** in the presence of 2,4,6-collidine. B_1: The ^{31}P NMR spectrum of the product obtained in phosphitylation of **5d** in the presence of triethylamine. B_2: The ^{31}P NMR spectrum of the product obtained in phosphitylation of **5d** in the presence of 2,4,6-collidine.

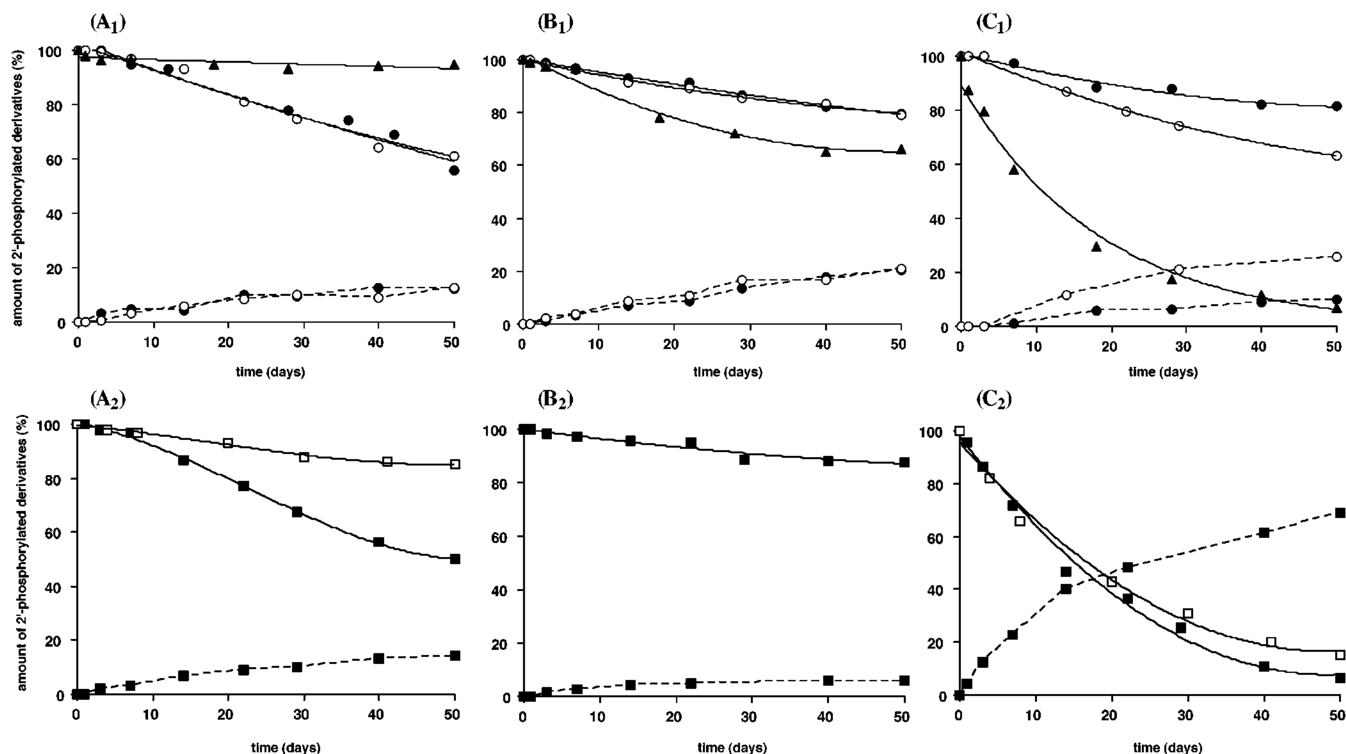
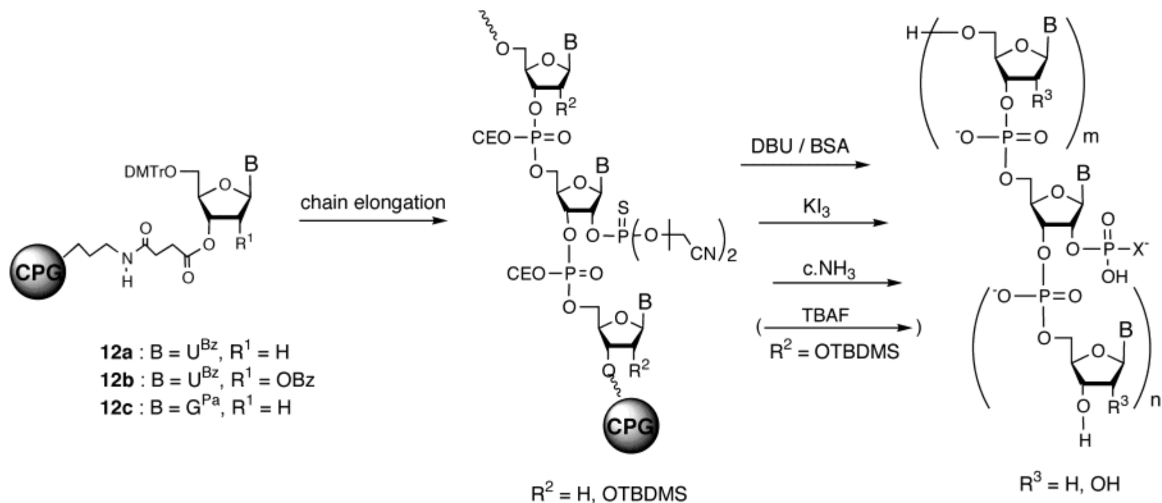


Figure 2. Stability of 2′-thiophosphorylated ribonucleoside derivatives **4a**−**e** in various solvents at 25 °C: solid dot, **4a**; open dot, **4b**; open square, **4c**; solid triangle, **4d**; solid squere, **4e**; solid line, the remaining amount of the starting material **4a**−**d**; dotted line, the amount of 3′-isomerized product; A: Each sample (0.06 mmol) was dissolved in Py-Py-d_5 (9:1, v/v, 0.6 mL). B: Each sample (0.06 mmol) was dissolved in CD_3OD (0.6 mL). C: Each sample (0.06 mmol) was dissolved in acetic acid−D_2O (4:1, v/v, 0.6 mL).

Scheme 4



oligomer bound to the polymer support by use of DBU in the presence of *N,O*-bis(trimethylsilyl)acetamide (BSA)[10] without release of the oligomer from the CPG. Oxidative conversion of the unprotected 2′-*O*-phosphorothioate monoester function to the corresponding phosphate was successively carried out by treatment with iodine, which was employed in the synthesis of U(2′-p)pU in the previous paper.[10] Finally, the succinate linker was cleaved by treatment with concentrated NH_3, and the crude material was analyzed by ion exchange HPLC.

As seen in Figure 3-A_1, a large broad peak at 23−33 min appeared, suggesting a cluster of many compounds having similar structures were formed. To clarify what happened during a series of treatments, each step was carefully checked. As a consequence, it was disclosed that the random 5-iodination of the uracil base with iodine occurred. About 30% of 2′-deoxyuridine was iodinated at the 5-position by 10 equiv of iodine after 48 h. Therefore, in the general polymer-supported synthesis, iodine has to be used carefully, especially if its reaction time is prolonged. In our previous paper[10] with regard to the synthesis of U(2′-p)pU, however, such an electrophilic substitution on the pyrimidine residue was not observed at all. It was assumed that this previous better result could be explained in terms of the electron-withdrawing effect by the N^3-protecting group which suppressed the side reaction. Accordingly, the N^3-benzoylated 2′-deoxyuridine phosphoramidite **11** was separately prepared,

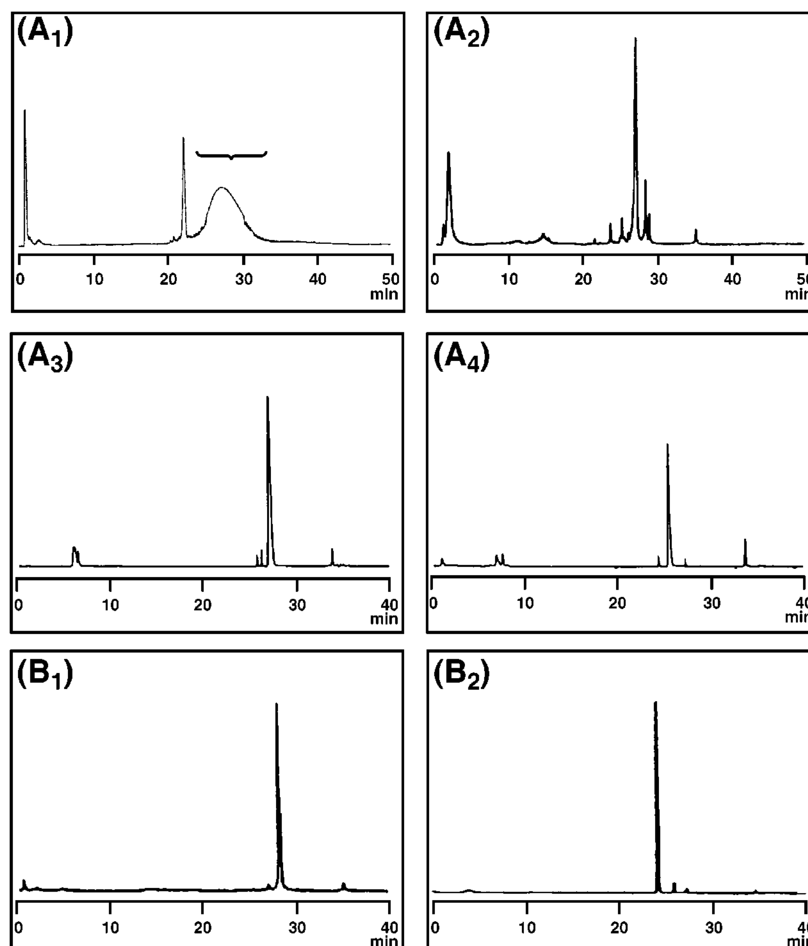
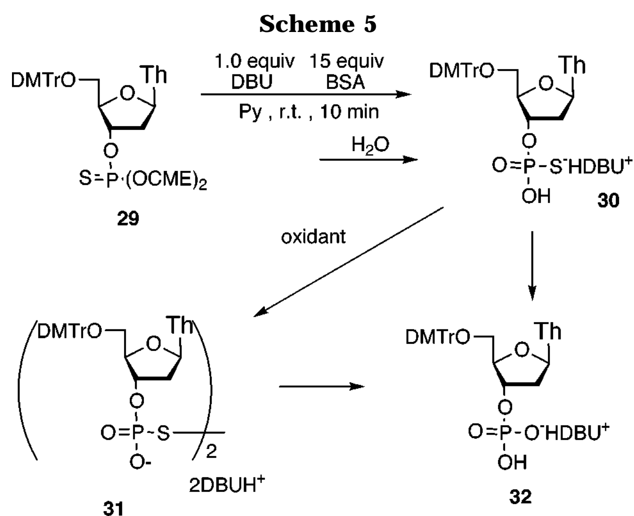


Figure 3. The ion-exchange HPLC profiles of uridylate 13mer containing a 2′-phosphoryl group. A_1: A crude material of **13** obtained after iodine oxidation followed by concd NH_3 treatment. A_2: A crude material of **16** obtained after KI_3-oxidation followed by concd NH_3 treatment. A_3: A purified material of **16.** A_4: A product obtained in dephosphorylation of **16** by treatment with alkaline phosphatase. (B_1) A purified material of **17.** (B_2) A product obtained in thermolysis of **17** at pH 7.0, 90 °C for 20 min.

and the synthesis of oligonucleotide **16** was performed in the same manner. However, the HPLC profile taken after treatment with concentrated NH_3 was as complicated as before in spite of the use of **11**. Since the oxidative S−O conversion by iodine not only took long (t_{comp} = 48 h) but also was accompanied 5-iodination, improvement of this reaction would require the use of an alternative to iodine.

