Studies on Steric and Electronic Control of 2′−3′ Phosphoryl Migration in 2′-Phosphorylated Uridine Derivatives and Its Application to the Synthesis of 2′-Phosphorylated Oligouridylates

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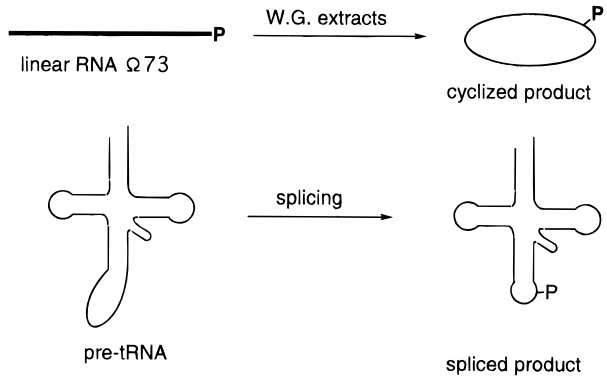
For the synthesis of 2′-phosphorylated oligouridylates by use of new phosphoramidite building units, several masked phosphoryl groups have been examined as 2′-phosphate precursors, which should not be migrated to the 3′ position when the 3′ hydroxy protecting group must be removed to introduce a phosphoramidite residue into the 3′-position. As a consequence, bis(2-cyano-1,1-dimethylethoxy)-thiophosphoryl (BCMETP) was found to be the most suitable 2′-phosphate precursor. This thiophosphoryl group could be introduced into the 2′-hydroxyl of 3′,5′-silylated uridine derivative **7** by phosphitylation with bis(2-cyano-1,1-dimethylethoxy)(diethylamino)phosphine followed by sulfurization. Treatment of the 2′-thiophosphorylated product **15** with $(HF)_x$·Py in THF gave exclusively the 3′,5′-unprotected uridine derivative **16a**. Compound **16a** was converted to the phosphoramidite unit **22** via a two-step reaction. This building block was used for the solution phase synthesis of U(2′-p)pU (**29**) and U(2′-ps)pU (**30**). Both the 2-cyano-1,1-dimethylethyl and 2-cyanoethyl groups were effectively removed from the fully protected derivative **25** by treatment with DBU in the presence of *N*,*O*-bis(trimethylsilyl)acetamide (BSA). The resulting 2′-thiophosphoryl group was successfully converted to a phosphoryl group by iodine treatment to give U(2′-p)pU (**29**). U(2′-ps)pU (**30**) was also synthesized by a modified procedure without the iodine treatment. Reaction of **29** with a new biotinylating reagent in aqueous solution in the presence of $MgCl_2$ gave a biotin-labeled product **35** having a pyrophosphate bridge at the 2′ position. Reaction of **30** with monobromobimane gave the 2′-S-alkylated product **33** in aqueous solution. Application of the phosphoramidite unit **22** to the solid phase synthesis using aminopropyl CPG gel gave successfully $[U(2'\text{-p})p]_nU$ (n = 1, 3, 5). It was found that stability of the succinate linker between the CPG and oligouridylates was unaffected by the treatment with DBU when BSA was present. Several enzymatic properties of the synthetic 2′-phosphorylated and 2′-thiophosphorylated oligouridylates are also described.

Introduction

In 1981, Konarska *et al*. discovered an unprecedented 2′-phosphomonoester, 3′−5′ phosphodiester linkage at the ligation site of a circular RNA which was formed by intramolecular cyclization of a linear RNA fragment Ω73 with wheat germ extracts having a unique RNA ligase capability.[1,2] A similar structure was also found in a circular RNA formed by ligation of a linearized viroid RNA by use of wheat germ or Chlamidomonas extracts.[3] Abelson *et al*. reported that the 38th G at the splice junction of the spliced yeast pre-$tRNA^{Leu3}$ is phosphorylated at the 2′-position.[4] Later, such 2′-phosphomonoester, 3′−5′ phosphodiester linkages have been generally found in a number of spliced pre-tRNAs (Scheme 1).[5,6]

Scheme 1



The 2′-phosphate group is removed by a tRNA specific phosphatase with the help of NAD to give mature tRNAs.[7] Phizicky has recently clarified the mechanism of this dephosphorylation, where the 2′-phosphate group attacks the anomeric carbon with elimination of the nicotinamide residue to produce an ADP-ribosylated 2′-phosphorylated tRNA intermediate which, in turn, docomposes to the normal tRNA and a 1″,2″-cyclic phosphate derivative of ADP-ribose.[8] Although the biological meaning and role of the 2′-phosphate group in the structure−

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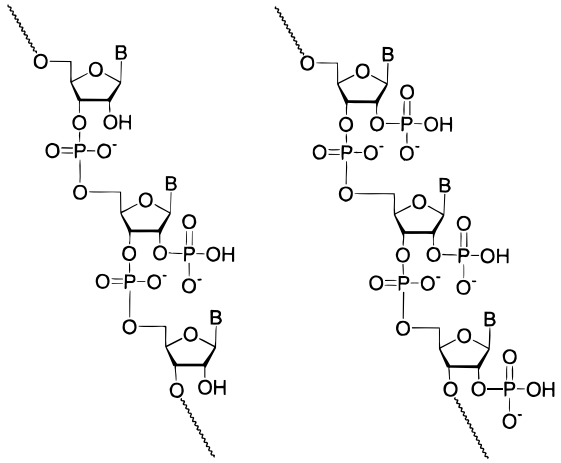
Scheme 2



2'-phosphorylated RNAs

function relationships of tRNAs is of great interest, no basic physicochemical studies have been reported regarding 2′-phosphorylated RNAs (Scheme 2). The chemical synthesis of this kind of RNAs is highly desired for such studies.

On the other hand, much attention has recently been paid to polyanion species such as cyclodextrin polysulfates[9] and heteropolyacids,[10] which have proved to have potent anti-HIV activities. In connection with these studies, oligoribonucleotides having 2′-phosphates at each 2′-position are of interest as a new type of polyanion species.

In connection with recent studies in antisense chemistry,[11] oligonucleotides having a 2′-phosphate group would be used as a new tool for introduction of a variety of functional groups such as fluoresence probes and biotin by the use of the intrinsic property of a phosphomonoester type of 2′-phosphate group which can be selectively modified.

Recently, Szostak and co-workers[12] have reported artificial RNA ribozymes capable of catalyzing RNA ligation and ATP(-γS)-dependent (thio)phosphorylation. Interestingly, they found that these ribozymes thiophosphorylate their internal 2′-hydroxyls. More recently, Szostak's research group has reported reversetranscription of RNA containing a 2′-thiophosphate at a unique internal site with AMV and MMLV H^- reverse transcriptases, showing that these reverse transcriptases pause before the 2′-thiophosphate.[13]

These intriguing findings about 2′-phosphorylated or hitherto unknown 2′-thiophosphorylated RNAs led us to explore the chemical synthesis of such RNAs. Previously, we have reported the first method for the synthesis of 2′-phosphorylated oligoribonucleotides using the *tert*-butyl group as the 2′-phosphate protecting group.[14]

In this paper, we report an improved method for the synthesis of 2′-phosphorylated and 2′-thiophosphorylated oligouridylates by use of highly effective steric and electronic control of the 2′−3′ phosphoryl migration in 2′-phosphorylated ribonucleosides.

Results and Discussion

Strategies for the Synthesis of 2′-Phosphorylated RNAs. For the synthesis of 2′-phosphorylated RNAs, there would be two main strategies: One is the approach in which appropriately protected monomer units having a 2′-masked phosphate group are used as starting materials. In fact, we have employed this strategy in our previous paper.[14] This kind of method has also been utilized by us for the synthesis of branched RNAs.[15]

The other involves the introduction of 2′-phosphate groups into oligoribonucleotide blocks at a later stage. If the protecting groups of the internucleotidic phosphates and 2′-hydroxy groups can be selectively removed to produce the unprotected 2′-hydroxyl group, one can introduce a protected phosphoryl group at each 2′-position in a manner similar to that used for the synthesis of branched RNAs[16] reported by Kierzek and Caruthers,[17] Chattopadhyaya,[18] and Imbach.[19] In fact, quite recently, Kierzek[20] has adopted this type of approach to synthesize N(2′-p)pN, which has been utilized as a reference material for elucidation of the biochemical course of NAD-promoted dephosphorylation of a 2′-phosphorylated tRNA species,[8] as mentioned in the foregoing text.

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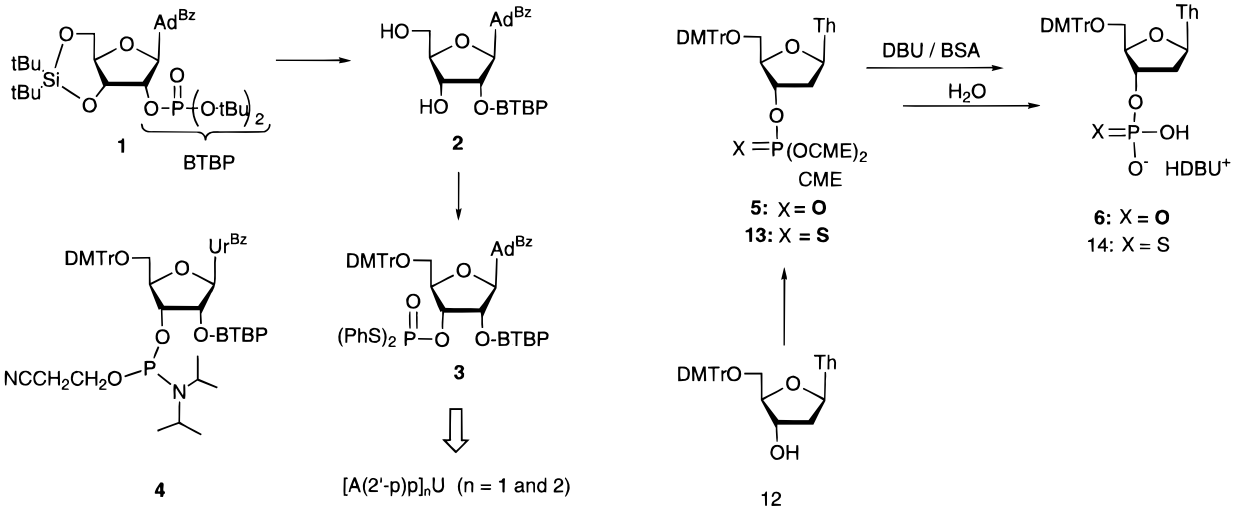
Scheme 3



In our present study, we have focused our efforts on the former strategy. In our approach, 3′,5′-unprotected 2′-monophosphorylated ribonucleoside derivatives are required as key synthetic intermediates, but such species are known to be extremely unstable to undergo easy cyclization and/or migration of the phosphoryl group owing to the neighboring group participation of the proximal OH group.[21−23] Regulation of the cyclization and migration of such a phosphoryl group is a serious problem, if this strategy is chosen. We have recently found the first clue to a general solution to this long-standing problem, namely, that treatment of a 2′-phosphorylated adenosine derivative **1** with $(HF)_x$·Py gave exclusively a 3′,5′-unprotected product **2**, without 2′−3′ migration of the 2′-(di-*tert*-butoxyphosphoryl) (BTBP) group.[14] On the basis of this result, a new class of RNAs, *i.e.*, 2′-*O*-phosphorylated oligoribonucleotides, A(2′-p)pU and A(2′-p)pA(2′-p)pU, have been synthesized using the BTBP group as the 2′-phosphate precursor (Scheme 3).[14]

However, deprotection of the two *tert*-butyl groups from the BTBP group required rather drastic conditions (20% trifluoroacetic acid in acetic acid at rt for 3−5 h) which are apparently unsuitable for the synthesis of long oligomers. In fact, attempts to synthesize $[U(2'\text{-p})p]_6U$ in the solid phase approach on a CPG gel by the use of a newly prepared uridine phosphoramidite unit **4** having the BTBP group at the 2′-position gave complex mixtures. Another drawback in this method is the unsatisfactory yield of **1**. The reason why such a sluggish phosphitylation occurred is now rationalized in terms of Watanabe's recent finding[24] that 1*H*-tetrazole used as activator at the 2′-phosphitylation step partially decomposes the resulting di-*tert*-butyl phosphite intermediate by protonation with formation of a hydrogen phosphonate derivative. Therefore, a more ideal strategy capable of complete avoidance of the phosphoryl migration as well as facile 2′-phosphitylation and deprotection should be developed.

Synthesis of 2′-Phosphorylated Oligouridylates by Use of the BCMETP Group. To avoid the acidic conditions used for removal of the *tert*-butyl group as well as to avoid the protonation on the phosphorus by 1*H*-tetrazole at the stage of phosphitylation, we have searched for sterically hindered protecting groups which should be removed under more suitable basic conditions. The 2-cyano-1,1-dimethylethyl (CME) group[25] was first introduced to nucleic acid chemistry as a sterically hindered phosphate protecting group by van Boom, who showed that the CME group could be removed by the action of ammonia. It was expected that the presence of the two electron-withdrawing cyano groups causes less protonation on the phosphorus of a phosphite intermediate. Quite recently, we have also reported an effective method for simultaneous removal of the two CME groups from bis(2-cyano-1,1-dimethylethyl) 5′-*O*-dimethoxytritylthymidine 3′-phosphate (**5**) by use of DBU in the presence of *N*,*O*-bis(trimethylsilyl)acetamide (BSA), which gave the deprotected material (**6**) after desilylation by addition of water.[26] Evans[27] and Bartlett[28] have reported similar strategies for the simultaneous removal of phosphate-protecting groups capable of β-elimination.