Conversion of **30** as a model compound to **32** by other oxidants was examined (Scheme 5, Table 1). After removal of the two CME groups from the thymidine derivative **29** thiophosphorylated at the 3′-position and successive hydrolysis of the silyl ester, the oxidant was added in situ, and the time-dependent product distribution was pursued by ^{31}P NMR (see Supporting Information). Most of the reagents were found to promote formation of a byproduct **31** (δp: ca. 15 ppm) having the disulfide bond rather than desulfurization, but only iodine and KI_3 could gradually convert the once-formed **31** to the desired product **32**. (See Supporting Information) Although the desulfurization by mCPBA was too rapid to observe any intermediates, a small amount of pyrophosphate derivative (δ_P: ca. −10 ppm) was formed; therefore, this reaction is unsuitable for application to solid-phase synthesis. Reagents tBuOOH, H_2O_2, and K_3-$Fe(CN)_6$[17] readily accumulated a large amount of a disulfide derivative, and the reaction mixture became

Scheme 5



more complicated. Among them, the triiodide reagent KI_3, which was reported by Nussbaum et al.[18] in activation of the P−SEt bond of phosphorothioate derivatives, gave exclusively the desired product **32** during a relatively short period (t_{comp} = 6 h), and more importantly it was

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(18) Poonian, M. S.; Nowoswiat, E. F.; Nussbaum, A. L. *J. Am. Chem. Soc.* **1972**, *94*, 3992−3997.

Table 1. Oxidative Desulfurization of 30 to 32

oxidant (10 equiv)	t_{comp}[a]
I_2	48 h
KI_3	6 h
mCPBA	1 h[b]
tBuOOH	–[c]
H_2O_2	–[c]
$K_3Fe(CN)_6$	–[c]

[a] t_{comp} refers to the time when the reaction has completely finished. [b] A small amount of pyrophosphate was formed. [c] A considerable amount of S–S bond was formed.

Table 2. Isolated Yields and T_m Values of Oligomers Containing the 2′-*O*-Phosphoryl Groups

sequence	yield (%)	T_m (°C)	ΔT_m (°C)
d(UUUU**U**UUUUU) (**13**)			
d(UUUUUUUUUUUUU) (**14**)	–	30.9[a]	
d(UU**U**UUUUUUUUUU) (**15**)	20	28.8[a]	−2.1
d(UUUUUU**U**UUUUUU) (**16**)	28	26.2[a]	−4.7
d(UUUUUU$\underline{U}$UUUUUU (**17**)	19	25.5[a]	−5.4
d(UU**U**UUU**U**UUU**U**UU) (**18**)	–	–	–
UUUUUUUUUUUUU (**19**)			
UUUUUUUUUUUUU (**20**)	–	35.1[a]	
UU**U**UUUUUUUUUU (**21**)	7	27.8[a]	−7.3
UUUUUU**U**UUUUUU (**22**)	6	23.6[a]	−11.5
d(CGCGAAUUCGCG) (**23**)	–	55.7[b]	
d(C**G**CGAAUUCGCG) (**24**)	16	53.3[b]	−2.4
d(CGC**G**AAUUCGCG) (**25**)	29	55.7[b]	0
d(CGCGA**A**UUCGCG) (**26**)	22	41.2[b]	−14.5
d(CGCGAA**U**UCGCG) (**27**)	20	40.5[b]	−15.2
d(CGCGAAUU**C**GCG) (**28**)	27	51.3[b]	−4.4

[a] In the T_m measurement of oligomers **14**–**22**, dA_{13} or A_{13} was used as a complementary strand. Conditions: 10 mM sodium phosphate, 0.1 mM EDTA, 1 M NaCl, pH 7.0. [b] In the T_m measurement of self-complementary oligomers **23**–**28**. Conditions: 10 mM sodium phosphate, 0.1 mM EDTA, 150 mM NaCl, pH 7.0. Bold and underlined letters in sequences refer to the 2′-phosphorylated and 2′-thiophosphorylated ribonucleotide, respectively. $\underline{U}$ refers to 2′-*O*-thiophosphorylated uridine.

found to be inert against the electrophilic substitution on the uracil moiety. Therefore, we chose to apply this KI_3-oxidation procedure to the preparation of oligonucleotides involving a 2′-*O*-phosphorylated ribonucleoside.

To obtain the 13mer **16** incorporating a 2′-*O*-phosphorylated uridine having no base-protecting group, the usual protocol involving chain elongation, removal of the CME groups, and oxidation by use of KI_3 was performed. As shown in the HPLC profile after treatment with concentrated NH_3 (Figure 3-A_2), a main peak corresponding to $dU_6U(2'\text{-p})dU_6$ appeared without the 5-iodination. After purification by HPLC, this main product was isolated and characterized by treatment with alkaline phosphatase. As expected, this enzymatic treatment gave the 2′-dephosphorylated product dU_6UdU_6 (Figure 3-A_3 and 3-A_4), which was further characterized by enzymatic assay using snake venom phosphodiesterase. In a similar way, a tridecadeoxyuridylates **15** incorporating a 2′-*O*-phosphorylated uridine at another site was synthesized. Furthermore, self-complementary dodecadeoxynucleotides **24**–**28** with the Drew–Dickerson sequence containing the four common nucleobases could also be prepared in good yields using phosphoramidites **6a**–**d** (Table 2). Therefore, compounds **6a**–**d** have proved to be generally useful building blocks capable of incorporating a 2′-*O*-phosphorylated ribonucleoside (The HPLC profiles of the crude materials obtained after cleavage of the succinate linker are summarized in Supporting Information). Moreover, we attempted to synthesize oligonucleotides $dU_2U(2'\text{-p})dU_3U(2'\text{-p})dU_3U(2'\text{-p})dU_2$ **18** and $[U(2'\text{-p})]_{12}U$ **19** bearing a higher number of 2′-*O*-phosphorylated nucleosides. However, HPLC analysis suggested that these reactions were complicated by intra- or intermolecular formation of disulfide bonds to give dimerization-, trimerization-, and larger products (see Supporting Information). These S–S bonds in relatively long oligonucleotides were difficult to convert to the corresponding phosphate because of steric hindrance.

Furthermore, a tridecaeoxyuridylate **17** bearing a 2′-*O*-thiophosphorylated uridine was also synthesized by a modified procedure without the KI_3 treatment and characterized by the facile 2′-dethiophosphorylation using thermolysis at pH 7.0 and 90 °C, which was recently reported by us,[19] to give dU_6UdU_6 in nearly quantitative yield without appreciable internucleotidic bond cleavage. Interestingly, this 2′-*O*-thiophosphate group was eliminated in only 20 min, and the first-order rate constant of this hydrolysis was $1.9 \times 10^{-3}\ s^{-1}$, which was greater than any other values such as that of U(2′-ps)pU ($1.4 \times 10^{-3}\ s^{-1}$, $t_{comp} = 1$ h).

The synthetic method described above was applied to incorporation of a 2′-*O*-phosphorylated nucleoside into tridecauridylates. These modified oligoribonucleotides are a simple motif of the ligation site of tRNA splicing intermediates. To prepare the 13mers **21** and **22**, compound **6a** and the normal uridine phosphoramidite, in which the 2′-hydroxyl was masked by the *tert*-butyldimethylsilyl group, were employed for chain elongation. In these syntheses, an additional treatment with TBAF at the last step was carried out. Finally, the crude material was purified by HPLC to give the oligomers **21** and **22**, having a 2′-*O*-phosphorylated uridine at the same positions as **15** and **16**, respectively.

As the results, we were able to establish the solid-phase synthesis for DNA and RNA oligomers with a 2′-*O*-phosphoryl group using the phosphoramidites **6a**–**d**.

Duplex Stability of Oligonucleotides Containing 2′-*O*-Phosphorylated Ribonucleoside. The UV–melting curves for duplexes between these oligomers and complementary strands were measured to study the effect of the 2′-*O*-phosphoryl group on the duplex stability.

These T_m measurements of the duplexes formed by oligouridylates and oligoadenylates were carried out by mixing them in a 1:1 ratio. As shown in the figure of Supporting Information, the melting curves of both modified and unmodified nucleotide duplexes clearly suggested two states in all cases. Therefore, we concluded that these oligonucleotides formed duplexes under the condition described in the present paper. The T_m values obtained are summarized in Table 2.

In the case of DNAs hybridized with dA_{13} as complementary strand, the T_m value of the duplex **15**/dA_{13} (28.8 °C) was lower by 2.1 °C than that of unmodified dU_{13} **14**/dA_{13} (30.9 °C). This difference was rather smaller than expected since the electrostatic repulsion due to negative charge of the 2′-*O*-phosphomonoester was estimated to be more detrimental for duplex formation. We previously reported the MD simulation of the duplex of $dA_7/dU_3U(2'\text{-p})dU_3$, suggesting that 2′-*O*-phosphorylated uridine U(2′-p) of $dU_3U(2'\text{-p})dU_3$ has a rigid C2′-endo conforma-

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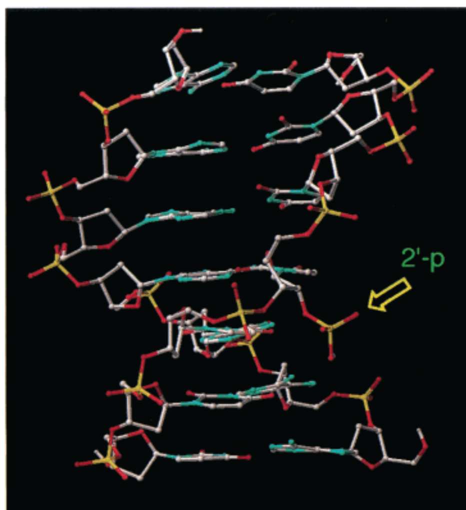

	control	2'-p oligomer
DNA duplex	5' d(UUUUUUU) 3' 3' d(AAAAAAA) 5' **33**	5' d(UUU**U**UUU) 3' 3' d(AAAAAAA) 5' **34**
RNA duplex	5' UUUUUUU 3' 3' AAAAAAA 5' **35**	5' UUU**U**UUU 3' 3' AAAAAAA 5' **36**

Figure 4. Snapshot of DNA duplex having a 2′-phosphorylated ribonucleoside for 1 ns-MD simulation and sequences of DNA and RNA duplex used in this simulation. Bold letters refer to 2′-phosphorylated ribonucleoside.

tion.[11] The present result also suggests that conformation of the 2′-O-phosphorylated uridine was more favorable for the B-DNA duplex than normal deoxyribonucleosides. On the other hand, the T_m experiment of **16**/dA_{13} (26.2 °C) suggested that the duplex stability decreased less significantly when a 2′-O-phosphoryl group was introduced at a position closer to the center of the strand. The duplex of **17**/dA_{13} was destabilized more significantly by 5.4 °C than that of **14**. This may be explained in terms of more unfavorable steric disruption by the bigger-sized sulfur atom as well as stronger electrostatic repulsion due to the 2′-O-thiophosphate dianion species. It is known that the pK_a values of monoalkyl esters of thiophosphoric acids are generally 0.5 unit lower than those of the corresponding monoalkyl esters of phosphoric acids so that the electrostatic charge distribution of the 2′-O-thiophosphate group is greater than that of the 2′-phosphate at pH 7.0.