In consideration of these facts, a phosphitylating reagent, bis(2-cyano-1,1-dimethylethoxy)(diethylamino)-phosphine (CMEAP), was prepared in situ by the reaction of dichloro(diethylamino)phosphine with 2-cyano-1,1-dimethylethanol in an attempt to use this CME group as the 2′-protecting group in our strategy mentioned above. The purity of this reagent was about 60% (^{31}P NMR). Since the other byproducts were inert species having a pentavalent phosphorus atom, this reagent underwent smooth phosphitylation with nucleosides in spite of its relatively low purity. Reaction of N^3-benzoyl-3′,5′-*O*-(di-*tert*-butylsilanediyl)uridine (**7**)[29] with 1.2 equiv of CMEAP in the presence of 1.8 equiv of 1*H*-tetrazole in CH_2Cl_2 for 2 h followed by the oxidation of the resulting phosphite intermediate **8** with *tert*-butyl hy-

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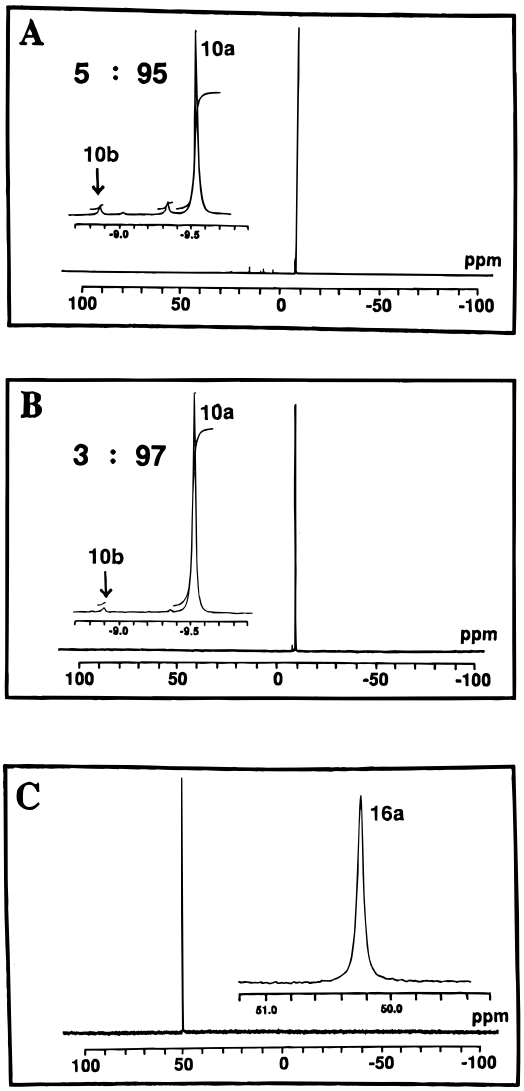

Figure 1. 2′−3′ isomerization of the 2′-phosphoryl group of **9** and **15** upon desilylation by HF-pyridine. A: The ^{31}P NMR spectrum of the product obtained by desilylation of **9** with $(HF)_x$·Py−Py in THF. B: The ^{31}P NMR spectrum of the product obtained by desilylation of **9** with $(HF)_x$·Py−Py in THF in the presence of acetic acid. C: The ^{31}P NMR spectrum of the product obtained by desilylation of **15** with $(HF)_x$·Py−Py in THF.

droperoxide[30] gave the 2′-phosphorylated product **9**. Further treatment of **9** with $(HF)_x$·Py[14] in THF at rt for 10 min gave a 95:5 mixture of 3′,5′-unprotected 2′-phosphorylated species **10a** and the 3′-regioisomer **10b** in an overall yield of 72% yield from **7**. The ^{31}P NMR spectrum of the mixture is shown in Figure 1A.

Compared with the BTBP group, the bis(2-cyano-1,1-dimethylethoxy)phosphoryl (BCMEP) group caused phosphoryl migration to a degree of 5% owing to addition of two electron-withdrawing cyano groups to the BTBP group at the β-positions. When removal of the di-*tert*-butylsilanediyl (DTBS) group from **7** was carried out in the presence of acetic acid[31,32] as a proton source that neutralizes the 3′-oxide ion generated upon the fluoride ion-mediated Si−O3′ bond cleavage, a slightly suppressed phosphoryl migration was observed (Figure 1B) but the yield of **10a**,**b** was considerably decreased to 46%. Several efforts to isolate **10a** free from **10b** by silica gel column chromatography have failed. In an attempt to separate the two regioisomers **10a** and **10b** at the next step, dimethoxytritylation of a mixture of **10a** and **10b** was conducted (Scheme 4). However, it was impossible to separate the new products **11a** and **11b** by chromatographic techniques.

Therefore, we tried to suppress the 2′−3′ phosphoryl migration by changing the electronic environment on the phosphorus as electrophilic center to avoid intramolecular attack of the proximal 3′-OH group. It was thought that displacement of the P═O bond with a P═S bond would result in the suppression of the 2′−3′ phosphoryl migration since sulfur is lesser electron negative than oxygen so that the phosphorus should become a poorer electrophilic center. It is also known that thiophosphoric acid monoesters can be converted into phosphoric acid monoesters by treatment with iodine in aqueous solution.[33] Therefore, we synthesized 2′-phosphorylated oligoribonucleotides via an indirect route involving an additional step of oxidative S → O conversion, using bis-(2-cyano-1,1-dimethylethoxy)thiophosphoryl (BCMETP) as the 2′-phosphoryl precursor.

However, it is unknown whether the CME group can be completely removed from ROP(S)$(OCME)_2$ (R = nucleoside residue) by treatment with DBU/BSA under conditions similar to those used for removal of the CME group from ROP(O)$(OCME)_2$.[26] Therefore, a model compound **13** was synthesized from **12** to study detailed conditions for complete removal of the two CME groups to give compound **14**.

It was found that both CME groups could be more rapidly removed from **13** in 3 min than those of the corresponding phosphoryl compound **5**, which required 1.5 h as reported previously by us.[26] This marked difference in elimination of the CME group between **5** and **13** is explained by the difference in leaving ability between the phosphoryl and thiophosphoryl residues of the two silylated species, ROP(O)(OCME)$(OSiMe_3)$ and ROP(S)(OCME)$(OSiMe_3)$, respectively. In general, dialkyl phosphorothioates have lesser pK_a values by approximately 0.5 unit than the corresponding dialkyl phosphates. Therefore, the (thiophosphoryl)oxy group should be stronger as a leaving group than the phosphoryloxy group so that the CME group of **13** was eliminated faster than that of **5**. Since the oxygen has a stronger electronegative atom than the sulfur, it seems that the phosphoryloxy group should be more readily removable than the (thiophosphoryl)oxy group. However, this is not correct since a thiophosphate anion once generated can be isomerized to a more stable tautomer in which an anion charge is located at the sulfur atom. It is likely that, because of this further stabilization, preferential elimination of the thiophosphoryl residue takes place over the phosphoryl residue.

Since simultaneous rapid removal of the CME groups from **13** was achieved, sulfurization of intermediate **8** was examined under various conditions. Consequently, treatment of **8** with S_8 in CS_2[34] gave the corresponding

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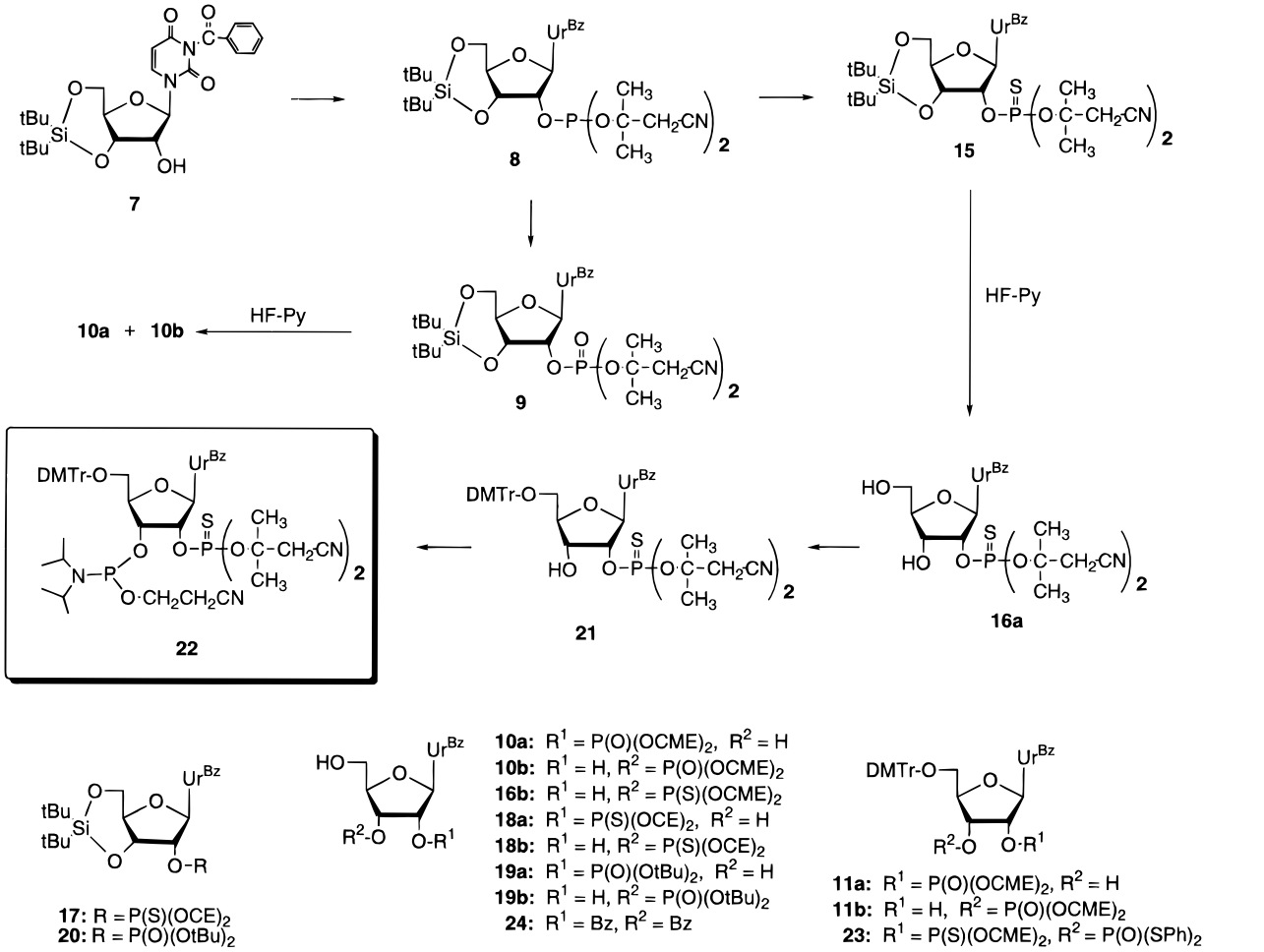
Scheme 4



thiophosphorylated species **15**. $Et_2NC(S)SS(S)NEt_2$[35] was less effective as the sulfurization reagent.

In situ desilylation of **15** thus formed was carried out. As expected, treatment of **15** with $(HF)_x$·Py in THF afforded exclusively the 2′-thiophosphorylated product **16a**, as shown in Figure 1C. The overall yield of **16a** from **7** was 69%. The ^1H, ^{13}C, and ^{31}P NMR spectra of **16a** suggested that this compound was essentially free from its 3′-regioisomer **16b**.

To evaluate the importance of both the steric and electronic effects of substituents, attached to the phosphorus, on the suppression of phosphoryl migration, a 2′-thiophosphorylated uridine derivative **17** having a less hindered 2-cyanoethyl group than the CME group was synthesized and treated similarly with $(HF)_x$·Py in THF. Consequently, TLC analysis suggested that the 2′-thiophosphorylated product **18a** was predominantly formed over **18b** when the reaction was completed, but very rapid 2′−3′ phosphoryl migration occurred even during extraction to give ultimately a nearly 1:1 mixture of **18a** and **18b**. These results clearly indicated that both the steric and electronic effects are very essential in the present study.