On the contrary, the T_m values of RNA duplexes **21**/A_{13} and **22**/A_{13} were markedly decreased by the existence of the 2′-O-phosphate group compared with those of DNA duplexes **15**/dA_{13} and **16**/dA_{13} with identical sequences. The reason for this destabilization might be ascribed to the essential incompatibility between the A-type RNA backbone structure and the B-type favored 2′-O-phosphorylated uridine as well as electrostatic repulsion. Accordingly, incorporation of 2′-O-phosphorylated uridine caused conformational change of the A-type duplex structure to a more disordered one.

The duplexes formed by the self-complementary DNAs **24**−**28** involved a 2′-O-phosphorylated nucleoside in each strand. Therefore, it was anticipated that the stability of these duplexes should be influenced more considerably than that of oligomers **15**−**17**/dA_{13}. Indeed, most of the T_m values of these modified self-complimentary duplexes decreased more significantly than that of the unmodified duplex **23**/**23**. Particularly, the duplex **27**/**27** was extremely destabilized by 15.2 °C. To our surprise, the T_m value of the duplex **25**/**25** having a 2′-O-phosphorylated guanosine was the same (55.7 °C) as that of the control **23**/**23**. In nucleic acid chemistry, the phosphate group has been generally thought as an electrostatically disadvantageous function as far as stability of duplexes is concerned. The present study, however, indicates the possibility that this is not always the case. In **28**/**28**, the duplex was not destabilized only by 4.4 °C compared with that of the unmodified duplex.

Conformational Analysis by Constrained Molecular Dynamics Simulation. The 2′-O-phosphate group inhibits various tRNA-processing enzymes from recognizing tRNA intermediates.[4] We speculated that considerable destabilization of RNA duplexes containing a 2′-O-phosphorylated nucleoside as described above was ascribed to distortion caused by unfitting of the 2′-O-phosphorylated nucleoside for the A-type duplex. First, the CD spectra and native 20% PAGE of single- and double-stranded oligonucleotides containing a 2′-O-phosphorylated nucleoside were analyzed. However, they did not give any helpful information with respect to the 3D-structure of the modified duplexes (see the Supporting Information).

To study the structural effects of the 2′-O-phosphorylated nucleoside on the duplex in more detail, computational molecular dynamics (MD) simulations were carried out for 1 ns by using the duplexes containing a 2′-O-phosphorylated nucleoside or not (Figure 4).

Indeed, $dU_3U(2'\text{-}p)dU_3/dA_7$ **34** and dU_7/dA_7 **33** as DNA duplexes and $U_3U(2'\text{-}p)U_3/A_7$ **36** and U_7/A_7 **35** as RNA duplexes were simulated. Figure 5 shows the MD trajectory of distinctive torsion angles (the ϵ and ζ angles defined in nucleic acid chemistry[20]) and phase angle (P), of the ribose in the fourth uridine residue which is or is not phosphorylated at the 2′-position.

With respect to the DNA duplex **34**, the structural parameters of this duplex did not change significantly after simulation. In detail, the phase angle (P) of the unmodified fourth deoxyuridine in **33** was relatively flexible and frequently changed from C2′-endo (137° < P < 194°) to C3′-endo conformer (−1° < P < 34°) and vice versa. On the contrary, the C2′-O-phosphorylated sugar in **34** moved sluggishly only within the range of the C2′-endo conformer during the 1 ns-MD simulation. (Scheme 6)

This result was in agreement with the fact that the ribose residue of 2′-O-phosphorylated dinucleotides was found to have a rigid C2′-endo conformation as evidenced by NMR analysis.[11] Therefore, the 2′-O-phosphorylated nucleoside could entropically stabilize the B-DNA duplex. As a unique structure, it was found that the ϵ angle of the 2′-O-phosphorylated uridine in **34** was sometimes transferred to g^- conformation although that of the unmodified fourth uridine has trans conformation during

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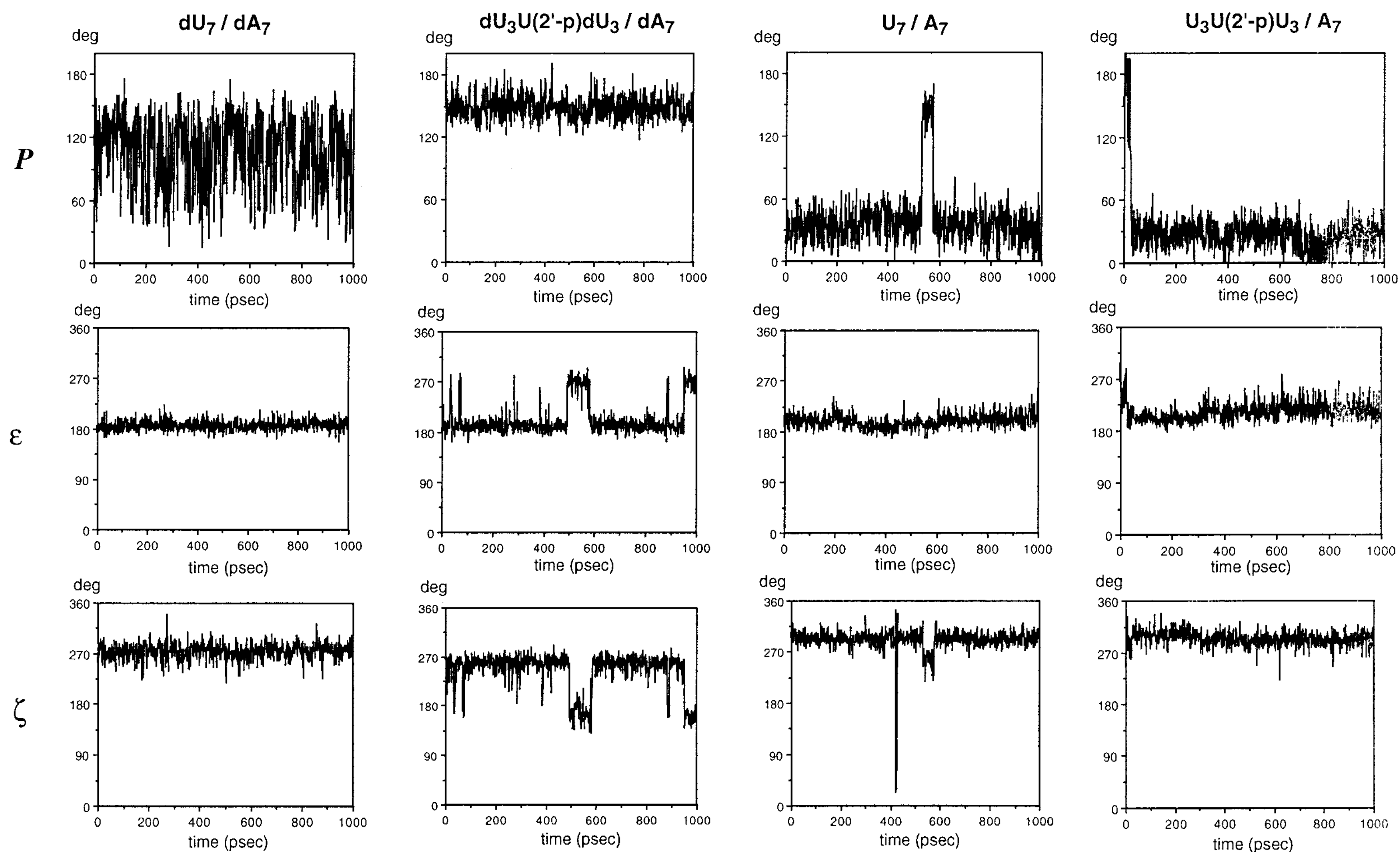
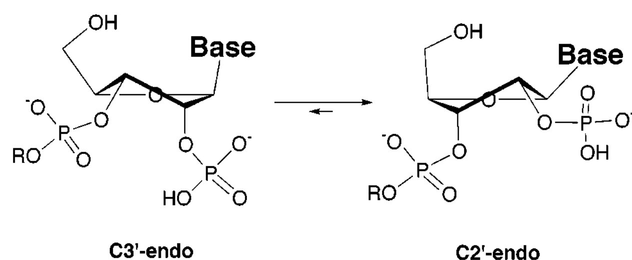


Figure 5. The trajectory of phase angles (P) and tortion angles (ϵ and ζ) of the fourth uridines, which were phosphorylated at the 2′-position or not, in the duplexes **33**, **34**, **35**, and **36** in molecular dynamics simulation at 300 K for 1 ns.

Scheme 6



this simulation like the ordinary case.[20] However, the ζ angle of the 2′-O-phosphorylated uridine simultaneously transferred from normal g^- conformation to trans so as to cancel the distortion generated at the ϵ angle. In consideration of these results, since the ribose residue of the 2′-O-phosphorylated uridine incorporated into **34** exists rigidly in a C2′-endo conformation, it seemed that the total stability of **34** did not decrease despite the electrostatic repulsion and change in the ϵ and the ζ angle.

During simulation of RNA duplexes U_7/A_7 **35** and $U_3U(2'\text{-p})U_3/A_7$ **36**, having the same sequence described above, the fourth 2′-O-phosphorylated uridine first seemed to have a rigid C2′-endo conformation similar to that of the typical DNA duplex. However, this ribonucleoside was unexpectedly found to behave as a C3′-endo conformer. Its ϵ and ζ angles also gave the same conformation as those of the normal ribonucleosides in the A-type RNA duplex, which has trans and g^- conformations, respectively. In this 1 ns-MD simulation, it was irrelevant whether the used initial structure of the fourth 2′-O-phosphorylated nucleoside was C2′- or C3′-endo. Since A-RNA duplexes are structurally rigid, tight, and conservative, as is well-known,[20] probably the 2′-O-phosphorylated uridine could not come to a C2′-endo conformation because of stronger pressure of the neighbor A-type circumstance regardless of its propensity to the C2′-endo conformation so that the duplex was extremely destabilized, especially at the modified nucleoside point. Consequently, the DNA duplex containing a 2′-O-phosphorylated nucleoside is stable due to rigid C2′-endo puckering despite the existence of difference in the two torsional angles compared with the normal ribonucleoside, while the RNA duplex is entropically destabilized by the expelled structural force prone to the C3′-endo form. Unexpectedly, the total structure of the RNA duplex **36** containing a 2′-O-phosphorylated nucleoside did not change significantly in the present simulation. In a tRNA intermediate during splicing, however, a 2′-O-phosphorylated nucleoside was located at the border between the tRNA anticodon stem and loop like an A-type helical structure. Therefore, it is necessary to examine a partial structure having an actual 2′-O-phosphorylated tRNA sequence, if we want to confirm the structural effect of tRNA intermediates on biological activities.