Next, the relative stability of the $P(O)(OCME)_2$ (in **10a**), and $P(S)(OCME)_2$ (in **16a**), $P(O)(O\text{-}t\text{-}Bu)_2$ (in **19a**) groups attached to the 2′-hydroxyl of N^3-benzoyluridine was systematically examined. To obtain substrate **19a** having a $P(O)(O\text{-}t\text{-}Bu)_2$ group, compound **7** was allowed to react with di-*tert*-butyl phosphonate in the presence

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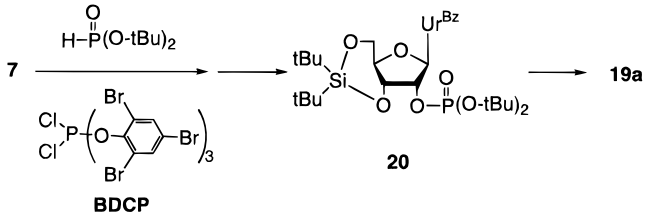
Scheme 5



of tris(2,4,6-tribromophenoxy)dichlorophosphorane (BDCP)[36] in pyridine followed by oxidation with *t*-BuOOH. This reaction gave 2′-phosphorylated derivative **20**. Compound **20** was in situ desilylated in a manner similar to that described for **16a** to give **19a** as a crystalline material in 58% yield (Scheme 5).

The 2′-phosphorylated uridine derivatives **10a**, **16a**, and **19a** were kept in pyridine and in 80% acetic acid at 25 °C. These solutions were analyzed by ^{31}P NMR to monitor the phosphoryl migration. These results are shown in Figure 2. In pyridine, an equilibrium between **10a** and **10b** was reached in ca. 50 days. About 0.7% of phosphoryl migration occurred in 4 h, the time of which is usually necessary for completion of the 5′-*O*-dimethoxytritylation in pyridine. This result is not acceptable.

(36) Hotoda, H.; Wada, T.; Sekine, M.; Hata, T. *Tetrahedron Lett.* **1987**, *28*, 1681. Wada, T.; Hotoda, H.; Sekine, M.; Hata, T. *Tetrahedron Lett.* **1988**, *29*, 4143. Matsuzaki, J.; Hotoda, H.; Sekine, M.; Hata, T. *Nucleosides Nucleotides* **1989**, *8*, 367. Wada, T.; Kato, R.; Hata, T. *J. Org. Chem.* **1991**, *56*, 1243. Ohuchi, S.; Singh, R. K.; Wada, T.; Hata, T. *Chem. Lett.* **1992**, 1505.

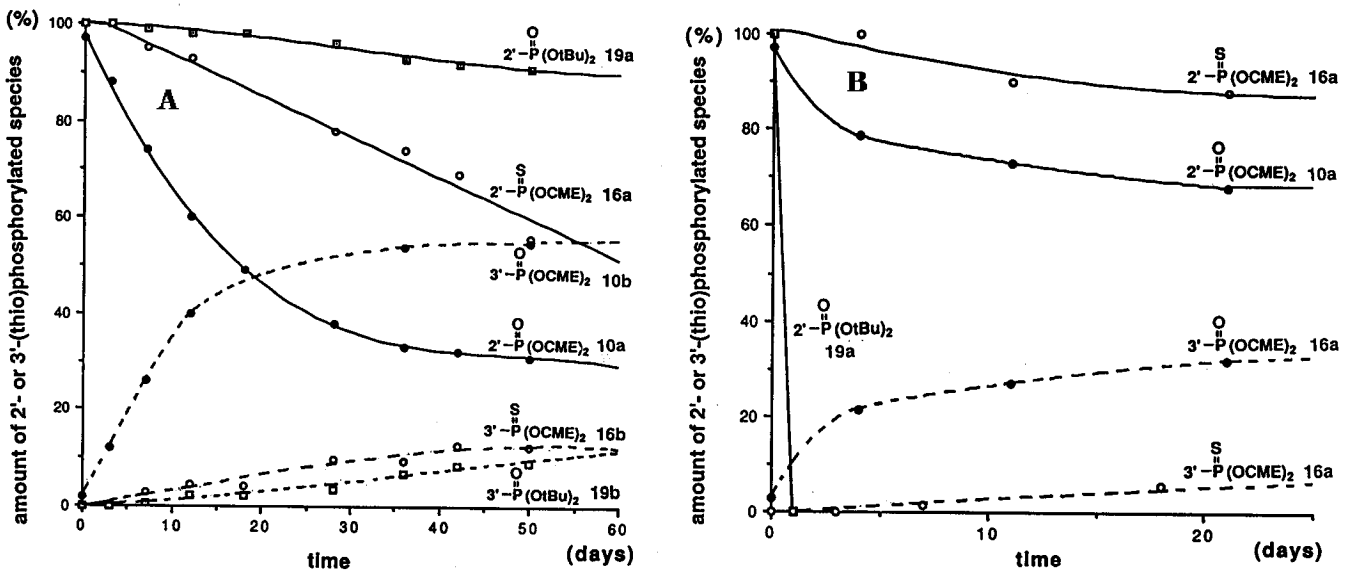


Figure 2. A: Stability of 2′-(thio)phosphorylated uridine derivatives **10a**, **16a**, and **19a** in pyridine. Each sample (0.06 mmol) was dissolved in Py−Py-d_5 (9:1, v/v, 0.6 mL), and the solution was kept at 25 °C: solid dot, **10a** and **10b**; open dot, **16a** and **16b**; open square, **19a** and **19b**; solid line, the remaining amount of the starting material **10a**, **16a**, or **19a**; dotted line, the amount of the isomerized product **10b** and **16b**. B: Stability of 2′-(thio)phosphorylated uridine derivatives **10a**, **16a**, and **19a** in 80% acetic acid. Each sample (0.06 mmol) was dissolved in acetic acid−D_2O (4:1, v/v, 0.6 mL), and the solution was kept at 25 °C: solid dot, **10a** and **10b**; open dot, **16a** and **16b**; open square, **19a** and **19b**; solid line, the remaining amount of the starting material **10a**, **16a**, or **19a**; dotted line, the amount of the isomerized product **10b**, **16b**, or **19b**.

Compound **11a** protected with the DMTr group at the 5′-position was similarly migrated to the regioisomer **11b**. There was almost no difference in the rate of the isomerization between **10a** and **11a**. On the other hand, the P(S)(OCME)$_2$ group in **16a** was so slowly migrated to the 3′-position that an equilibrium mixture of **16a** and **16b** was not obtained even after 60 days. During the dimethoxytritylation of **16a**, phosphoryl migration was expected to occur to a degree of 0.1%. This figure is negligible. Actually, the ^{31}P NMR spectrum of the 5′-*O*-dimethoxytritylated product (**21**) obtained from **16a** showed a single resonance signal at 50.82 ppm. The P(O)(O-*t*-Bu)$_2$ group in **19a** was extremely resistant to the migration, which occurred to a degree of only 5% even in 30 days.

Compound **16a** was the most stable in 80% acetic acid among these three compounds, and more than 80% of **16a** remained unchanged even after 1 month. In contrast to this, the *tert*-butyl group in **19a** rapidly decomposed. This difference is due simply to the destabilizing effect of the carbonium cation by the electron-withdrawing 2-cyanoethyl group.

A phosphoramidite unit **22** was synthesized in an overall yield of 78% from **16a** via compound **21** by a two-step reaction. The steric hindrance around the 2′-thiophosphoryl residue of **16a** did not affect phosphitylation of **21**. A phosphotriester unit **23** was obtained in 85% yield by phosphorylation with cyclohexylammonium *S*,*S*-diphenyl phosphorodithioate in the presence of DDS.[37] During these reactions, no 2′−3′ phosphoryl migration was detected. Condensation of 1.3 equiv of **22** with N^3,2′,3′-*O*-tribenzoyluridine (**24**) in the presence of 1*H*-tetrazole in acetonitrile at rt followed by oxidation with *t*-BuOOH gave the protected dimer **25** in 90% yield. Treatment of **25** with 0.1 M DBU (3 equiv) and BSA (23 equiv)[26] in pyridine at rt for 3 min resulted in quantitative formation of the silyl ester **26** with simultaneous elimination of the two CME groups and one CE group. The silyl ester **26** was hydrolyzed by addition with water to give a free acid **27**. Treatment of **27** with I_2 in pyridine−water (9:1, v/v) at rt for 40 h resulted in quantitative S → O conversion[38] to give compound **28**. It was confirmed that the S → O conversion proceeded very cleanly as evidenced by ^{31}P NMR (Figure 3), which suggests that quantitative reactions occurred at all three steps. Thus, the BCMETP group could be readily converted into a thiophosphoryl or a phosphoryl group under mild conditions which are compatible with those used in the current oligoribonucleotide synthesis.

Finally, successive treatments of **28** with concd NH_3 (rt, 18 h) and 80% acetic acid (rt, 15 min) gave the desired product **29**, which was isolated in 72% yield (Scheme 6).

The 2′-thiophosphorylated derivative **30** was also synthesized in 80% yield from **25** by the following procedure: (1) 1% trifluoroacetic acid in CH_2Cl_2 at rt for 15 min for removal of the DMTr group; (2) 0.1 M DBU−BSA in pyridine at rt for 10 min; (3) concd NH_3−pyridine (9:1, v/v) at rt for 18 h. It was observed that compound **30** is gradually air-oxidized to give **31**. The compound **30** isolated by paper chromatography always contained an appreciable amount of **31**. However, when compound **30** was isolated by HPLC and preserved at −30 °C, it was stable for several months. It is expected that compounds like **31** having a S−S linkage could be used for physicochemical studies of DNA (or RNA)−DNA (or RNA) cross-linking which can be released by reductive cleavage using DTT or $NaBH_4$. In fact, we confirmed that reaction of **31** with $NaBH_4$ gave quantitatively **30**.

Since thiophosphate monoesters allow facile S-alkylation with alkyl halides without affecting the base residues of nucleotides, the presence of such a reactive functional group would be also useful for introduction of a variety of reporter groups to RNA fragments via the 2′-position.

(37) Sekine, M.; Matsauzaki, J.; Hata, T. *Tetrahedron* **1985**, 5279.

(38) Burgers, P. M. J.; Eckstein, F. *Biochemistry* **1979**, *18*, 450.

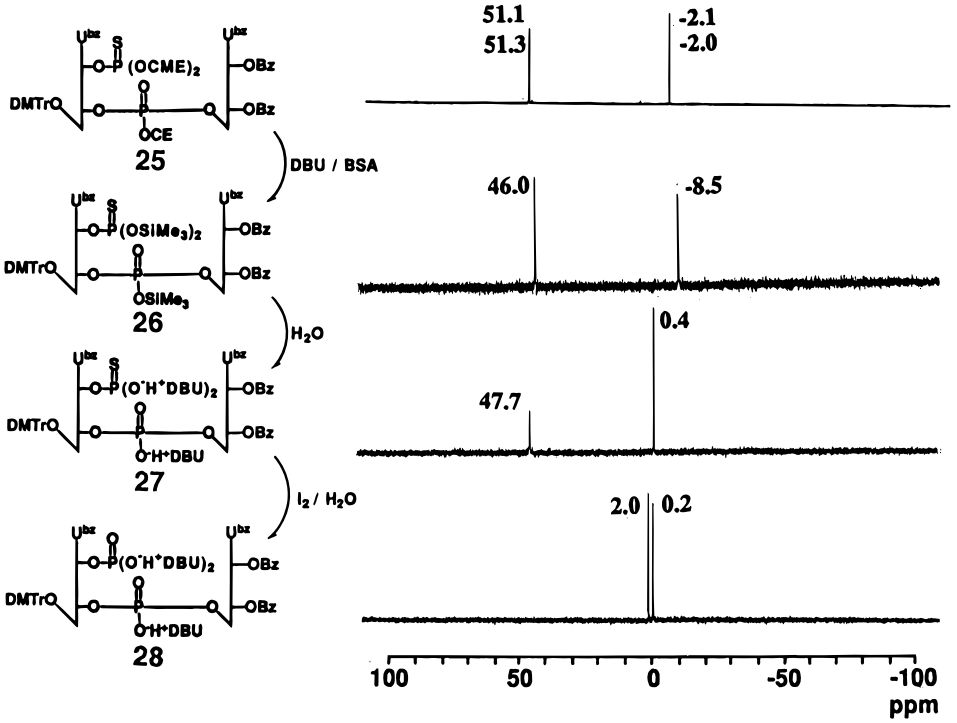
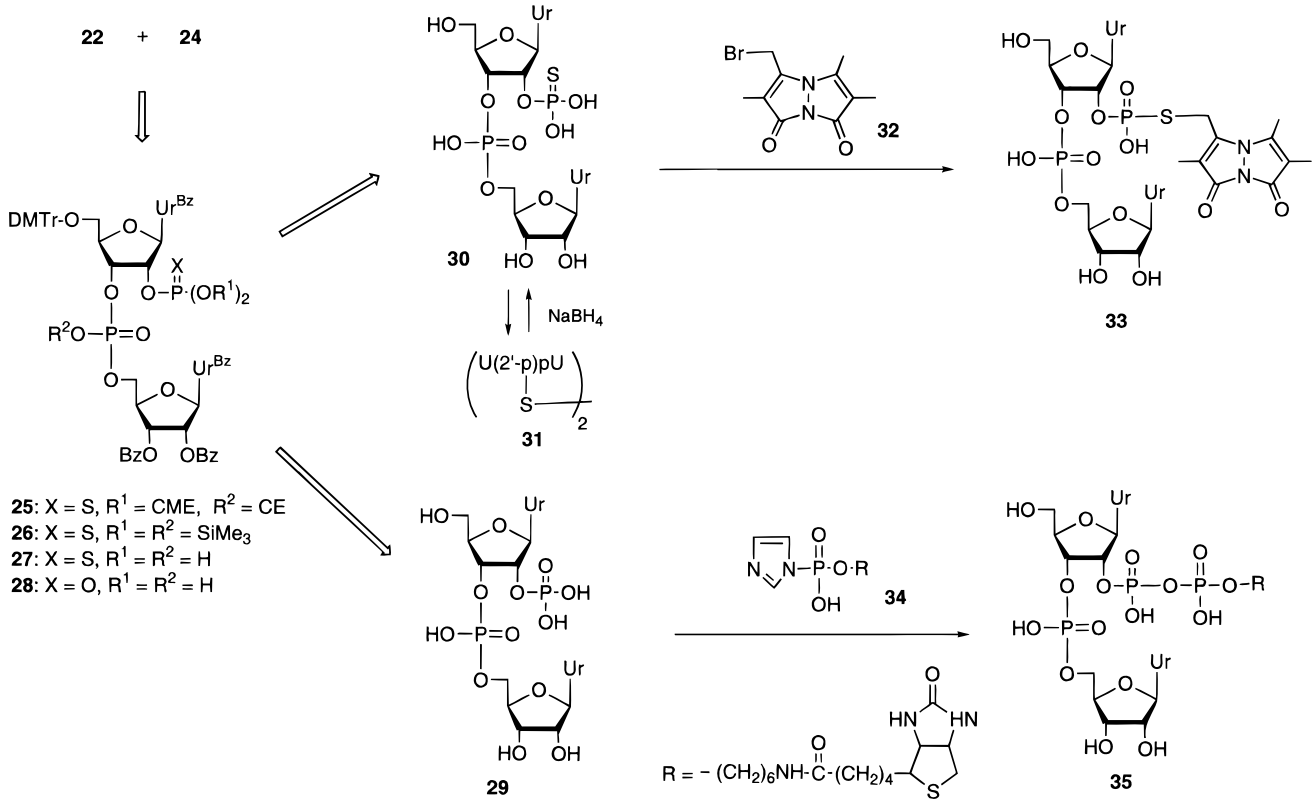


Figure 3. ^{31}P NMR spectra (Py−Py-d_5, 9:1, v/v) of the mixtures obtained at each stage by a three-step reaction for conversion of **25** to **28**.

Scheme 6



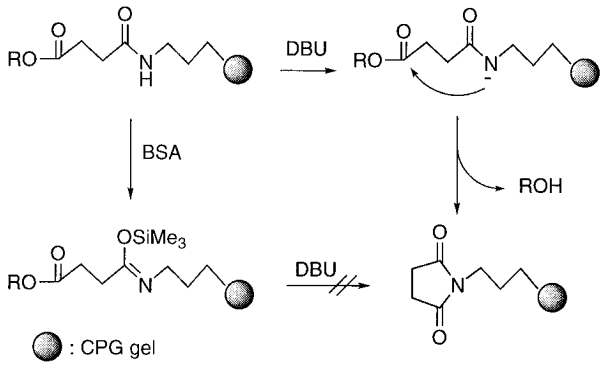
For example, a fluorescent group of bimane[39] could be easily introduced to the 2′-thiophosphoryl group via a P−S bond by treatment of **30** with monobromobimane **32** in aqueous acetonitrile to give the product **33** in 60% yield. The presence of this fluorescence group was confirmed by a fluorescence detector attached to HPLC.

We have recently developed a new method for selective biotin labeling of oligonucleotides having a 5′-monophosphate group by use of 6-(*N*-biotinylamino)hexyl phosphorimidazolidate **34**.[40] This reagent allows facile pyrophosphate bond formation with such a terminal phosphate group in an aqueous medium without affecting the base moieties and hydroxyl functions. The 2′-phosphorylated

(39) Cosstick, R.; McLaughlin, L. W.; Eckstein, F. *Nucleic Acids Res.* **1984**, *12*, 1791.

(40) Sekine, M. *et al.* Manuscript in preparation.

Scheme 7



dimer **29** could be selectively biotin-labeled at the 2′-phosphoryl group via a pyrophosphate linkage by treatment with **34** in aqueous solution in the presence of $MgCl_2$ by using Sawai's reaction[41] to give the product **35** in good yield. These new modified oligoribonucleotides would be utilized as RNA probes or antisense RNAs as well as site specific modulators for termination of reversetransriptase-mediated DNA chain elongation.[13]

Polymer-Supported Synthesis of [U(2′-p)p]$_n$U (n = 1, 3, 5, 7, and 9). Finally, we applied our new method to the solid phase synthesis of oligouridylates [U(2′-p)p]$_n$U (n = 1, 3, 5, 7, and 9). Pfleiderer[42] and Brown[43] reported that part of the oligodeoxyribonucleotide chain was eliminated from the CPG gel because of the intramolecular cyclization of the succinate linker which was catalyzed by DBU as depicted in Scheme 7. Accordingly, the succinate linker must have been hitherto avoided when DBU was used as reagent for removal of the (4-nitrophenyl)ethyl group or 2-cyanoethyl group. Indeed, they have used a sarcosine linker in place of the usual succinate to avoid such undesirable side reactions.

Therefore, it was checked to see if the 2′-phosphorylated oligouridylates were eliminated from CPG gel upon treatment with DBU/BSA as prescribed for removal of the internal CE and 2′-CME groups when the succinate linker was used. We tested two kinds of CPG gels, *i.e.*, aminopropyl CPG gel (AP-CPG) and long-chain alkylamino CPG gel (LCAA-CPG), from which DMTr(2′- or 3′-Bz)U-suc-AP-CPG and DMTr(2′- or 3′-Bz)U-suc-LCAA-CPG were prepared, respectively. To gauge the instability of the succinate linker, the amounts of DMTr(2′- or 3′-Bz) released from these CPG gels were measured by the DMTr cation assay. These results are shown in Figure 4. As reported by Pfleiderer, DMTr-U(2′- or 3′-Bz) was eliminated from both DMTr-U(2′- or 3′-Bz)-suc-AP-CPG and DMTr-U(2′- or 3′-Bz)-suc-LCAA-CPG. Interestingly, we found that DMTr(2′- or 3′-Bz)U-suc-AP-CPG was more stable than DMTr(2′- or 3′-Bz)U-suc-LCAAP-CPG when they were treated with 0.2 M of DBU in pyridine. Furthermore, elimination of DMTr(2′- or 3′-Bz)U from both resins was largely avoided when BSA was added as shown in Figure 4. Approximately 10% of DMTr-U(2′- or 3′-Bz) was lost after 24 h in the case of LCAA-CPG gel. In particular, it should be noted that DMTr(2′- or 3′-Bz)U remained almost intact in the case of DMTr(2′- or 3′-Bz)U-suc-AP-CPG even even after 100 h. From these results, we finally chose AP-CPG gel for our study.

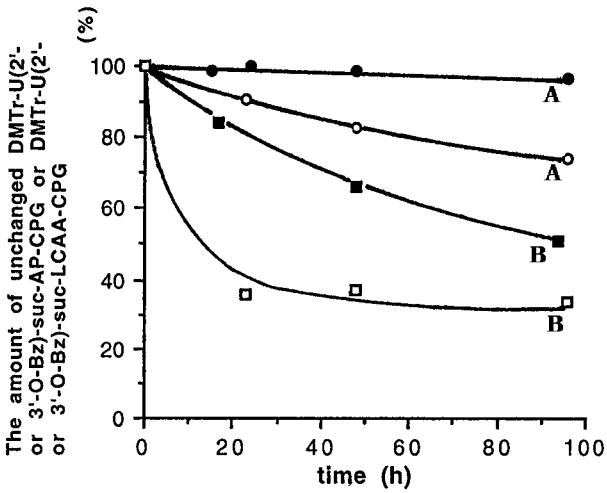


Figure 4. A: Stability of DMTr-U(2′- or 3′-Bz)-suc-AP-CPG under the conditions prescribed for removal of the CE and CME groups. Solid dot: after DMTr-U(2′- or 3′-O-Bz)-suc-AP-CPG (38.3 μmol/g, 28 mg, 1 μmol) was suspended in a solution of BSA (200 μL, 0.8 mmol) in pyridine (188 μL) at room temperature for 30 min, DBU (12 μL, 80 μmol) was added. Open dot: DMTr-U(2′- or 3′-Bz)-suc-AP-CPG (1 μmol) was suspended in pyridine (388 μL), and DBU (12 μL, 80 μmol) was added. B: Stability of DMTr-U(2′- or 3′-O-Bz)-suc-LCAA-CPG under the conditions prescribed for removal of the CE and CME groups. Solid square: after DMTr-U(2′- or 3′-Bz)-suc-LCAA-CPG (30.1 μmol/g, 38 mg, 1 μmol) was suspended in a solution of BSA (200 μL, 0.8 mmol) in pyridine (188 μL) at room temperature for 30 min, DBU (12 μL, 80 μmol) was added. Open square: DMTr-U(2′- or 3′-Bz)-suc-LCAA-CPG (1 μmol) was suspended in pyridine, (388 μL) and DBU (12 μL, 80 μmol) was added.

Table 1. Protocol of the Solid-Phase Synthesis of [U(2′-p)p]$_n$U

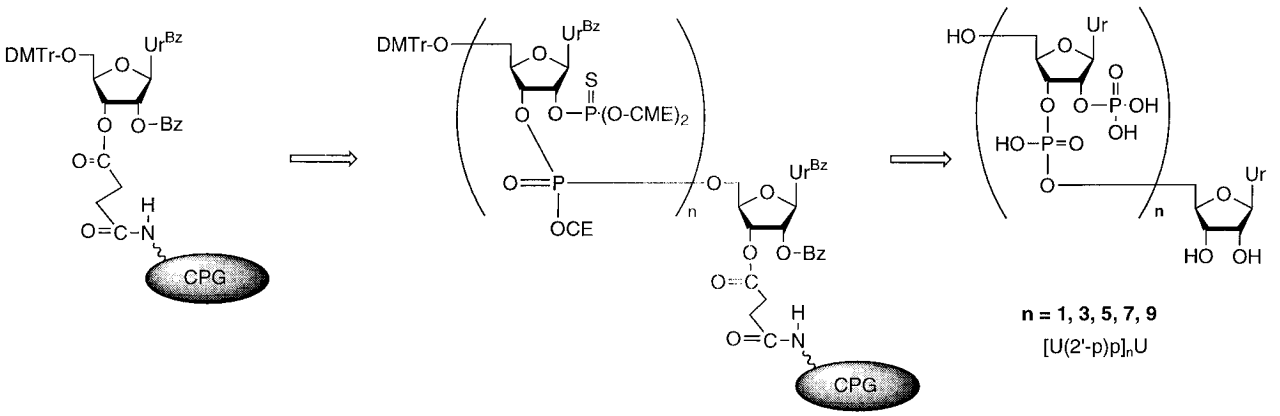
step	manipulation	
1. detritylation	1% TFA/CH_2Cl_2	
2. wash	pyridine	
3. dry up	CH_3CN	10 min
4. condensation	U-unit (0.15 M, 30 equiv), 1*H*-tetrazole (1.5 M, 300 equiv)/CH_3CN	20 min
5. wash	pyridine	
6. oxidation	0.1 M I_2/THF−Py−H_2O (10:10:1, v/v/v)	2 min
7. wash	pyridine	
8. capping	Ac_2O−Py (1:9, v/v), 0.1 M DMAP	2 min

To obtain [U(2′-p)p]$_n$U on AP-CPG gel, N^3,2′-*O*-dibenzoyl-5′-*O*-(4,4′-dimethoxytrityl)uridine was attached to AP-CPG gel via a succinate linker in the usual manner.[44] The chain elongation was performed as shown in Table 1. Since the steric effect of the bulky 2′-thiophosphoryl group was expected, the uridine unit **22** was used at a concentration of 0.15 M which is more than the usual (0.1 M). The condensation was performed for 20 min. First, the synthesis of oligouridylates U(2′-p)pU on CPG gel was tried. The coupling yield was 97% which was estimated by the DMTr cation assay (Scheme 8).

Removal of the CE and CME groups and the successive S−O conversion were carried out on CPG gel to facilitate

(41) Shimizu, M.; Shinozuka, K.; Sawai, H. *Tetrahedron Lett.* **1990**, *31*, 235.
(42) Strengele, K.; Pfleiderer, W. *Tetrahedron Lett.* **1990**, 31, 2549. Resmini, M.; Pfleiderer, W. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 1909.
(43) Brown, T.; Pritchard, C. E.; Turner, G.; Salisbury, S. A. *J. Chem. Soc., Chem. Commun.* **1989**, 891.
(44) Tanimura, H.; Maeda, M.; Fukazawa, T.; Sekine, M.; Hata, T. *Nucleic Acids Res.* **1987**, *17*, 8135.