Conclusion

In recent studies on new antisense oligonucleotides, many 2′-modified ribonucleoside derivatives, such as 2′-halogenated and 2′-O-alkylated nucleosides, have been synthesized and their conformational properties have been investigated in great detail. These studies disclosed that ribonucleoside derivatives, substituted by electron-withdrawing groups at the 2′-position, have predominantly the C3′-endo conformation and stabilize the A-type double helix when incorporated into DNA strands and hybridized with the target mRNA.[21]

It is known that the population of the C3′-endo conformer is in proportion to the strength of electronegativity of a 2′-substituent.[20] Chattopadhyaya and coworkers have recently reported that the electronegativity of a 3′-substituent has a close connection with the ΔH value obtained by the equilibrium between the C2′-endo and C3′-endo conformers, and concluded that this essential factor is explained in terms of the gauche effect between the 3′-substituent and the oxygen of the sugar ring.[22] A similar discussion was deduced in the case of 2′-substituted nucleosides.

We previously showed that 2′-O-phosphorylated nucleosides interestingly prefer the C2′-endo conformer to the C3′-endo though the monoester-type phosphate group is generally regarded as a weak electron-withdrawing group. In pre-tRNA splicing products, the 2′-O-phosphorylated nucleosides are mostly located at the border between the tRNA anticodon stem and loop, which involves the typical A-type single strand. Therefore, it is interesting to estimate how the 2′-phosphate group affects the local conformation in this region. In the present study we could show that, if a 2′-phosphate group exists in an RNA duplex, the duplex stability is significantly destabilized due to incompatibility between the unique DNA-like property of the 2′-O-phosphorylated nucleoside and the conservative A-type RNA strand. These results apparently indicate that a 2′-O-phosphorylated nucleoside of the pre-tRNA splicing product causes the distortion of the strictly arranged A-type strand in the anticodon loop region.

An increasing number of MD simulation studies in nucleic acid chemistry have recently been reported.[23−27] MD simulation is, indeed, a powerful tool to estimate the thermodynamic behavior of a modified nucleoside incorporated into a DNA or RNA strand. In this study, we also showed that the 2′-O-phosphorylated uridine in $dU_3U(2'\text{-p})dU_3/dA_7$ preserves its favored C2′-endo conformation and fits the B-type helix through a simultaneous change in the two torsion angles. This result is a typical case which shows the very flexibility of the DNA backbone.

Furthermore, we have recently found that the 2′-O-phosphate group is eventually hydrolyzed more rapidly than expected due to the neighboring participation of the proximal 3′,5′-phosphodiester moiety, and suggested a novel possibility as a catalytic function in RNA.[19] Actually, Chattopadhyaya reported a new finding that 2′-O-phosphorylated cyclic RNA species has a catalytic activity

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of 2′-*O*-phosphodiester.[28] Two vicinal phosphate groups, such as branched RNA and 2′-phosphorylated RNA, might have more unique chemical properties.

Through our continuous studies, we have realized that oligonucleotides incorporating a 2′-*O*-phosphorylated nucleoside at the specific position of the sequence having the four common nucleobases could be effectively synthesized. Compounds of this kind would be promising tools to elucidate the mechanism of tRNA processing in molecular biology.

Experimental Section

General Methods. ^{1}H NMR spectra were measured at 270 and 400 MHz with TMS (for $CDCl_3$) or DDS (for D_2O) as internal standard. ^{13}C NMR spectra were obtained at 67.8 and 100.6 MHz, respectively, with $CDCl_3$ (for $CDCl_3$) as internal standard or DDS (for D_2O) as external standard. ^{31}P NMR spectra were recorded at 109 MHz with the external reference of 85% H_3PO_4. UV spectra were taken on a U-2000 spectrophotometer. Reversed phase HPLC was performed on a Waters LC module 1 with a μBondasphere 5 μm C18 100 Å (3.9 × 150 mm) column using a linear gradient of acetonitrile (0−30%) in 0.1 M ammonium acetate buffer (pH 7.0) for 30 min at flow rate of 1 mL/min at 50 °C. Ion exchange HPLC was carried out at a flow rate of 1 mL/min for 30 min at 50 °C on a Whatman Partisil 10 SAX WCS analytical column (4.6 × 250 mm) using a linear gradient of 0−100% solution A (20% CH_3CN in 0.5 M KH_2PO_4) in solution B (20% CH_3CN in 0.005 M KH_2PO_4) Paper chromatography was carried out by use of a descending technique with Whatman 3MM Chr papers using *i*PrOH−concd NH_3−H_2O (6:1:3, v/v/v). Thin-layer chromatography was performed using Merck Kieselgel 60F-254 (0.25 mm) with developing solvent of CH_2Cl_2−CH_3OH (9:1, v/v) or *i*PrOH-concd NH_3−H_2O (7:1:2, v/v/v). Column chromatography was performed with silica gel C-200 purchased from Wako Co. Ltd., and a minipump for a goldfish bowl was conveniently used to attain sufficient pressure for rapid chromatographic separation. Elemental analyses were performed by the Microanalytical Laboratory, Tokyo Institute of Technology, at Nagatsuta.

3′,5′-*O*-(Di-*tert*-butylsilanediyl)-4-*N*-isobutyrylcytidine (2c). 4-*N*-Isobutyrylcytidine (**1c**) (313 mg, 1 mmol) was rendered anhydrous by repeated coevaporation with dry dimethylformamide (3 mL × 2) and dissolved with dimethylformamide (3 mL). After addition of silver nitrate (170 mg, 1 mmol) and di-*tert*-butylsilyl bis(trifluoromethanesulfonate) (360 μL, 1.1 mmol), the solution was stirred vigorously at room temperature for 30 min. The mixture was added with triethylamine (1.25 mL, 9 mmol), and the solvent was removed under reduced pressure. The residue was dissolved with CH_2Cl_2 (50 mL) and washed with saturated $NaHCO_3$ and water. The organic layer was collected, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The crude material was chromatographed on a column of silica gel eluted with CH_2Cl_2−MeOH to give **7**: ^{1}H NMR (270 MHz, TMS) δ 1.01−1.13 (18H), 1.23 (6H, d, J = 6.9 Hz, 6H), 2.61 (1H, sept), 3.91 (1H, dd, J = 4.6, 9.6 Hz), 4.05 (1H, dd, J = 8.9 Hz, 10.2 Hz), 4.24−4.29 (2H, m), 4.55 (1H, dd, J = 5.0 Hz, 8.9 Hz), 5.72 (1H, s), 7.52 (1H, d, J = 7.5 Hz), 7.76 (1H, d, J = 7.5 Hz); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 18.64, 18.71, 20.20, 22.52, 26.85, 27.08, 36.34, 67.26, 73.55, 74.66, 75.58, 93.82, 97.13, 143.34, 155.65, 162.93, 177.93. Anal. Calcd for $C_{21}H_{35}N_3O_6Si\cdot 2H_2O$: C, 51.51; H, 7.21; N, 8.58. Found: C, 51.65; H, 7.35; N, 8.27.

6-*O*-(2-Cyanoethyl)-2-*N*-phenylacetylguanosine (1d). 2-*N*-Phenylacetylguanosine[29] (10.3 g, 25 mmol) was rendered anhydrous by repeated coevaparation with dry pyridine and suspended in acetonitrile (220 mL). Hexamethyldisilazane (26.4 mL, 125 mmol) was added, and the mixture was refluxed for 1 h. The resulting clear solution was evaporated under reduced pressure, and the last traces of pyridine were removed by coevaporation with ethanol. The residue was dissolved in dry CH_2Cl_2 (220 mL). To the solution were added successively (dimethylamino)pyridine (153 mg, 1.25 mmol), triethylamine (13.9 mL, 100 mmol), and mesitylenesulfonyl chloride (6.56 g, 30 mmol). After being stirred at room temperature for 1 h, the mixture was cooled to 0 °C. To the solution was added pyrolidine (26.0 mL, 250 mmol). After the mixture was stirred at 0 °C for 40 min, 2-cyanoethanol (17.1 mL, 250 mmol) and DBU (11.2 mL, 75 mmol) were added, and stirring was continued for 30 min. CH_2Cl_2−MeOH and phosphate buffer (pH 6.0) were added. The organic layer was collected, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was dissolved in a 1:1 mixture of CH_2Cl_2−MeOH 500 mL), and trifluoroacetic acid (5 mL) was added. After being stirred for 30 min, the mixture was evaporated under reduced pressure. The residue was chromatographed on a column of silica gel with CH_2Cl_2−MeOH to give **1d** (4.5 g, 40%): ^{1}H NMR (270 MHz, TMS) δ 2.96 (2H, t, J = 6.6 Hz), 3.76 (1H, dd, J = 2.6, 12.5 Hz), 3.84 (2H, s), 3.88 (1H, dd, J = 3.0, 12.5 Hz), 4.23 (1H, m), 4.47 (1H, dd, J = 3.0, 5.0 Hz), 4.63 (1H, dd, J = 5.0, 5.6 Hz), 4.71 (2H, t, J = 6.6 Hz), 5.91 (1H, d, J = 5.6 Hz), 7.32−7.41 (5H, m), 8.14 (1H, s); ^{13}C NMR (67.8 MHz, α-proton of pyridine-d_5) δ 18.37, 44.48, 62.43, 72.12, 76.27, 79.74, 87.43, 90.09, 118.52, 118.66, 127.34, 129.05, 130.23, 136.27, 136.31, 142.07, 152.84, 152.87, 153.75. 160.01, 170.84. Anal. Calcd for $C_{21}H_{22}N_6O_6\cdot 1.2\ H_2O$: C, 52.98; H, 5.21; N, 17.65. Found: C, 53.29; H, 4.76; N, 16.94.

3′,5′-*O*-(Di-*tert*-butylsilanediyl)-2-*N*-phenylacetyl-6-*O*-(2-cyanoethyl)guanosine (2d). 2-*N*-Phenylacetyl-6-*O*-(2-cyanoethyl)guanosine (**1d**) (181 mg, 0.4 mmol) was coevaporated with dimethylformamide (2 mL × 2) and dissolved in dimethylformamide (2 mL). To the solution were added silver nitrate (190 mg, 1.12 mmol) and di-*tert*-butyldichlorosilane (110 μL, 0.52 mmol). After being stirred vigorously at 0 °C for 15 min, the mixture was diluted with CH_2Cl_2−pyridine (2:1, v/v, 10 mL). The solvent was removed under reduced pressure, and the residue was partitioned between CH_2Cl_2 and water. The organic phase was collected, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was chromatographed on a column of silica gel eluted with CH_2Cl_2−MeOH to give **2d** (220 mg, 92%): ^{1}H NMR (270 MHz,-$CDCl_3$, TMS) δ 1.04 (9H, s), 1.09 (9H, s), 2.92 (2H, t, J = 6.4 Hz), 3.43 (1H, s), 3.95−4.13 (4H, m), 4.46 (1H, dd, J = 3.5 Hz, J = 7.9 Hz), 4.54 (1H, dd, J = 7.9 Hz, J = 5.0 Hz), 4.70 (1H, d, J = 5.0 Hz), 5.97 (1H, d), 7.27−7.38 (5H, m), 7.89 (1H, s), 8.27 (1H, s); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 17.65, 20.06, 22.36, 26.88, 27.05, 43.87, 53.30, 61.44, 67.05, 73.23, 74.45, 75.72, 90.57, 116.93, 117.84, 126.90, 138.41, 129.18, 134.02, 140.61, 151.41, 151.99, 159.28, 169.36. Anal. Calcd for $C_{29}H_{38}N_6O_6Si$: C, 58.57; H, 6.44; N, 14.13. Found: C, 58.33; H, 6.37; N, 13.91.