Scheme 8



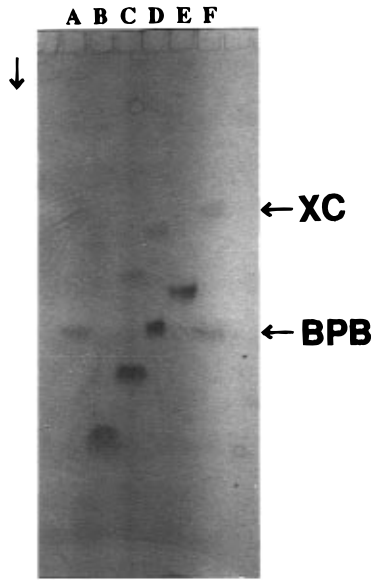


Figure 5. 20% Polyacrylamide gel electrophoresis of $[U(2'\text{-p})p]_nU$ (n = 3, 5, 7, and 9): lane A, xylene cyanole and bromophenol blue; lane B, $[[U(2'\text{-p})p]_3U$; lane C, $[U(2'\text{-p})p]_5U$; lane D, $[U(2'\text{-p})p]_7U$; lane E, $[U(2'\text{-p})p]_9U$.

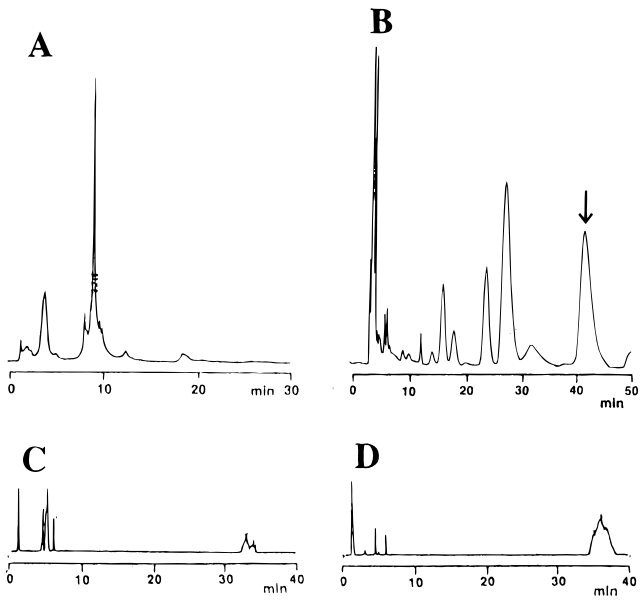


Figure 6. HPLC profiles of the mixtures obtained by polymer-supported synthesis of $[U(2'\text{-p})p]_nU$ (n = 3 (A), 5 (B), 7 (C), and 9 (D)). In the case of A, reversed-phase HPLC was carried out. In the case of B, ion-exchange HPLC using a SAX column was carried out. In the case of C and D, ion-exchange HPLC using a FAX column was performed. In panels A−D, the ordinate is UV absorbance at 254 nm.

removal of the excess reagents and the eliminated species. Treatment with concentrated ammonia was performed to release the oligomer from CPG gel as well as to remove the benzoyl and succinyl groups. At the final stage, treatment of the released product with 80% acetic acid gave U(2′-p)pU by HPLC.

The best conditions for removal of the CE and CME groups and for the S−O conversion were examined in detail by using the CPG gel having a dimer UpU sequence. As a result, successive treatments of the gel with 0.2 M of DBU and BSA in pyridine for 40 h and with iodine in aqueous pyridine for 48 h gave satisfactory results as suggested by HPLC analysis. The isolated yield of U(2′-p)pU was 75%.

On the basis of these results, the synthesis of longer oligouridylates $[U(2'\text{-p})p]_nU$ (n = 3, 5, 7, and 9) was examined on CPG gel. The average coupling yield was usually ca. 97% in each synthesis. These crude reaction mixtures obtained after deprotection were analyzed by polyacrylamide gel electrophoresis (PAGE). As seen in Figure 5, it seemed that the oligomers $[U(2'\text{-p})p]_nU$ moved as main bands in PAGE keeping a constant distance among them. To our surprise, however, more detailed HPLC analysis (Figure 6A−D) of the crude materials by reversed-phase or ion-exchange HPLC showed that some byproducts were simultaneously formed with the desired 2′-phosphorylated oligouridylates. It was possible to use reversed-phase C_{18} HPLC for isolation of 2′-phosphorylated tetrauridylate $[U(2'\text{-p})p]_3U$ as described in the case of U(2′-p)pU. In the case of $[U(2'\text{-p})p]_5U$, however, anion-exchange HPLC using a SAX column was neccesary for its better separation as shown in Figure 6B. In the case of n = 7 or 9, ion exchange HPLC using a FAX column was performed because of the increasing anion charge. As shown in Figure 6C and D, a cluster of multipeaks around the expected retention time appeared so that it was extremely difficult to isolate the desired 2′-phosphorylated products. As far as estimation of the purity of 2′-phosphorylated oligoribonucleotides is concerned, the PAGE analysis is unsuitable. The essential factor of the mobility of oligonucleotides in PAGE is not the number of the anion charges but the size of the substrates.[45] Indeed, Brownlee et al. have recently reported the difficulty in separating oligoribonucleotides having a 5′-

(45) Martin, R. *Gel Electrophoresis: Nucleic Acids*; B10S Scientific Pub. Ltd: Oxford, 1996.

terminal diphosphate from oligoribonucleotides having a 5′-terminal monophosphate by PAGE.[46]

We also observed that, as the chain becomes long, the retention time of ion exchange HPLC is considerably prolonged. In contrast, these polyanion charged oligouridylates were not retained on reversed-phase HPLC columns. In the case of the synthesis of $[U(2'\text{-}p)p]_3U$ and $[U(2'\text{-}p)p]_5U$, these compounds were fortunately obtained as the main products as shown in Figure 6A,B. The isolated yields of $[U(2'\text{-}p)p]_3U$ and $[U(2'\text{-}p)p]_5U$ were 19% and 12%, respectively, from the fully protected 2′-phosphorylated uridylates bound to CPG gel. The structure of this highly negative-charged oligouridylates was confirmed by enzymatic assay as described in a later section.

Our finding strongly suggests that BSA reacted with the amide group of the succinyl linker to give a O-$SiMe_3$ derivative which lost the NH proton responsible for the side reaction as depicted in Scheme 7. This also implies that the more accessible succinate linker can be used even in the usual oligonucleotide synthesis using (4-nitrophenyl)ethyl groups if silylating agents such as BSA are present.

Enzyme Assay of 2′-Phosphorylated Oligouridylates. U(2′-p)pU was digested with snake venom phosphodiesterase to give U(2′-p) and pU while this dimer was resistant to nuclease P1 and spleen phosphodiesterase. In the enzymatic internucleotide bond cleavage of U(2′-p)pU with snake venom phosphodiesterase, the peak corresponding to uridine was observed as a minor product. This reaction required a relative excess of this enzyme compared with the usual internucleotidic bond hydrolysis. Accordingly, it is likely that the appearance of uridine is due to the phosphatase acitivity contaminated in snake venom phosphodiesterase. Treatment of U(2′-p)pU with calf intestinal alkaline phosphatase (CIAP) gave the usual UpU dimer, which was digestable with nuclease P1 to give U and pU. These enzymatic properties are consistent with those of pG(2′-p)pA reported previously.[4] In the case of $[U(2'\text{-}p)p]_3U$ and $[U(2'\text{-}p)p]_5U$, dephosphorylation also occurred upon treatment with CIAP. The stability of the 2′-phosphoryl group to CIAP increased with an increase in length of the oligomer. Contrary to these results, the 2′-thiophosphorylated uridine dimer U(2′-ps)pU **30** was found to be completely resistant to CIAP.

Conclusion

Apart from the synthesis of 2′-phosphorylated RNAs, the present strategy would provide a new promising tool for the regioselective phosphorylation (thiophosphorylation) of not only ribonucleosides but also the neighboring OH-containing phosphate compounds such as and *myo*-inositol 1,4,5-triphosphates and phospholipids where similar problems of phosphoryl isomerization have been encountered.[47] Quite recently, Heeb[48] reported that the bis(2-cyanoethoxy)thiophosphoryl [$P(S)(OCE)_2$] group remained almost intact upon acid-catalyzed removal of the isopropylidene group from 1,2-*O*-isopropylidene-3-*O*-[bis-(2-cyanoethoxy)thiophosphoryl]glycerin. As mentioned before, compound **18a** having a $P(S)(OCE)_2$ group is rapidly isomerized to the 3′-regioisomer **18b** even under neutral conditions. Therefore, it is expected that the use of our $P(S)(OCME)_2$ group in phospholipids would result in complete avoidance of the phosphoryl migration. These results imply that thiophosphorylated species of acyclic 1,2-diols do not migrate to the proximal hydroxyl group compared with those of ribonucleoside derivatives which have more rigid conformations due to the presence of a five membered ring.

Now we have achieved an approach to the solid phase synthesis of 2′-phosphorylated oligouridylates. However, we have encountered certain limitations to the present method because of the considerable increase in relative amounts of byproducts which comigrate with the desired oligomers in PAGE. The HPLC analysis indicates that the present method is limited to the synthesis of 2′-phosphorylated oligoribonucleotides up to the level of six mers. This limitation is due essentially to the multiple complexity with the increasing number of the BCMETP group involved in the synthetic intermediate, although at the dimer level the amount of such byproducts is not significant.

The synthesis of oligoribonucleotides or oligodeoxyribonucleotides having a 2′-phosphate group at the specific positions in the sequence is also of interest. Such phosphate-bearing oligonucleotides would be utilized in antisense DNA/RNA chemistry as described before. These one-point modified oligomers would be more easily prepared by a combined use of the phosphoramidite unit **22** and the commercially available standard phosphoramidite units,[49] since the conditions prescribed for removal of the CME and the subsequent S−O conversion are compatible with the 2′-TBDMS group which is used in the usual phosphoramidite units.

Further study on the chemical synthesis of 2′-(thio)-phosphate-containing oligonucleotides using four kinds of synthetic units is now in progress. Our preliminary results suggest that all four phosphoramidite units can be constructed in a similar manner and a partial structure of 2′-phosphorylated RNA species can be synthesized by the present strategy.[49] The chemical, physicochemical, and biochemical properties of mono- and multi-2′-(thio)-phosphorylated oligoribonucleotides will be reported in the near future.

Experimental Section

General Experimental Procedures. Melting points were determined and are uncorrected. ^1H NMR spectra were recorded at 270 and 500 MHz with Me_4Si (for water-insoluble materials) or DSS (for water-soluble materials) as the external reference. ^{13}C NMR spectra were measured at 67.8 MHz with TMS as the internal standard. Paper chromatography was performed by use of a descending technique with Whatman 3MM papers and Toyo Roshi 51 papers using the following solvent system: 2-propanol−concd aqueous ammonia−water, 6:1:3, 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. Reversed-phase column chromatography was performed by the use of μBondapak C-18 silica gel (Prep S-500, Waters). TLC was performed on precoated TLC plates of silica gel 60 F-254 (Merck). Reversed-phase HPLC was performed using a μBondasphere C-18 column with a linear gradient starting from 0.1 M NH_4OAc, pH 7.0 and applying CH_3CN at a flow rate of 1.0 mL/min for 30 min. Ion-exchange high-performance liquid chromatography (IE HPLC) was carried out at a flow rate of 1mL/min at 50 °C on a Whatman PARTISIL 10 SAX WCS

(46) Brownlee, G. G.; Foder, E.; Pritlove, D. C.; Ground, K. G.; Dalluge, J. J. *Nucleic Acids Res.* **1995**, *23*, 2641.

(47) Watanabe, Y.; Ogasawara, T.; Nakahira, H.; Matsuki, T.; Ozaki, S. *Tetrahedron Lett.* **1988**, *29*, 5259 and references cited therein.

(48) Heeb, N. V.; Nambiar, K. P. *Tetrahedron Lett.* **1993**, *34*, 6193.

(49) Sekine, M. *et al.* Manuscript in preparation.

analytical column (4.6 × 250 mm) or a Waters Gen-Pak FAX column (4.6 × 100 mm). Analysis by SAX-HPLC was performed using a linear gradient of 0−100% solution B (20% CH_3CN in 0.005 M KH_2PO_4) in solution A (20% CH_3CN in 0.005 M KH_2PO_4) for 20 min followed by maintaining the volume in 100% solution B for 20 min. In the case of FAX-HPLC analysis, elution buffer by using 10−77% linear gradient of solution C (1 M NaCl, 25 mM phosphate buffer, pH 6.0) in solution D (25 mM phosphate buffer, pH 6.0) for 50 min was employed. Uridine was purchased from Yamasa Co., Ltd. Pyridine was distilled two times from *p*-toluenesulfonyl chloride and from calcium hydride and then stored over molecular sieves 4A. Elemental analyses were performed by the Microanalytical Laboratory, Tokyo Institute of Technology, at Nagatsuta.

2-Chloro-1,1-dimethylethanol. 3-Chloro-2-methylpropene (315 mL, 3 mol) was cooled at 0 °C, and 80% H_2SO_4 (230 mL), which was prepared in advance by addition of concentrated H_2SO_4 (168 mL) to water (62 mL), was added. The mixture was stirred at room temperature for 2.5 h. The orange viscous solution was diluted with water (1.3 L) cooled at 0 °C. The mixture was steam distilled to give a mixture containing the product, which was extracted with ether (500 mL × 3). The extract was dried over Na_2SO_4, filtered, and concentrated under reduced pressure. Distillation gave the title compound (145 g, 44%): bp 125−127 °C [lit.[50] 126.7 °C]; ^{1}H NMR (270 MHz, $CDCl_3$, TMS) δ 1.35 (6H, s, CH_3), 2.28 (1H, s, OH), 3.54 (2H, s, CH_2); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 26.29, 54.65, 70.01.

2-Cyano-1,1-dimethylethanol. 2-Chloro-1,1-dimethylethanol (31 mL, 0.3 mol) was dissolved in ethanol (688 mL) and water (112 mL) and sodium cyanide (17.6 g, 0.36 mol). The mixture was refluxed for 1 h. The brownish solution was concentrated under reduced pressure and saturated NaCl (150 mL) and water (500 mL) were added. The residue was extracted with ether (500 mL × 5). The ether extracts were combined, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was distilled to give the title compound (15.4 g, 51%): bp 118 °C/38 mmHg (lit.[49] 114 °C/30 mmHg); ^{1}H NMR (270 MHz, $CDCl_3$, TMS) δ 1.39 (6H, s, CH_3), 2.33 (1H, s, OH), 2.52 (2H, s, CH_2); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 28.88, 32.65, 69.13, 117.70.

***N*^{3}-Benzoyl-3′,5′-*O*-(di-*tert*-butylsilanediyl)uridine (7).** N^3-Benzoyluridine (1.04 g, 3 mmol) was rendered anhydrous by repeated coevaporation with dry pyridine (10 mL × 3) and toluene (10 mL × 3) and finally dissolved in dimethylformamide (30 mL). To the mixture were added silver nitrate (1.33 g, 7.8 mmol) and di-*tert*-butyldichlorosilane (0.824 mL, 3.9 mmol). The resulting mixture was stirred vigorously at room temperature for 30 min. The mixture was quenched at 0 °C by addition of triethylamine (1.25 mL, 9 mmol), and the solvent was removed under reduced pressure. 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 **7** (1.39 g, 95%): ^{1}H NMR (270 MHz, $CDCl_3$, TMS) δ 1.00−1.08 (18H, m, CH_3), 4.00−4.03 (2H, m, 5′-H and 5″-H), 4.11−4.18 (1H, m, 4′-H), 4.43 (1H, d, $J_{2'H-3'H}$ = 4.3 Hz, 3′-H), 4.47 (1H, d, $J_{2'H-3'H}$ = 4.3 Hz, 2′-H), 5.61 (1H, s, 1′-H), 5.86 (1H, d, J_{5H-6H} = 8.3 Hz, 5-H), 7.32 (1H, d, J_{5H-6H} = 8.3 Hz, 6-H), 7.48−7.53 (2H, m, *m*-ArH), 7.64−7.69 (1H, m, *p*-ArH), 7.91−7.95 (2H, m, *o*-ArH); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 20.18, 22.45, 27.01, 27.15, 66.94, 73.16, 74.50, 75.65, 93.96, 102.19, 129.06, 130.35, 131.03, 135.17, 140.81, 148.63, 161.87, 168.28. Anal. Calcd for $C_{17}H_{26}N_2O_6Si$: C, 41.79; H, 5.36; N, 5.73. Found: C, 41.85; H, 5.01; N, 5.42.

***N*^{3}-Benzoyl-2′-*O*-(di-*tert*-butoxyphosphoryl)uridine (19a). Method A.** A mixture of N^3-benzoyl-3′,5′-*O*-(di-*tert*-butylsilanediyl)uridine (**7**) (348 mg, 0.71 mmol) and 1*H*-tetrazole (140 mg, 2.0 mmol) was rendered anhydrous by repeated coevaporation with dry pyridine (5 mL × 5), dry toluene (5 mL × 2), and CH_2Cl_2 (5 mL × 2) and was dissolved in CH_2Cl_2 (10 mL). To the mixture was added di-*tert*-butyl *N*,*N*-diethylphosphoramidite (1.1 mL, 4.5 mmol). After the mixture was stirred at room temperature for 3 h, *tert*-butyl hydroperoxide (about 80% solution in *t*-BuOO-*t*-Bu, 1.5 mL, 1.5 mmol) was added. The solution was stirred at room temperature for 15 min, diluted with CH_2Cl_2, washed twice with 5% $NaHCO_3$ and with water, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue containing compound **20** was dissolved in THF (4 mL), and a mixture of (HF)$_x$·Py (0.519 mL)−Py (2.9 mL) was added. The mixture was stirred at room temperature for 10 min and then partitioned between CH_2Cl_2 and 5% $NaHCO_3$. The organic phase was collected, washed with 5% $NaHCO_3$ and water, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was chromatographed on a column of silica gel (12 g) with CH_2Cl_2−MeOH containing 0.5% pyridine to give **19a** (172 mg, 56%): mp 116 °C dec from CH_2Cl_2−toluene (9:1, v/v); ^{1}H NMR (270 MHz, $CDCl_3$, TMS) δ 1.47(18H, m, CH_3 of *t*-Bu), 3.71 (1H, dd, $J_{4'H-5''H}$ = 2.3 Hz, $J_{5'H-5''H}$ = 12.2 Hz, 5″-H), 3.95 (1H, dd, $J_{4'H-5''H}$ = 2.3 Hz, $J_{5'H-5''H}$ = 12.2 Hz, 5′-H), 4.20 (1H, m, 4′-H), 4.46 (1H, dd, $J_{2'H-3'H}$ = 3.5 Hz, $J_{3'H-4'H}$ = 5.0 Hz, 3′-H), 4.92 (1H, m, $J_{1'H-2'H}$ = 5.9 Hz, $J_{2'H-3'H}$ = 3.5 Hz, 2′-H), 5.87 (1H, d, J_{5H-6H} = 7.9 Hz, 5-H), 5.95 (1H, d, $J_{1'H-2'H}$ = 5.9 Hz, 1′-H), 7.46−7.52 (2H, m, ArH), 7.62−7.67 (1H, m, ArH), 7.70 (1H, d, 6-H), 7.96−7.93 (2H, m, ArH); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 29.49, 29.53, 29.58, 61.42, 70.01, 76.52, 76.98, 77.47, 78.20, 84.57, 84.69, 84.80, 84.92, 85.37, 87.62, 87.69, 87.78, 87.84, 102.50, 129.00, 130.37, 131.18, 135.02, 140.97, 149.20, 162.14, 168.37; ^{31}P NMR (109 MHz, $CDCl_3$, 85% H_3PO_4) δ −8.97. Anal. Calcd for $C_{24}H_{33}N_2O_{10}P$·1/2H_2O: C,52.46; H, 6.24; N, 5.10. Found: C, 52.70; H, 6.17; N, 4.99.

Method B. BDCP[36] (7.0 g, 6.4 mmol) was dissolved in dry pyridine (40 mL), and di-*t*-butyl phosphonate (0.956 mL, 4.8 mmol) was added. The mixture was stirred at room temperature for 15 min and then was added to N^3-benzoyl-3′,5′-*O*-(di-*tert*-butylsilanediyl)uridine (**7**) (1.95 g, 4.0 mmol) which was rendered anhydrous by repeated coevaporation with dry pyridine (20 mL × 5). The resulting solution was stirred at room temperature for 1 h, and then *tert*-butyl hydroperoxide (2 mL, 20 mmol) was added. After being stirred at room temperature for an additional 1 h, the mixture was diluted with CH_2Cl_2, washed with 5% $NaHCO_3$ and water, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was dissolved in THF (16 mL), and a mixture of (HF)$_x$·Py (2.1 mL)−Py (10.3 mL) was added. The mixture was stirred at room temperature for 15 min. A workup similar to that described above gave **19a** (1.26 g, 58%).

2′-*O*-Bis[(2-cyano-1,1-dimethylethoxy)phosphoryl]-*N*^{3}-benzoyluridine (10a). First, the phosphitylating reagent of bis(2-cyano-1,1-dimethylethyl) *N*,*N*-diethylphosphoramidite was prepared as follows: To a solution of (diethylamino)dichlorophosphine (4.13 mL, 26.3 mmol) in dry ether (18 mL) was added dropwise a mixture of 2-cyano-1,1-dimethylethanol (5.12 mL, 50 mmol) and triethylamine (6.94 mL, 50 mmol) in dry ether (12.5 mL) with cooling at 0 °C from −10 °C over a period of 2 h. The solution was warmed to room temperature and stirred for an additional 3.5 h. The white precipitate was removed by filtration and washed with ether. The filtrate and washing were combined and concentrated under reduced pressure. The residue was extracted with ether and 5% $NaHCO_3$. The organic extract was dried over Na_2SO_4 and filtrated. The solution was concentrated in vacuo. The purity of the residue containing the phosphitylating reagent was estimated by measurement of its ^{31}P NMR spectrum (135.72 ppm in $CDCl_3$), which showed that the purity varied usually from 50−70%. Then, this residue was diluted by addition of CH_2Cl_2 so as to obtain a 0.3−0.5 M solution of the reagent, which was stocked in the presence of molecular sieves 3A in a refrigerator at −20 °C. N^3-Benzoyl-3′,5′-*O*-(di-*tert*-butylsilanediyl)uridine (**7**) (488 mg, 1.0 mmol) and 1*H*-tetrazole (126 mg, 1.8 mmol) were rendered anhydrous by repeated coevaporation with dry pyridine (5 mL × 5), dry toluene (5 mL × 2), and CH_2Cl_2 (5 mL × 2) and dissolved in CH_2Cl_2 (7.5 mL). To the mixture was added bis(2-cyano-1,1-dimethylethyl) *N*,*N*-diethylphosphoramidite (purity 58%, 2.4 mL, 1.2 mmol). After the mixture was stirred at room temperature for 2 h, *tert*-butyl hydroperoxide (1 mL, 10 mmol) was added. The solution was

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stirred at room temperature for 10 min, diluted with CH_2Cl_2, 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−AcOH (1:1, v/v, 4 mL), and a mixture of $(HF)_x$·Py (0.52 mL)−Py (2.9 mL) was added. The mixture was stirred at room temperature for 10 min, and then an excess amount of pyridine (5 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_2SO_4, filtered, and evaporated under reduced pressure. The residue was chromatographed on a column of silica gel (18 g) with CH_2Cl_2−MeOH containing 0.5% pyridine to give **10a** (purity 97%, 269 mg, 46%). When the solvent for removal of the DTBS group was changed from THF−AcOH to THF, **10a** was obtained in 72% yield with a purity of 95%. **10a**: ^{1}H NMR (270 MHz, $CDCl_3$, TMS) δ 1.59−1.69 (12H, m, CH_3 of CME), 2.63−3.05 (4H, m, CH_2 of CME), 3.87 (2H, s, 5′-H and 5″-H), 4.25 (1H, d, $J_{3'H-4H}$ = 1.65 Hz, 4′-H), 4.58 (1H, dd, $J_{3'H-4''H}$ = 1.65 Hz, $J_{2'H-3'H}$ = 4.62, 3′-H), 4.91 (1H, m, 2′-H), 5.91 (1H, d, J_{5H-6H} = 8.25 Hz, 5-H), 6.27 (1H, d, $J_{1'H-2'H}$ = 6.93 Hz, 1′-H), 7.47−7.52 (2H, m, *m*-ArH), 7.62−7.68 (1H, m, *p*-ArH), 7.97−7.98 (2H, m, *o*-ArH), 8.06 (1H, d, J_{5H-6H} = 8.25 Hz, 6-H); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 26.99, 27.03, 27.15, 27.21, 27.30, 27.37, 31.25, 31.32, 31.43, 31.50, 61.24, 69.88, 69.92, 79.01, 79.08, 81.82, 81.92, 85.07, 87.03, 87.15, 102.59, 116.78, 116.93, 129.06, 130.48, 131.11, 135.17, 140.59, 149.33, 162.16, 168.70; ^{31}P NMR (109 MHz, $CDCl_3$, 85% H_3PO_4) δ −9.51. Anal. Calcd for $C_{26}H_{31}N_4O_{10}P$·1/2H_2O: C, 52.09; H, 5.38; N, 9.34. Found: C, 51.95; H, 5.34; N, 9.17.