2′-*O*-Bis(2-cyano-1,1-dimethylethoxy)thiophosphoryl-6-*N*-benzoyladenosine (4b). 3′,5′-*O*-(di-*tert*-butylsilanediyl)-6-*N*-benzoyladenosine (**2b**) (205 mg, 0.4 mmol) and 1*H*-tetrazole (63 mg, 0.9 mmol) was rendered anhydrous by repeated coevaporation with dry pyridine (2 mL × 2) and dry toluene (2 mL × 1). To the mixture was added a 0.25 M solution of bis(2-cyano-1,1-dimethylethyl) *N,N*-diethylphosphoramidite (1.2 mmol) in CH_2Cl_2. After the mixture was stirred at room temperature for 2 h, a 1 M solution of S_8 in CS_2−pyridine (4 mL, 20 mmol) was added. The solution was stirred at room temperature for 2 h and then evaporated under reduced pressure. The residue was dissolved with CH_2Cl_2 (50 mL). The precipitate was removed by filtration. The filtrate was washed twice with 5% $NaHCO_3$ and with water, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was dissolved in THF (2 mL), and a mixture of (HF)·Py (231 μL)/Py (1.2 mL) was added. The mixture was stirred at room temperature for 10 min, and then an excess amount of pyridine (3 mL) was added. The mixture was partitioned between CH_2Cl_2 and 5% $NaHCO_3$. The organic phase was collected, washed with 5% $NaHCO_3$ and water, dried over Na_2-

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SO_4, filtered, and evaporated under reduced pressure. The residue was chromatographed on a column of silica gel with CH_2Cl_2−MeOH containing 0.5% pyridine to give **4b** (172 mg, 82%): ^{1}H NMR (270 MHz,$CDCl_3$, TMS) δ 1.42−1.59 (12H, m), 2.65−2.87 (4H, m,), 3.70−3.76 (1H, m), 3.91 (1H, dd, J = 1.6 Hz, J = 13.2 Hz), 4.32 (1H, s), 4.71 (1H, d, J = 4.6 Hz), 5.72 (1H, ddd, J = 7.2 Hz, J = 4.6 Hz, J = 11.9 Hz), 6.11 (1H, d, J = 7.2 Hz), 7.42−7.57 (3H, m), 8.14 (1H, s), 8.94 (2H, d), 8.73 (1H, s), 9.14 (1H, s); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 26.92, 26.97, 27.24, 27.31, 31.48, 62.28, 70.71, 78.40, 82.75, 82.86, 87.24, 87.69, 87.84, 116.55, 116.80, 123.76, 127.89, 128.05, 128.66, 132.79, 133.23, 142.98, 150.04, 150.84, 152.09, 165.00; ^{31}P NMR (109 MHz, $CDCl_3$, 85% H_3PO_4) δ 48.90. Anal. Calcd for $C_{27}H_{32}N_7O_7PS$: C, 51.5; H, 5.12; N, 15.57. Found: C, 51.65; H, 5.25; N, 15.41.

2′-*O*-Bis(2-cyano-1,1-dimethylethoxy)thiophosphoryl-4-*N*-isobutyrylcytidine (4c). 3′,5′-*O*-(Di-*tert*-butylsilanediyl)-4-*N*-isobutyrylcytidine (**2c**) (2.4 g, 5 mmol) was converted to **4c** (2.45 g, 86%) using the same method as described in **4b**. **4c**: ^{1}H NMR (270 MHz, DSS) δ 1.22 (6H, d, J = 6.9 Hz), 1.69−1.74 (12H, m), 2.62 (1H, sept, J = 6.9 Hz), 2.84−3.17 (4H, m), 3.94 (2H, m), 4.21 (1H, m), 4.64 (1H, m), 5.23 (1H, ddd, J = 4.3 Hz, J = 4.6 Hz, J = 11.2 Hz), 6.12 (1H, d, J = 4.3 Hz), 7.47 (1H, d, J = 7.6 Hz), 8.33 (1H, d, J = 7.6 Hz), 8.51 (1H, brs); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 18.65, 26.60, 26.74, 26.79, 26.85, 26.90, 26.96, 27.01, 27.14, 27.19, 31.09, 31.16, 35.94, 36.03, 60.16, 68.07, 68.14, 79.68, 82.43, 82.55, 82.59, 82.71, 84.55, 89.49, 89.52, 96.98, 116.87, 123.81, 148.63, 155.29, 162.68, 177.43; ^{31}P NMR (109 MHz, $CDCl_3$, 85% H3PO4) δ 49.725. Anal. Calcd for $C_{23}H_{34}N_5O_8PS{\cdot}3/2H_2O$: C, 46.14; H, 5.73; N, 11.70. Found: C, 46.43; H, 5.91; N, 11.42.

2′-*O*-Bis(2-cyano-1,1-dimethylethoxy)thiophosphoryl-2-*N*-phenylacetyl-6-*O*-(2-cyanoethyl)guanosine (4d). 3′,5′-*O*-(Di-*tert*-butylsilanediyl)-2-*N*-phenylacetyl-6-*O*-(2-cyanoethyl)-guanosine **2d** (214 mg, 0.36 mmol) was converted to **4d** (217 mg, 84%) using the same method as described in **4b**. **4d**: ^{1}H NMR (270 MHz,$CDCl_3$, TMS) δ 1.48−1.63 (12H, m), 2.77−2.99 (6H, m), 3.81 (1H, dd, J = 2.0 Hz, J = 12.9 Hz), 3.87 (2H, s) 3.94 (1H, dd, J = 2.0 Hz, J = 12.9 Hz), 4.31−4.33 (1H, m), 4.73 (2H, t, J = 6.2 Hz), 4.87 (1H, dd, J = 4.8 Hz, J = 1.7 Hz), 5.68 (1H, ddd, J = 6.9 Hz, J = 4.8 Hz, J = 9.8 Hz), 6.09 (1H, d, J = 6.9 Hz), 7.32−7.42 (5H, m), 8.02 (1H, s), 8.18 (1H, s); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 17.95, 21.37, 26.94, 27.05, 27.10, 27.46, 27.53, 31.43, 31.50, 44.55, 61.74, 62.32, 70.55, 70.60, 78.44, 78.51, 82.84, 82.93, 82.97, 87.39, 87.53, 116.55, 116.77, 116.87, 119.07, 125.21, 127.42, 128.14, 128.95, 129.42, 133.89, 142.32, 151.34, 152.45, 159.82, 161.71, 169.16; ^{31}P NMR (109 MHz, $CDCl_3$, 85% H3PO4) δ 48.60. Anal. Calcd for $C_{31}H_{37}N_8O_8PS{\cdot}1/2H_2O$: C, 51.59; H, 5.31; N, 15.52; S, 4.44. Found: C, 51.87; H, 5.27; N, 15.12; S, 4.93.

2′-*O*-Bis(2-cyano-1,1-dimethylethoxy)thiophosphoryl-4-*N*-benzoylcytidine (4e). A mixture of 3′,5′-*O*-(di-*tert*-butylsilanediyl)-4-*N*-benzoylcytidine **(2e)** (3.45 g, 7.07 mmol) and 1*H*-tetrazole (1.88 g, 26.9 mmol) was rendered anhydrous by repeated coevaporation with dry pyridine (2 mL × 2) and dry toluene (2 mL × 1). To the mixture was added a 0.25 M solution of bis(2-cyano-1,1-dimethylethyl) *N,N*-diethylphosphoramidite (17.7 mmol) in CH_2Cl_2. After the mixture was stirred at room temperature for 19 h, the solvent was removed in vacuo, and the residue was dissolved in 1 M solution of S_8 (18 g, 71 mmol) in CS_2−pyridine (1:1, v/v, 80 mL). The mixture was stirred at room temperature for 2 h and then evaporated under reduced pressure. After the residue was dissolved in CH_2Cl_2 (200 mL), the precipitate was removed by filtration. The filtrate was washed with 5% $NaHCO_3$ and water, dried over Na_2SO_4, and evaporated under reduced pressure. The residue was dissolved in THF (25 mL), and a mixture of pyridinium hydrogenfluoride (4.1 mL)/Py (18.3 mL) was added. After the mixture was stirred for 10 min, a large excess of pyridine (20 mL) was added. The mixture was partitioned between CH_2Cl_2 and 5% $NaHCO_3$, and the organic layer was collected, dried over Na_2SO_4, and evaporated under reduced pressure. The crude material was chromatographed on a column of silica gel with CH_2Cl_2−MeOH containing 0.5% pyridine to give **4e** (3.29 g, 77%): ^{1}H NMR (270 MHz,$CDCl_3$, TMS) δ 1.69 (6H, s), 1.73 (6H, s), 2.80−3.18 (4H, m), 3.90 (1H, dd, J = 1.7 Hz, J = 12.4 Hz), 4.00 (1H, dd, J = 1.7 Hz, J = 12.4 Hz), 4.24 (1H, dd, J = 4.3 Hz, J = 1.7 Hz), 4.63 (1H, t, J = 4.3 Hz, J = 4.3 Hz), 5.23 (1H, dt, J = 4.3 Hz, J = 4.3 Hz, J = 11.2 Hz), 6.19 (1H, d, J = 4.3 Hz), 7.15−7.63 (4H, m), 7.90 (2H, d), 8.40 (1H, d, J = 7.6 Hz); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 27.05, 27.10, 27.17, 27.35, 27.41, 27.55, 27.60, 31.25, 31.34, 31.94, 60.56, 68.64, 80.00, 80.06, 82.62, 82.75, 82.98, 83.09, 84.85, 89.60, 97.38, 117.05, 117.11, 123.79, 127.69, 128.84, 132.72, 133.14, 136.23, 146.33, 149.38, 162.73; ^{31}P NMR (109 MHz, $CDCl_3$, 85% H_3PO_4) δ 49.68. Anal. Calcd for $C_{26}H_{32}N_5O_8PS{\cdot}1/2H_2O$: C, 50.81; H, 5.41; N, 11.39; S, 5.22. Found: C, 50.56; H, 5.65; N, 11.31; S,5.47.