3′-*O*-[Bis(2-cyano-1,1-dimethylethoxy)thiophosphoryl]-5′-*O*-(4,4′-dimethoxytrityl)thymidine (13). A mixture of 5′-*O*-(4,4′-dimethoxytrityl)thymidine (545 mg, 1.0 mmol) and 1*H*-tetrazole (158 mg, 2.25 mmol) was dried by repeated coevaporation with dry pyridine (2 mL × 3), toluene (2 mL × 2), and CH_2Cl_2 (2 mL × 1) and finally dissolved in CH_2Cl_2 (6 mL). To the solution was added a 0.5 M solution of bis(2-cyano-1,1-dimethylethyl) *N*,*N*-diethylphosphoramidite in CH_2Cl_2 (purity 58%, 4 mL, 1.5 mmol) prepared above. After being stirred for 50 min, the mixture was treated with elemental sulfur (S_8) (1.78 g, 6 mmol) for another 12 h. Then, it was extracted by the use of CH_2Cl_2/H_2O. The organic layer was dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The crude material was purified by chromatography on a silica gel column eluted with CH_2Cl_2−CH_3OH containing 0.5% pyridine to give **13** (441 mg, 55%): ^{1}H NMR (270 MHz, $CDCl_3$, TMS) δ 1.43−1.71 (15H, m, CH_3 of CME and Th), 2.46−2.73 (2H, m, 2′-H and 2″-H), 2.79−3.07 (4H, m, CH_2 of CME), 3.45 (2H, d, $J_{4'H-5'H}$ = 2.31 Hz, 5′-H and 5″-H), 4.26 (1H, d, $J_{4'H-5'H}$ = 2.31 Hz, 4′-H), 5.42 (1H, m, 3′-H), 6.30 (1H, m, 1′-H), 6.83−6.88 (4H, m, ArH), 7.23−7.42 (9H, m, ArH), 7.57 (1H, s, 6-H), 8.50 (1H, br, NH); ^{13}C NMR (67.8 MHz, $CDCl_3$, TMS) δ 163.7, 158.65, 150.46, 144.04, 135.20, 135.04, 130.03, 128.12, 128.05, 127.96, 127.08, 116.53, 116.39, 113.26, 111.57, 97.08, 84.42, 84.33, 84.30, 82.41, 82.21, 79.12, 63.29, 55.17, 38.92, 38.85, 31.50, 31.43, 31.39, 31.34, 27.42, 27.39, 27.32, 27.13, 27.08, 11.61; ^{31}P NMR (109 MHz, $CDCl_3$, 85% H_3PO_4) δ 49.19; Anal. Calcd for $C_{41}H_{47}N_4O_9PS$·1/2H_2O: C, 60.66; H, 5.94; N, 6.89. Found: C, 60.40; H, 6.20; N, 7.33.

2′-*O*-[Bis(2-cyano-1,1-dimethylethoxy)thiophosphoryl]-*N*^{3}-benzoyluridine (16a). N^3-Benzoyl-3′,5′-*O*-(di-*tert*-butylsilanediyl)uridine (**7**) (1.02 g, 2.08 mmol) and 1*H*-tetrazole (328 mg, 4.68 mmol) were rendered anhydrous by repeated coevaporation with dry pyridine (5 mL × 5), dry toluene (5 mL × 2), and CH_2Cl_2 (5 mL × 2) and dissolved in CH_2Cl_2 (7.5 mL). 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 45 min, a 1 M solution of S_8 in CS_2 (20 mL) was added. The solution was stirred at room temperature for 2 h and then evaporated under reduced pressure. The residue was dissolved in CH_2Cl_2 (300 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 (8 mL), and a mixture of $(HF)_x$·Py (1.2 mL)−Py (5.4 mL) was added. The mixture was stirred at room temperature for 10 min, and then a excess amount of pyridine (10 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_2SO_4, filtered, and evaporated under reduced pressure. The residue was chromatographed on a column of silica gel (40 g) with CH_2Cl_2−MeOH containing 0.5% pyridine to give **16a** (864 mg, 69%): ^{1}H NMR (270 MHz, $CDCl_3$, TMS) δ 1.60−1.74 (12H, m, CH_3 of CME), 2.63−2.97 (4H, m, CH_2 of CME), 3.86 (2H, s, 5′-H and 5″-H), 4.26 (1H, s, 4′-H), 4.58 (1H, d, $J_{2'H-3'H}$ = 4.9 Hz, 3′-H), 5.13 (1H, ddd, $J_{1'H-2'H}$ = 6.9 Hz, $J_{2'H-3'H}$ = 4.9 Hz, $J_{2'H-P}$ = 11.5 Hz, 2′-H), 5.92 (1H, d, J_{5H-6H} = 8.3 Hz, 5-H), 6.30 (1H, d, $J_{1'H-2'H}$ = 6.9 Hz, 1′-H), 7.47−7.52 (2H, m, *m*-ArH), 7.62−7.68 (1H, m, *p*-ArH), 7.96−7.99 (2H, m, *o*-ArH), 8.10 (1H, d, J_{5H-6H} = 8.3 Hz, 6-H); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 26.88, 26.92, 27.35, 27.41, 27.48, 27.55, 28.36, 28.45, 31.16, 31.41, 31.47, 62.50, 71.45, 79.03, 79.10, 82.50, 82.62, 83.60, 83.72, 86.00, 86.11, 86.25, 103.22, 116.86, 117.27, 129.08, 130.64, 131.38, 135.04, 140.54, 149.54, 162.05, 168.55; ^{31}P NMR (109 MHz, $CDCl_3$, 85% H_3PO_4) δ 50.21. Anal. Calcd for $C_{26}H_{31}N_4O_9PS$·1/2H_2O: C, 50.73; H, 5.24; N, 9.10. Found: C, 50.71; H, 5.28; N, 9.01.

2′-*O*-[Bis(2-cyano-1,1-dimethylethoxy)thiophosphoryl]-*N*^{3}-benzoyl-5′-*O*-(4,4′-dimethoxytrityl)uridine (21). Compound **16a** (2.1 g, 3.5 mmol) was rendered anhydrous by repeated coevaporation with dry pyridine (20 mL × 3) and finally dissolved in dry pyridine (35 mL). To the mixture was added 4,4′-dimethoxytrityl chloride (1.78 g, 5.25 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 **21** (2.94g, 93%); ^{1}H NMR (270 MHz, $CDCl_3$, TMS) δ 1.61−1.75 (12H, m, CH_2 of CME), 2.72−2.92 (4H, m, CH_2 of CME), 3.41 (1H, dd, $J_{4'H-5''H}$ = 2.3 Hz, $J_{5'H-5''H}$ = 11.2 Hz, 5″-H), 3.52 (1H, dd, $J_{4'H-5'H}$ = 2.3 Hz, $J_{5'H-5''H}$ = 11.2 Hz, 5′-H), 4.25 (1H, d, $J_{4'H-5''H}$ = 2.3 Hz, 4′-H), 4.80 (1H, dd, $J_{2'H-3'H}$ = 4.6 Hz, $J_{3'H-4'H}$ = 2.0 Hz, 3′-H), 5.31−5.41 (2H, m, $J_{1'H-2'H}$ = 6.3 Hz, $J_{2'H-3'H}$ = 4.6 Hz, J_{5H-6H} = 8.2 Hz, 2′-H and 5-H), 6.30 (1H, d, $J_{1'H-2'H}$ = 6.3 Hz, 1′-H), 6.84−6.90 (4H, m, ArH), 7.14−7.67 12H, m, ArH), 7.93−8.02 (3H, m, ArH and 6-H); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 26.99, 27.39, 27.44, 27.51, 27.57, 31.47, 31.54, 31.88, 31.95, 55.20, 63.14, 70.65, 79.59, 79.66, 82.79, 82.89, 83.77, 83.90, 84.48, 85.66, 85.81, 87.51, 102.62, 113.42, 116.75, 127.17, 128.01, 128.14, 128.97, 129.06, 130.03, 130.12, 130.57, 131.30, 134.70, 135.04, 140.16, 144.02, 149.27, 158.69, 158.72, 161.70, 168.50; ^{31}P NMR (109 MHz, $CDCl_3$, 85% H_3PO_4) δ 50.82. Anal. Calcd for $C_{47}H_{49}N_4O_{11}PS$: C, 62.11; H, 5.43; N, 6.16. Found: C, 61.61; H, 5.46; N, 5.86.

2′-*O*-[Bis(2-cyano-1,1-dimethylethoxy)thiophosphoryl]-*N*^{3}-benzoyl-5′-*O*-(4,4′-dimethoxytrityl)uridine 3′-(2-Cyanoethyl-*N*,*N*-diisopropylphosphoramidite) (22). Compound **21** (2.27 g, 2.5 mmol) was rendered anhydrous by successive coevaporations with dry pyridine (20 mL × 3) and dry toluene (20 mL × 2) and finally dissolved in dry CH_2Cl_2 (25 mL). To the mixture were added triethylamine (1.39 mL, 10 mmol) and 2-cyanoethoxy (*N*,*N*-diisopropylamino)phosphorochloridite (1.09 mL, 5.0 mmol). The resulting mixture was stirred for 1 h and then extracted with CH_2Cl_2. The CH_2Cl_2 extract was washed with 5% $NaHCO_3$ and water. The usual workup followed by silica gel column chromatography eluted with hexane−ethyl acetate containing 0.5% pyridine gave **22** (2.34 g, 84%): ^{1}H NMR (270 MHz, $CDCl_3$, TMS) δ 1.07−1.76 (26H, m, CH_3 of CME and *i*-Pr), 2.40−3.05 (6H, m, CH_2CN of CE and CME), 3.48−3.97 (4H, m, CH of iPr, and 5′-H and 5″-H), 3.80 (6H, s, OCH_3 of DMTr), 4.28−4.35 (1H, m, 4′-H), 4.75−4.84 (3H, m, 3′-H), 5.34−5.51 (3H, m, $POCH_2$ of CN, 2′-H), 6.27−6.46 (1H, m, 1′-H), 6.85−6.91 (4H, m, ArH), 7.16−7.68 (13H, m, 6-H and ArH), 7.96−8.04 (2H, m, ArH); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 20.11, 20.36, 20.45, 24.49, 24.58, 24.71, 24.84, 26.97, 27.05, 27.33, 27.39, 27.51, 27.62, 27.68, 27.73, 31.48, 31.57, 31.63, 31.68, 43.13, 43.16, 43.36, 55.26, 63.31, 82.39, 82.46, 82.57, 83.56, 83.69, 84.46, 85.23, 85.30, 86.11, 87.69, 87.75, 102.66, 113.46, 116.50, 116.59, 117.07, 117.38, 117.83, 127.24, 128.07, 128.19, 129.06, 130.10, 130.15, 130.40, 130.66, 130.75, 130.80, 131.41, 134.81, 149.45, 158.78, 162.01, 168.55; ^{31}P NMR (109 MHz, $CDCl_3$, 85% H_3PO_4) δ 153.26, 151.29, 51.55, 51.21. Anal. Calcd for $C_{56}H_{66}N_6O_{12}P_2S$: C,

60.64; H, 6.00; N, 7.57; S, 2.89. Found: C, 60.91; H, 6.38; N, 7.50; S, 2.63.

***S,S*-Diphenyl 2′-*O*-[Bis(2-cyano-1,1-dimethylethoxy)-thiophosphoryl]-*N*^{3}-benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-uridine 3′-Phosphorodithioate (23).** A mixture of **21** (943 mg, 1.04 mmol), cyclohexylammonium *S,S*-diphenyl phosphorodithioate (633 mg, 1.66 mmol), and 1*H*-tetrazole (291 mg, 4.16 mmol) was rendered anhydrous by repeated coevaporation with dry pyridine (5 mL × 7) and finally dissolved in dry pyridine (10 mL). To the mixture was added isodurenedisulfonyl dichloride (1.10 g, 3.33 mmol). After being stirred at room temperature for 2 h, the mixture was partitioned between CH_2Cl_2−pyridine (99:1, v/v, 200 mL) and saturated $NaHCO_3$ (200 mL). The organic phase was collected, washed with saturated $NaHCO_3$ and water, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was chromatographed on a column of silica gel with hexane−ethyl acetate containing 0.5% pyridine to give **23** (1.03 g, 85%): ^{1}H NMR (270 MHz, $CDCl_3$, TMS) δ 1.55−1.76 (12H, m, CH_3 of CME), 2.63−2.90 (4H, m, CH_2 of CME), 3.31 (2H, s, 5′-H and 5″-H), 3.81 (6H, s, CH_3 of DMTr), 4.02 (1H, s, 4′-H), 5.42−5.48 (3H, m, 2′-H, 3′-H, and 5′-H), 6.31 (1H, d, $J_{1'H-2'H}$ = 6.9 Hz, 1′-H), 6.86−6.89 (4H, m, ArH), 7.24−7.67 (18H, m, ArH), 7.90 (1H, d, J_{5H-6H} = 8.6 Hz, 6-H), 8.02 (2H, d, J = 7.59 Hz, *o*-ArH); ^{13}C NMR (67.8 Mz, $CDCl_3$) δ 26.94, 26.97, 27.05, 27.10, 27.14, 27.19, 27.53, 27.60, 31.34, 31.43, 31.72, 31.79, 55.26, 63.00, 76.21, 76.78, 77.20, 83.97, 84.12, 84.67, 84.78, 87.98, 102.89, 113.53, 116.50, 116.60, 125.61, 125.73, 125.84, 127.35, 128.05, 128.27, 129.11, 129.52, 129.56, 129.65, 129.70, 129.78, 129.96, 130.05, 130.15, 130.76, 131.30, 134.55, 134.72, 135.13, 135.22, 135.45, 135.54, 140.00, 143.79, 149.38, 158.85, 161.87, 168.48; ^{31}P NMR (109 MHz, $CDCl_3$, 85% H_3PO_4) δ 51.20, 51.90. Anal. Calcd for $C_{59}H_{58}N_4O_{12}P_2S_2\cdot H_2O$: C, 59.94; H, 5.03; N, 4.73. Found: C, 59.77; H, 4.82; N, 4.74.