2′-*O*-Bis(2-cyano-1,1-dimethylethoxy)thiophosphoryl-5′-*O*-(4,4′-dimethoxytrityl)-6-*N*-benzoyladenosine (5b). Compound **4b** (1.3 g, 2.06 mmol) was rendered anhydrous by repeated coevaporation with dry pyridine (20 mL × 2) and finally dissolved in dry pyridine (20 mL). To the mixture was added 4,4′-dimethoxytrityl chloride (1.3 g, 3.75 mmol), and the solution was kept at room temperature for 4 h. Extraction was performed with CH_2Cl_2 and 5% $NaHCO_3$. The usual workup followed by silica gel column chromatography eluted with CH_2Cl_2−MeOH containing 0.5% pyridine gave **5b** (1.82 g, 95%): ^{1}H NMR (270 MHz,$CDCl_3$, TMS)δ 1.43−1.70 (12H, m), 2.62−3.14 (4H, m,), 3.41 (1H, d, J = 8.6 Hz), 3.50 (1H, d, J = 8.6 Hz), 3.77 (6H, s,), 4.33 (1H, s), 4.88 (1H, d, J = 6.3 Hz), 5.79 (1H, ddd, J = 6.3 Hz, J = 6.3 Hz, J = 10.9 Hz), 6.45 (1H, d, J = 6.3 Hz), 6.82 (4H, d), 7.14−7.58 (12H, m), 8.0 (2H, d), 8.33 (1H, s), 8.73 (1H, s), 9.23 (1H, s); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 26.56, 26.99, 27.03, 27.10, 27.35, 27.41, 31.29, 31.36, 31.57, 31.65, 55.08, 63.07, 770.35, 79.35, 79.43, 82.80, 82.91, 83.04, 84.39, 85.14, 86.70, 112.92, 113.14, 116.46, 116.71, 122.79, 126.83, 127.64, 127.71, 127.87, 127.96, 128.70, 129.94, 132.69, 133.41, 135.27, 135.35, 141.76, 144.24, 149.45, 151.73, 152.65, 158.38, 164.53; ^{31}P NMR (109 MHz, $CDCl_3$, 85% H_3PO_4) δ 49.91. Anal. Calcd for $C_{48}H_{50}N_7O_9PS$: C, 61.86; H, 5.41; N, 10.50. Found: C, 61.62; H, 5.54; N, 10.03.

2′-*O*-Bis(2-cyano-1,1-dimethylethoxy)thiophosphoryl-5′-*O*-(4,4′-dimethoxytrityl)-4-*N*-isobutyrylcytidine (5c). Compound **4c** (1.7 g, 3.0 mmol) was converted to **5c** (2.5 g, 96%) using the same method as described in **5b**. **5c**: ^{1}H NMR (270 MHz, DSS) δ 1.21 (6H, 2d, J = 6.9 Hz), 1.67−1.75 (12H, m), 2.55 (1H, sept, J = 6.9 Hz), 2.88−3.10 (4H, m), 3.53 (2H, m), 3.81 (6H, s), 4.20 (1H, m), 4.65 (1H, m), 5.15 (1H, ddd, J = 3.0 Hz, J = 3.9 Hz, J = 11.5 Hz), 6.24 (1H, d, J = 3.0 Hz), 6.81−6.88 (4H, m), 7.08 (1H, d, J = 7.6 Hz), 7.15−7.42 (9H, m), 8.14 (1H, brs), 8.36 (1H, d, J = 7.6 Hz); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 18.82, 18.89, 27.01, 27.05, 27.12, 27.17, 27.28, 27.33, 31.20, 31.27, 31.47, 31.54, 36.21, 55.02, 61.73, 68.91, 80.59, 80.67, 82.71, 82.84, 83.16, 83.29, 87.05, 87.94, 88.07, 96.80, 113.21, 116.78, 116.91, 126.97, 127.89, 128.05, 129.83, 129.96, 134.97, 135.33, 143.74, 144.80, 154.97, 158.49, 162.61, 176.87; ^{31}P NMR (109 MHz, $CDCl_3$, 85% H_3PO_4) δ 50.339. Anal. Calcd for $C_{44}H_{52}N_5O_{10}PS{\cdot}5/2H_2O$: C, 57.51; H, 5.70; N, 7.62. Found: C, 57.79; H, 5.90; N, 7.35.

2′-*O*-Bis(2-cyano-1,1-dimethylethoxy)thiophosphoryl-5′-*O*-(4,4′-dimethoxytrityl)-2-*N*-phenylacetyl-6-*O*-(2-cyanoethyl)guanosine (5d). Compound **4d** (2.08 g, 2.92 mmol) was converted to **5d** (2.9 g, 99%) using the same method as described in **5b**. **5d**: ^{1}H NMR (270 MHz, DSS) δ 1.40−1.69 (12H, m), 2.62−3.14 (6H, m), 3.30−3.48 (4H, m), 3.76 (6H, s), 4.28 (1H, s), 4.74 (2H, t, J = 6.4 Hz), 4.89 (1H, s), 5.29 (1H, m), 6.21 (1H, d, J = 6.6 Hz), 6.77 (2H, s), 6.80 (2H, s), 7.15−7.50 (14H, m), 8.07 (1H, s), 8.60−8.63 (1H, br); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 17.95, 26.63, 26.70, 26.74, 26.79, 26.99, 27.05, 27.51, 31.38, 31.43, 31.57, 31.63, 43.85, 55.13, 61.64, 63.29, 70.08, 82.79, 82.86, 82.89, 82.97, 84.49, 86.54, 113.19, 116.53, 116.80, 116.87, 118.19, 123.72, 126.92, 127.15, 127.94, 128.05, 128.12, 128.68, 128.93, 129.38, 130.01, 133.91, 135.51, 135.65, 141.51, 141.56, 144.55, 151.52, 153.03, 158.49, 159.66, 169.02; ^{31}P NMR (109 MHz, $CDCl_3$, 85% H_3PO_4) δ 49.58. Anal. Calcd for $C_{52}H_{55}N_8O_{10}PS$: C, 61.53; H, 5.46; N, 11.04; S, 3.16. Found: C, 61.53; H, 5.47; N, 10.75; S, 2.84.

2′-*O*-Bis(2-cyano-1,1-dimethylethoxy)thiophosphoryl-5′-*O*-(4,4′-dimethoxytrityl)-4-*N*-benzoylcytidine (5e). Com-

pound **4e** (2.5 g, 4.1 mmol) was converted to **5e** (3.4 g, 91%) in the same manner of **5b**. **5e**: ^{1}H NMR (270 MHz, DSS) δ 1.62 (6H, s), 1.76 (6H, s), 2.86–3.06 (4H, m), 3.55 (2H, d, J = 1.7 Hz), 3.77 (6H, s), 4.22 (1H, dd, J = 1.7 Hz, J = 6.3 Hz), 4.69 (1H, dd, J = 3.3 Hz, J = 6.3 Hz), 5.18 (1H, dt, J = 3.3 Hz, J = 3.3 Hz, J = 11.6 Hz), 6.27 (1H, d, J = 3.3 Hz), 6.87 (2H, s), 6.90 (2H, s), 7.16–7.65 (13H, m), 7.88 (2H, d), 8.44 (1H, d, J = 7.3 Hz); ^{13}C NMR (67.8 MHz, $CDCl_3$) d 27.15, 27.28, 27.42, 31.36, 31.43, 31.61, 31.70, 55.15, 61.64, 68.97, 80.63, 80.67, 80.70, 82.88, 82.98, 83.27, 83.47, 83.60, 87.24, 88.00, 88.07, 113.33, 113.46, 116.78, 117.02, 123.74, 125.19, 127.12, 127.51, 128.05, 128.12, 128.93, 129.06, 129.99, 130.06, 132.94, 132.94, 132.97, 133.10, 135.00, 135.35, 143.90, 144.87, 144.91, 144.98, 145.01, 158.62, 162.28; ^{31}P NMR (109 MHz, $CDCl_3$, 85% H3PO4) δ 50.27. Anal. Calcd for $C_{47}H_{50}N_5O_{10}PS{\cdot}H_2O$: C, 60.96; H, 5.66; N, 7.56. Found: C, 60.83; H, 5.43; N, 7.19.

2′-*O*-Bis(2-cyano-1,1-dimethylethoxy)thiophosphoryl-5′-*O*-(4,4′-dimethoxytrityl)-4-*N*-benzoylcytidine-3′-(2-cyanoethyl)-morpholinophosphoramidite (6f). Compound **5e** (2.1 g, 2.2 mmol) was rendered anhydrous by successive coevaporations with dry pyridine (10 mL × 3) and dry toluene (10 mL × 2) and finally dissolved in dry CH_2Cl_2 (22 mL). To the mixture were added triethylamine (1.22 mL, 8.8 mmol) and chloro(2-cyanoethoxy)morpholinophosphine (0.69 mL, 4.4 mmol). After being stirred for 40 min, the reaction mixture was diluted with CH_2Cl_2 (20 mL). The CH_2Cl_2 extract was washed with 5% $NaHCO_3$ and water. The usual workup followed by silica gel column chromatography eluted with hexanes–ethyl acetate containing 1% pyridine gave **6f** (1.54 g, 61%): ^{1}H NMR (270 MHz, $CDCl_3$, TMS) δ 1.72–1.76 (12H, m), 2.40–2.69 (2H, m), 2.81–3.20 (8H, m), 3.42–4.09 (8H, m), 3.82 (6H, s), 4.28–4.33 (1H, m), 4.74–4.78 (1H, m), 5.20–5.26 (1H, m), 6.32–6.38 (1H, m), 6.87 (2H, s), 6.90 (2H, s), 7.15–7.63 (12H, m), 7.88 (2H, m), 8.44–8.48 (1H, m), 8.53–8.76 (1H, brs); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 20.07, 20.16, 20.22, 20.31, 21.35, 26.97, 27.03, 27.14, 27.26, 27.32, 27.39, 27.46, 27.49, 27.60, 27.64, 31.04, 31.07, 31.14, 31.54, 31.61, 42.98, 43.13, 43.24, 43.36, 53.39, 55.19, 58.22, 58.51, 58.98, 59.23, 61.31, 61.44, 67.62, 67.69, 67.78, 67.85, 78.56, 78.65, 78.73, 82.28, 82.41, 82.52, 82.57, 82.71, 83.11, 83.18, 83.24, 83.31, 87.35, 87.48, 87.66, 87.71, 113.32, 116.71, 116.77, 117.07, 117.25, 117.57, 125.19, 127.22, 127.33, 127.49, 128.01, 128.12, 128.30, 128.41, 128.91, 129.99, 130.06, 130.15, 130.21, 130.28, 133.08, 134.75, 134.90, 135.08, 137.74, 143.77, 144.94, 145.00, 158.63, 158.72, 158.78, 162.26; ^{31}P NMR (109 MHz, $CDCl_3$, 85% H_3PO_4) δ 147.25, 146.83, 50.60, 50.50. Anal. Calcd for $C_{54}H_{61}N_7O_{12}P2S{\cdot}1/2H_2O$: C, 58.8; H, 5.66; N, 8.89. Found: C, 59.07; H, 5.77; N, 8.44.

2′-*O*-Bis(2-cyano-1,1-dimethylethoxy)thiophosphoryl-5′-*O*-(4,4′-dimethoxytrityl)-4-*N*-isobutyrylcytidine-3′-(2-cyanoethyl)-*N,N*-diisopropylphosphoramidite (6c). Compound **5c** (175 mg, 0.2 mmol) was rendered anhydrous by successive coevaporations with dry pyridine (2 mL × 2) and dry toluene (2 mL × 1) and finally dissolved in dry CH_2Cl_2 (2 mL). To the mixture were added 2,4,6-collidine (158 mL, 1.2 mmol) and chloro(2-cyanoethoxy)diisopropylaminophosphine (86 μL, 0.4 mmol). After being stirred over period of 1 h, the mixture was diluted with CH_2Cl_2 (10 mL) and washed with 5% $NaHCO_3$ and water. The organic layer was collected and concentrated in vacuo. The residue was dissolved in CH_2Cl_2 (1 mL), and the mixture was added dropwise to a solution of hexanes–ether containing 1% pyridine (8:2, v/v, 100 mL) with vigorous stirring. The precipitate was collected and washed with hexanes–ether (8:2, v/v) containing 1% pyridine. The usual workup followed by silica gel column chromatography eluted with hexanes–ethyl acetate containing 1% pyridine gave the title compound **6c** (155 mg, 72%): ^{1}H NMR (270 MHz, $CDCl_3$, TMS) δ 1.00–1.29 (18H, m), 1.66–1.80 (12H, m), 2.40–2.70 (3H, m), 2.85–3.20 (4H, m), 3.40–3.98 (10H, m), 4.25–4.35 (1H, m), 4.58–4.72 (3H, m), 5.10–5.40 (1H, m), 6.35, 6.45 (1H, 2d, J = 5.3, J = 4.3 Hz), 6.83–6.90 (4H, m), 6.97–7.21 (1H, m), 7.26–7.46 (9H, m), 8.15–8.20 (1H, br); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 18.71, 19.01, 19.97, 20.22, 24.37, 24.46, 24.57, 24.78, 24.89, 26.99, 27.046, 27.19, 27.37, 27.66, 31.09, 31.16, 31.36, 36.43, 42.98, 43.16, 55.10, 58.83, 59.09, 62.43, 78.58, 82.19, 82.30, 82.41, 82.86, 82.98, 83.11, 83.22, 87.24, 87.30, 96.64, 96.71, 113.17, 113.23, 116.59, 116.82, 116.93, 117.02, 117.31, 117.79, 127.08, 127.91, 127.96, 128.12, 128.25, 130.06, 130.14, 134.72, 134.88, 135.02, 135.11, 143.65, 145.03, 145.14, 155.02, 158.56, 162.44, 176.68; ^{31}P NMR (109 MHz, $CDCl_3$, 85% H_3PO_4) δ 152.41, 151.90, 50.70, 50.35. Anal. Calcd for $C_{53}H_{69}N_7O_{11}P_2S{\cdot}3/2H_2O$: C, 57.81; H, 6.31; N, 8.90. Found: C, 57.92; H, 6.42; N, 8.52.

2′-*O*-Bis(2-cyano-1,1-dimethylethoxy)thiophosphoryl-5′-*O*-(4,4′-dimethoxytrityl)-2-*N*-phenylacetyl-6-*O*-(2-cyanoethyl)guanosine-3′-(2-cyanoethyl)-*N,N*-diisopropylphosphoramidite (6d). Compound **5d** (1.62 g, 1.6 mmol) was rendered anhydrous by successive coevaporations with dry pyridine (5 mL × 2) and dry toluene (5 mL × 1) and finally dissolved in dry CH_2Cl_2 (4.8 mL). To the mixture were added 2,4,6-collidine (1.27 mL, 9.6 mmol) and chloro(2-cyanoethoxy)-diisopropylaminophosphine (0.7 mL, 3.2 mmol). After being stirred for 1 h, the mixture was diluted with CH_2Cl_2 (50 mL) and washed with 5% $NaHCO_3$ and water. The organic layer was collected and concentrated in vacuo. The usual workup followed by silica gel column chromatography eluted with hexanes–ethyl acetate containing 1% pyridine gave the title compound **6d** (1.95 g, 74%): ^{1}H NMR (270 MHz, $CDCl_3$, TMS) δ 0.97–1.70 (26H, m), 2.49–3.04 (6H, m), 3.22–4.03 (6H, m), 3.76 (6H, s), 4.30–4.40 (1H, m), 4.71–4.79 (3H, m), 6.02–6.12 (1H, m), 6.19–6.28 (1H, m), 6.79–6.84 (4H, m), 7.08–7.82 (14H, m), 8.10–8.12 (1H, m); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 17.94, 20.34, 20.42, 21.33, 24.37, 24.46, 24.57, 24.67, 24.80, 26.67, 26.74, 26.90, 26.96, 27.21, 27.26, 31.23, 31.39, 31.47, 43.00, 43.18, 43.40, 43.63, 55.13, 59.23, 59.46, 61.65, 63.06, 63.25, 82.30, 82.34, 82.41, 82.45, 82.48, 82.59, 82.70, 84.64, 84.67, 84.96, 85.09, 85.39, 86.69, 86.78, 113.26, 116.32, 116.41, 116.62, 116.86, 117.23, 118.04, 118.17, 125.18, 126.97, 127.03, 128.03, 128.10, 128.16, 128.50, 128.55, 128.91, 129.38, 129.97, 130.12, 133.98, 135.38, 135.51, 135.72, 141.53, 144.46, 144.60, 151.55, 153.28, 153.33, 158.54, 159.62, 159.66, 168.86; ^{31}P NMR (109 MHz, $CDCl_3$, 85% H3PO4) δ 153.01, 150.85, 50.24, 49.95. Anal. Calcd for $C_{61}H_{72}N_{10}O_{11}P_2S$: C, 60.29; H, 5.97; N, 11.53. Found: C, 59.94; H, 6.07; N, 11.11.

2′-*O*-Bis(2-cyano-1,1-dimethylethoxy)thiophosphoryl-5′-*O*-(4,4′-dimethoxytrityl)-6-*N*-benzoyladenosine-3′-(2-cyanoethyl)-*N,N*-diisopropylphosphoramidite (6b). Compound **5b** (129 mg, 0.135 mmol) was rendered anhydrous by successive coevaporations with dry pyridine (2 mL × 2) and dry toluene (2 mL × 1) and finally dissolved in dry CH_2Cl_2 (1.5 mL). To the mixture were added 2,4,6-collidine (71 μL, 0.54 mmol) and chloro(2-cyanoethoxy)diisopropylaminophosphine (63 mg, 0.27 mmol). After being stirred for 1 h, the mixture was diluted with CH_2Cl_2 (5 mL) and washed with 5% $NaHCO_3$ and water. The organic layer was collected and concentrated in vacuo. The usual workup followed by silica gel column chromatography eluted with hexanes–ethyl acetate containing 1% pyridine gave the title compound **6b** (110 mg, 72%): ^{1}H NMR (270 MHz, $CDCl_3$, TMS) δ 1.06–1.78 (26H, m), 2.36–3.04 (6H, m), 3.30–4.06 (4H, m), 3.76 (6H, s), 3.37–4.44 (1H, m), 4.34–4.94 (3H, m), 5.77–5.97 (1H, m), 6.416.52 (1H, m), 6.79–6.84 (4H, m), 7.19–7.64 (12, m), 8.03–8.09 (2H, m), 8.37–8.42 (1H, 2s), 8.76–8.79 (1H, 2s), 9.05–9.07 (1H, 2brs); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 16.85, 16.97, 17.91, 17.94, 18.02, 18.29, 18.39, 20.71, 20.75, 20.80, 22.37, 22.45, 22.56, 22.69, 22.80, 24.65, 25.04, 25.11, 25.28, 25.62, 29.32, 29.43, 41.05, 41.23, 43.10, 43.19, 53.09, 55.64, 56.00, 57.12, 57.35, 60.87, 61.05, 69.55, 74.57, 75.05, 75.25, 75.52, 75.72, 80.48, 80.58, 80.67, 80.78, 82.26, 82.72, 82.87, 82.97, 84.77, 84.86, 111.15, 114.29, 114.38, 114.62, 115.28, 115.79, 120.76, 120.87, 124.84, 125.71, 125.87, 125.99, 126.08, 126.69, 126.87, 127.97, 130.61, 131.51, 1332.22, 133.27, 133.36, 140.06, 142.17, 142.24, 147.48, 149.91, 150.65, 156.45, 162.49; ^{31}P NMR (109 MHz, $CDCl_3$, 85% H_3PO_4) δ 152.84, 151.31, 50.78, 50.31. Anal. Calcd for $C_{57}H_{67}N_9O_{10}P_2S{\cdot}1/2H_2O$: C, 59.50; H, 6.05; N, 10.95. Found: C, 60.00; H, 6.59; N, 10.52.

3-*N*-Benzoyl-2′-deoxyuridine (8). Hexamethyldisilazane (25 mL, 0.12 mol) was added to a solution of 2′-deoxyuridine (6.85 g, 30 mmol) in CH_3CN (300 mL). After being refluxed for 1 h, the solvent was removed under reduced pressure to

give a colorless foam. To the residue dissolved in CH_2Cl_2 (500 mL) − 0.2 M sodium carbonate solution (500 mL) were added tetrabutylammonium bromide (0.8 g, 2.4 mmol) and benzoyl chloride (5.2 mL, 45 mmol) with vigorous stirring. After 3 h, the organic layer was extracted with water, collected, dried over Na_2SO_4, and concentrated in vacuo. The resulting residue was refluxed in $CHCl_3$ (300 mL) for 1 h and evaporated under reduced pressure. The residue was dissolved in methanol (300 mL) containing 6 mL of trifluoroacetic acid. After being stirred for 20 min, the mixture was neutralized by addition of pyridine (20 mL). Extraction was performed with CH_2Cl_2−pyridine (2:1, v/v, 300 mL) and saturated $NaHCO_3$. The crude material was chromatographed on a column of silica gel with CH_2Cl_2−methanol to give **8** (9.2 g, 92%): ^{1}H NMR (270 MHz, C_5D_5N) δ 2.52−2.69 (2H, m), 4.10 (1H, dd, J = 2.6 Hz, J = 12.9 Hz), 4.20 (1H, dd, J = 3.0 Hz, J = 12.9 Hz), 4.43 (1H, m), 4.97 (1H, m), 5.93 (1H, d, J = 7.9 Hz), 6.81 (1H, dd, J = 6.3 Hz, J = 6.3 Hz), 7.34−7.40 (2H, m), 7.49−7.53 (1H, m), 8.15−8.18 (2H, m), 8.59 (1H, d, J = 7.9 Hz); ^{13}C NMR (67.8 MHz, C_5D_5N) δ 39.68, 59.80, 68.91, 84.06, 86.99, 99.89, 127.42, 128.68, 130.21, 133.15, 139.41, 160.41, 167.89. Anal. Calcd for $C_{16}H_{16}N_2O_6$: C, 57.83; H, 4.85; N, 8.43. Found: C, 57.33; H, 4.87; N, 8.38.

5′-*O*-(4,4′-Dimethoxytrityl)-3-*N*-Benzoyl-2′-deoxyuridine (9). N^3-Benzoyl-2′-deoxyuridine **8** (5.0 g, 15 mmol) was converted to **9** in the usual manner in 99% yield. **9**: ^{1}H NMR (270 MHz, $CDCl_3$) δ 2.23−2.43 (2H, m), 3.43 (1H, dd, J = 3.0 Hz, J = 11.2 Hz), 3.48 (1H, dd, J = 3.3 Hz, J = 11.2 Hz), 3.79 (6H, s), 3.98−4.01 (1H, m), 4.53−4.58 (1H, m), 5.46 (1H, d, J = 8.3 Hz), 6.26 (1H, dd, J = 5.9 Hz, J = 6.3 Hz), 6.83−6.88 (4H, m), 7.15−7.63 (12H, m), 7.90−7.94 (3H, m); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 41.33, 55.22, 62.75, 71.21, 85.34, 86.09, 87.08, 101.98, 112.83, 113.10, 123.86, 127.17, 127.75, 127.85, 128.01, 128.05, 128.99, 129.11, 129.49, 130.03, 130.21, 130.24, 130.31, 130.46, 131.36, 135.06, 135.11, 135.22, 135.29, 136.24, 140.09, 144.17, 144.17, 149.15, 158.69, 162.16, 168.82. Anal. Calcd for $C_{37}H_{34}N_2O_8$: C, 70.02; H, 5.34; N, 4.41. Found: C, 69.71; H, 5.35; N, 4.47.

5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-succinyl-3-*N*-Benzoyl-2′-deoxyuridine (10). Compound **9** was converted to **10** in the usual method in 98% yield. **10**: ^{1}H NMR (270 MHz, $CDCl_3$) δ 2.38−2.60 (6H, m), 3.44 (1H, dd, J = 2.3 Hz, J = 10.9 Hz), 3.54 (1H, dd, J = 2.6 Hz, J = 10.9 Hz), 3.79 (6H, s), 4.19−4.20 (1H, m), 5.41 (1H, d, J = 8.2 Hz), 5.48−5.50 (1H, m), 6.32 (1H, dd, J = 5.9 Hz, J = 7.9 Hz), 6.84−6.89 (4H, m), 7.15−7.66 (12H, m), 7.87−7.93 (3H, m); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 29.85, 30.26, 38.42, 55.17, 63.29, 74.50, 84.21, 85.09, 87.21, 102.21, 113.21, 127.15, 128.01, 128.01, 128.12, 129.06, 130.01, 130.39, 131.36, 134.83, 135.04, 139.93, 144.04, 149.13, 158.64, 161.96, 168.71, 172.58, 176.28. Anal. Calcd for $C_{41}H_{38}N_2O_{11}\cdot 3/4H_2O$: C, 67.41; H, 6.12; N, 4.75. Found: C, 67.87; H, 5.97; N,4.37.

5′-*O*-(4,4′-Dimethoxytrityl)-3-*N*-Benzoyl-2′-deoxyuridine-3′-(2-cyanoethyl)-*N,N*-diisopropylphosphoramidite (11). Compound **9** (4.44 g, 7 mmol) was rendered anhydrous by coevaporations with dry pyridine (5 mL × 2) and dry toluene (5 mL × 1) and finally dissolved in dry CH_2Cl_2 (70 mL). To the mixture were added diisopropylethylamine (2.44 mL, 14 mmol) and chloro(2-cyanoethoxy)diisopropylaminophosphine (1.67 mL, 6.7 mmol). After being stirred for 1 h, the mixture was diluted with CH_2Cl_2 (100 mL) and washed with 5% $NaHCO_3$ and water. The organic layer was collected and concentrated in vacuo. The usual workup followed by silica gel column chromatography eluted with hexanes−ethyl acetate containing 1% pyridine gave the title compound **11** (5.41 g, 93%): ^{1}H NMR (270 MHz, $CDCl_3$) δ 1.07−1.19 (12H, m), 2.34−2.63 (4H, m), 3.43−3.84 (12H, m), 4.14−4.19 (1H, m), 4.71−4.76 (1H, m), 5.44−5.45 (1H, 2d, J = 8.3 Hz, J = 8.3 Hz), 6.27−6.34 (1H, m), 6.84−6.89 (4H, m), 7.18−7.66 (12H, m), 7.93−8.01 (3H, m); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 20.06, 20.18, 20.29, 24.30, 24.33, 24.44, 24.49, 40.20, 40.27, 40.40, 42.98, 43.02, 43.16, 43.22, 53.35, 55.11, 57.83, 58.10, 62.23, 62.41, 72.18, 72.42, 72.63, 72.89, 85.19, 85.28, 85.34, 85.54, 85.59, 56.88, 101.92, 101.96, 113.14, 117.32, 117.48, 127.04, 127.87, 128.01, 128.86, 128.99, 130.35, 131.32, 134.86, 134.93, 135.08, 135.11, 139.89, 139.95, 144.04, 144.08, 149.06, 149.11, 158.58, 161.98, 168.77; ^{31}P NMR (109 MHz, $CDCl_3$) δ 149.37, 149.76. Anal. Calcd for $C_{46}H_{51}N_4O_9P$: C, 66.18; H, 6.16; N, 6.71. Found: C, 66.05; H, 6.12; N, 6.35.

General Method for Polymer Supported Synthesis of Oligonucleotides. Synthesis of dU$_m$U(2′-p)dUn (15−17). A CPG gel having deoxyuridine (1 μmol, 25 μmol/g) as the 3′-terminus was used. The synthetic unit **6a** obtained above and deoxyuridine phosphoramidite (or **11**) were used for condensation. Chain extension was performed according to the protocol described in our previous paper.[18] After all operations were over (DMTr off), the CPG gel was evaporated with acetonitrile and suspended in a solution of bis(trimethylsilyl)acetamide−pyridine (0.4 mL, 1:1, v/v), and DBU (12 μL, 0.08 mmol) was added. The mixture was slowly rotated in a vessel at room temperature for 2 h. The CPG gel was then filtered and washed with pyridine (1 mL × 3). The CPG gel was treated with a 0.1 M solution of KI_3 in pyridine−water (0.3 mL, 9:1, v/v) at room temperature for 24 h. Filtration was performed, and the CPG gel was washed with pyridine (1 mL × 7). The CPG gel was collected and transferred to a round flask. Concentrated ammonia−pyridine (20 mL, 9:1, v/v) was added, and the suspension was slowly rotated by a rotary evaporator for 18 h. The CPG gel was removed by filtration and washed with water. The filtrate and washings were combined and evaporated under reduced pressure. The residue was purified by ion exchange and reversed phase HPLC to give the desired products. (**15**: 18 A_{260}, 28%; **16**: 13 A_{260}, 20%)

Synthesis of dU$_6$U(2′-ps)dU$_6$ (17). A CPG gel having deoxyuridine (1 μmol, 25 μmol/g) as the 3′-terminus was used. The same procedure to synthesize **15** was carried out without the KI_3 treatment. The crude material was purified by ion exchange and reversed phase HPLC to give the desired products **17** (13 A_{260}, 19%).

Synthesis of U$_m$U(2′-p)Un (21, 22). A CPG gel having uridine (1 μmol, 25 μmol/g) as the 3′-terminus was used. The same procedure to synthesize **15** was carried out until treatment with concentrated ammonia−pyridine (20 mL, 9:1, v/v) and removal of CPG gel. After evaporation under reduced pressure, the residue was dissolved in 1 M TBAF/THF (1 mL, 1 mmol). After being stirred for 15 h, the solution was directly chromatographed by Sephadex G-15. The appropriate fraction was collected and concentrated. The residue was purified by ion exchange and reversed phase HPLC to give the desired products (**21**: 3 A_{260}, 7%; **22**: 4 A_{260}, 6%).

Enzymatic Assay of Oligonucleotides (15−17, 21, 22). Treatment with Calf Intestinal Alkaline Phosphatase. An oligonucleotide (0.4 A_{260}) was dissolved in 50 mM Tris-HCl buffer (pH 8.5, 200 μL) containing 0.1 mM EDTA. To the mixture was added a solution (10 μL) of CIAP (4 units/μL). The incubation was done at 50 °C for 1 h. After being heated at 100 °C for 1 min, the mixture was analyzed by ion exchange HPLC.

General Method for T_m Measurement. An appropriate trideca(deoxy)uridylate (**14**−**22**) (0.603 A_{260}, 5 nmol) and trideca(deoxy)adenylate (0.673 A_{260}, 5 nmol) were dissolved in 2.5 mL of buffer solution (10 mM sodium phosphate, 0.1 mM EDTA, 1 M NaCl, pH 7.0). The solution was heated at 65 °C for 5 min and annealed from 65 °C to 0 °C at a decreasing rate of 1 °C/min. After being kept at 0 °C for 10 min, the solution was heated to 65 °C at an increasing rate of 1 °C/min. The UV-melting curve was measured at 254 nm as temperature was increasing, and the T_m value was determined by calculation of transition point. (In the case of a self-complementary DNA, 2.0 A_{260} of oligonucleotide was used, and the following conditions were used: 10 mM sodium phosphate buffer, 0.1 mM EDTA, 150 mM NaCl (pH 7.0))

General Method for Molecular Dynamics Simulation. The present MD simulation was calculated on MacroModel 5.0/Batchmin software using Indigo 2 workstation of Silicon Graphics Inc.

MD Simulation of U$_7$/dA$_7$ (33) and dU$_3$U(2′-p)dU$_3$/dA$_7$ (34). To construct the duplex **34**, a typical B-DNA duplex of dU$_7$/dA$_7$ was generated on MacroModel 5.0 and the α-side 2′-hydrogen of the fourth deoxyuridine in the duplex was replaced by a phosphate group in the monoanion form. (In the simula-

tion of **33**, a typical B-DNA duplex was used as an initial structure.) The length of the hydrogen bonds of both 3′- and 5′-terminus base pairs was constrained on 1.9 Å. The energy minimization of **34** was carried out using the united atom mode on the Amber* force field and the GB/SA water model[30] to give a local minimized structure. It was used as the initial structure in MD simulation. The first simulation was broadly performed for 20 ps to equilibrate energy of the duplex. The detailed simulation at the second step was carried out using the initial structure obtained by the first simulation. The detailed simulation condition was summarized as described below.

First Step. Mode: stochastic dynamics; time step: 1.5 fs; shake on (H/Lp only); simulation temperature: 300 K; dielectric constant: 1; solvation: GB/SA water model; simulation time: 20 ps.

Second Step. Mode: stochastic dynamics, time step: 0.5 fs, shake off, simulation temperature: 300 K, dielectric constant: 1, sampling: 1000 conformer, solvation: GB/SA water model; simulation time: 1000 ps.

MD Simulation of U_7/A_7 (35) and $U_3U(2'\text{-}p)U_3/A_7$ (36). To construct the duplex **36**, a typical A-RNA duplex of U_7/A_7 was generated on MacroModel 5.0, and the 2′-hydroxyl of the fourth uridine in duplex was attached to a phosphate group in the monoanion form. (In the simulation of **35**, a typical A-RNA duplex was used as an initial structure.) The other simulation method and conditions were the same as described in the case of **34.**

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Supporting Information Available: Copies of ^{1}H and ^{13}C NMR data for **2c**, **2d**, **8**, **9**, **10**; ^{1}H, ^{13}C, and ^{31}P NMR data for **4b**, **4c**, **4d**, **4e**, **5b**, **5c**, **5d**, **5e**, **6b**, **6c**, **6d**, **6f**, and **11**; ^{31}P NMR data with respect to oxidative desulfurization; HPLC profiles of oligomer **21**, **22**, and **24**−**28**. This material is available free of charge via the Internet at http://pubs.acs.org.

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