Fully Protected 2′-Phosphorylated Dimer 25. A mixture of 2′,3′,5′-tri-*O*-benzoyluridine (**24**) (224 mg, 0.4 mmol) and 1*H*-tetrazole (56 mg, 0.8 mmol) was rendered anhydrous by repeated coevaporation with dry pyridine (5 mL × 5), dry toluene (5 mL × 3), and dry acetonitrile (5 mL × 2) and finally dissolved in acetonitrile (4 mL). To the mixture was added the phosphoramidite unit **22** (592 mg, 0.52 mmol), and the solution was stirred at room temperature for 3.5 h. *tert*-Butyl hydroperoxide (0.3 mL, 2.4 mmol) was added, and stirring was continued for an additional 50 min. The solvent was removed under reduced pressure, and extraction was performed with CH_2Cl_2−5% $NaHCO_3$. The CH_2Cl_2 extract was washed with 5% $NaHCO_3$ and water. The usual workup followed by silica gel column chromatography eluted with CH_2Cl_2−MeOH containing 0.5% pyridine gave **25** (570 mg, 90%): ^{1}H NMR (270 MHz, $CDCl_3$, TMS) δ 1.57−1.79 (12H, m, CH_2 of CME), 2.67−3.01 (6H, m, CH_2 of CME and CE), 3.64−4.98 (2H, m, 5′-H and 5″-H of Up), 3.78−3.80 (6H, m, CH_3 of DMTr), 4.23−4.56 (6H, m, 4′-H of Up, 4′-H and 5′-H and 5″-H of pU, $POCH_2$ of CE), 5.37−5.44 (2H, m, 5-H and 3′-H of Up), 5.50−5.68 (2H, m, 2′-H of Up and pU), 5.66−5.75 and 5.77−5.81 (1H, m, 3′-H of diasteromeric pU), 5.88−5.94 (1H, m, 5-H of pU), 6.17 and 6.24 (1H, d, $J_{1'H-2'H}$ = 5.9 Hz, 1′-H of diastereomeric pU), 6.34−6.39 (1H, m, 1′-H of Up), 6.86−6.90 (4H, m, *m*-ArH of DMTr), 7.27−7.63 (22H, m, 6-H of pU and *p*- and *m*-ArH), 7.83−7.99 (9H, m, 6-H of Up and *o*-ArH); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 19.52, 19.55, 19.63, 21.37, 26.79, 26.85, 27.17, 27.23, 31.54, 31.59, 31.65, 31.95, 32.04, 55.22, 62.93, 63.02, 63.07, 63.15, 67.23, 67.28, 70.35, 70.48, 73.35, 80.97, 81.08, 83.12, 83.23, 83.29, 83.33, 83.81, 83.92, 84.04, 85.39, 88.00, 88.03, 102.80, 103.29, 103.45, 113.39, 113.50, 116.42, 116.60, 116.68, 116.80, 116.86, 125.21, 127.33, 128.07, 128.14, 128.23, 128.36, 128.45, 128.50, 128.61, 128.95, 129.06, 129.09, 129.70, 129.74, 129.81, 130.01, 130.17, 130.28, 130.33, 130.46, 130.58, 130.69, 131.12, 131.29, 133.78, 134.41, 134.72, 135.06, 139.95, 140.11, 143.76, 149.24, 149.29, 158.83, 161.49, 161.60, 161.81, 165.19, 165.30, 165.48, 168.18, 168.30, 168.37; ^{31}P NMR (109 MHz, $CDCl_3$, 85% H_3PO_4) δ −2.12, −2.04, 51.14, 51.25. Anal. Calcd for $C_{80}H_{75}N_7O_{22}P_2S\cdot 3.5H_2O$: C, 58.46; H, 5.03; N, 5.97. Found: C, 58.36; H, 4.88; N, 5.91.

Synthesis of U(2′-p)pU (29). Compound **25** (48 mg, 0.03 mmol) was rendered anhydrous by repeated coevaporation with dry pyridine (5 mL × 3), and BSA (0.167 mL, 0.675 mmol) and a 0.1 M solution (0.9 mL, 0.09 mmol) of DBU in pyridine were added. After being stirred at room temperature for 10 min, the mixture was quenched by addition of water (0.2 mL). Iodine (38 mg, 0.3 mmol) was added, and the mixture was stirred at room temperature for 40 h. The mixture was diluted with pyridine−water (5 mL, 1:1, v/v), and the excess iodine was removed by extraction with ether (5 mL × 7). The aqueous phase was collected and evaporated under reduced pressure. The residue was dissolved in concentrated ammonia (45 mL)−pyridine (5 mL), and the mixture was stirred at room temperature for 18 h. After the solvent was removed under reduced pressure, the residue was coevaporated with water (5 mL × 2) and treated with 80% acetic acid (5 mL) at room temperature for 15 min. The solvent was removed under reduced pressure, and the residue was partitioned between water and ether. The aqueous layer was further washed with ether (5 mL × 2) and concentrated under reduced pressure. Paper chromatography using Whatman 3MM papers developed with 2-PrOH−concd NH_3−H_2O (6:1:3, v/v/v) gave **29** (421A_{260}, 72%): R_f = 0.19; UV(H_2O) λ_{max} 260 nm, λ_{min} 229 nm; ^{1}H NMR (270 MHz, D_2O, DSS, 40 °C) δ 3.82 (2H, d, $J_{4'H-5''H}$ = 3.3 Hz, 5′-H and 5″-H of Up), 4.08−4.27 (4H, m, 3′-H, 4′-H, and 5′-H and 5″-H of pU), 4.31−4.40 (2H, m, 2′-H of pU and 4′-H of Up), 4.73−4.78 (1H, m, 3′-H of Up), 4.83−4.90 (1H m, 2′-H of Up), 5.99 (1H, d, J_{5H-6H} = 8.2 Hz, 5-H of Up), 5.94−5.97 (2H, m, J_{5H-6H} = 8.3 Hz, $J_{1'H-2'H}$ = 4.0 Hz, 5-H and 1′-H of pU), 6.04 (1H, d, $J_{1'H-2'H}$ = 6.6 Hz, 1′-H of Up), 7.82 (1H, d, J_{5H-6H} = 8.2 Hz, 6-H of Up), 7.92 (1H, d, J_{5H-6H} = 8.3 Hz, 6-H of pU); ^{13}C NMR (67.8 MHz, D_2O, dioxane) δ 61.87, 65.84, 65.91, 70.37, 74.44, 74.75, 74.84, 74.97, 84.08, 84.13, 84.18, 85.08, 88.50, 88.55, 89.43, 103.37, 103.46, 142.42, 143.39, 152.23, 152.61, 166.95, 166.99; ^{31}P NMR (109 MHz, D_2O, 85% H_3PO_4) δ 0.153, 0.180; MS (FAB-) calcd for $C_{18}H_{23}O_{17}N_4P_2$ (M^+ − H) 629.0547, found 629.0558.

Synthesis of U(2′-ps)pU (30). Compound **25** (48 mg, 0.03 mmol) was dissolved in a 1% solution (2 mL) of trifluoroacetic acid in CH_2Cl_2, and the mixture was stirred at room temperature for 15 min. The mixture was quenched by addition of MeOH−pyridine (5 mL, 1:1, v/v) and extracted with CH_2Cl_2. The CH_2Cl_2 extract was washed successively with 5% $NaHCO_3$ and water, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was rendered anhydrous by repeated coevaporation with dry pyridine (5 mL × 3), and *N,O*-bis(trimethylsilyl)acetamide (0.167 mL, 0.675 mmol) and a 0.1 M solution (0.9 mL, 0.09 mmol) of DBU in pyridine were added. After being stirred at room temperature for 10 min, the solution was evaporated under reduced pressure. The residue was dissolved in concentrated ammonia (45 mL)−pyridine (5 mL), and the mixture was stirred at room temperature for 18 h. After the solvent was removed under reduced pressure, the residue was coevaporated with water (5 mL × 2) and partitioned between water and ether. The aqueous layer was further washed with ether (5 mL × 2) and concentrated under reduced pressure. Paper chromatography using Whatman 3MM papers developed with 2-propanol−concentrated ammonia−water (6:1:3, v/v/v) gave **30**, which was passed through a column of Dowex 50 W × 8 (sodium salt form, 1.5 × 15 cm) (472A_{260}, 80%): UV(H_2O) λ_{max} 260 nm, λ_{min} 229 nm; ^{1}H NMR (270 MHz, D_2O, DSS) δ 3.83 (2H, d, $J_{4'H-5'H}$ = 3.3 Hz, 5′-H and 5″-H of Up), 4.19−4.28 (3H, m, 4′-H and 5′-H and 5″-H of pU), 4.36−4.43 (2H, m, 3′-H of pU and 4′-H of Up), 4.46−4.50 (1H, m, 2′-H of pU), 4.78−5.00 (2H, m, 2′-H and 3′-H of up), 5.88 (1H, d, J_{5H-6H} = 7.6 Hz, 5-H of Up), 5.94−5.99 (2H, m, 1′-H and 5-H of pU), 6.10 (1H, d, $J_{1'H-2'H}$ = 7.3 Hz, 1′-H of Up), 7.89 (1H, d, J_{5H-6H} = 7.6 Hz, 6-H of Up), 7.96 (1H, d, J_{5H-6H} = 8.3 Hz, 6-H of pU); ^{13}C NMR (67.8 MHz, D_2O, dioxane) δ 62.24, 66.03, 66.12, 70.46, 74.39, 75.77, 75.85, 84.49, 84.61, 85.48, 88.41, 88.48, 89.23, 103.21, 103.48, 142.48, 144.16, 152.99, 153.15, 167.43, 167.32; ^{31}P NMR (109 MHz, D_2O, 85% H_3PO_4) δ 0.244, 46.14; MS (FAB-) calcd for $C_{18}H_{22}O_{16}N_4P_2SNa$ (M^+ − H) 667.0124, found 667.0134.

Compound **30** was gradually air-oxidized upon standing to give **31**: ^{1}H NMR (270 MHz, $CDCl_3$, TMS) δ 3.84−3.87 (4H, m, 5′-H and 5″-H of Up), 4.19−4.30 (6H, m, 4′-H, 5′-H, 5′-H of pU), 4.39−4.45 (6H, m, 4′-H of Up and 2′-H, 3′-H of pU), 4.77−

4.90 (4H, m, 3′-H of Up and 2′-H of pU), 5.93−6.00 (6H, m, 5-H of Up and 1′-H, 5-H of pU), 6.17 (2H, 2d, $J_{1'H-2'H}$ = 6.9 Hz, 1′-H of Up) 7.92−7.95 (4H, m, 6-H of Up, pU); ^{31}P NMR (109 MHz, D_2O, 85% H_3PO_4) δ −0.15, 16.00.

U(2′-pp-biotin)pU (35). Compound **29** (50A_{260}, 2.55 mmol) was dissolved in 2 M *N*-ethylmorpholine buffer (pH 7.0, 10 μL). To the mixture were added water (30 μL), 1 M $MgCl_2$ (20 μL), and 0.25 M biotin-HA-pIm (40 μL). The mixture was stirred at 37 °C for 40 h. Paper chromatography on Whatman 3MM papers developed with 2-propanol−concentrated ammonia−water (6:1:3, v/v/v) gave title compound **32** (12A_{260}, 23%): UV(H_2O) λ_{max} 258 nm, λ_{min} 228 nm; ^{1}H NMR (270 MHz, D_2O, DSS) δ 1.27−1.73 (14H, m, CH_2), 2.24 (2H, t, J = 7.26 Hz, CH_2C(O)), 2.77 (1H, d, J_{Ha-Hb} = 12.9 Hz, Ha of SCH_2), 2.96 (1H, dd, J_{Ha-Hb} = 12.9 Hz, J_{Hb-CHN} = 4.6 Hz, Hb, of CH_2S), 3.14−3.19 (2H, m, CH_2NH), 3.30−3.35 (1H, m, SCH), 3.79−3.86 (4H, m, $POCH_2$ and 5′-H and 5″-H of Up), 4.19−4.43 (7H, m, 4′-H of Up, 2′-H, 3′-H, 4′-H, 5′-H, and 5″-H of pU and CHC*H*NH), 4.60 (1H, d, J_{5H-6H} = 8.2 Hz, 5-H of Up), 5.94 (1H, d, J_{5H-6H} = 7.9 Hz, 5-H of pU), 6.00 (1H, d, $J_{1'-2'}$ =4.95 Hz, 1′-H of pU), 6.09 (1H, d, $J_{1'H-2'H}$ = 6.3 Hz, 1′-H of Up), 7.83 (1H, d, J_{5H-6H} = 8.2 Hz, 6-H of Up), 7.91 (1H, d, J_{5H-6H} = 7.9 Hz, 6-H of pU); ^{31}P NMR (109 MHz, D_2O, 85% H_3PO_4) δ −0.014, 9.828 (d, J_{P-P} = 18.5 Hz), −11.45 (d, J_{P-P} = 18.5 Hz); MS (FAB-) calcd for $C_{34}H_{51}O_{22}N_7P_3S$ (M^+ − H) 1034.2021, found 1034.2037.

U(2′-ps-bimane)pU (33). Compound **30** (59A_{260}, 3 μmol), which contained a small amount of **31**, was dissolved in ethanol (0.5 mL)−water (0.3 mL), and $NaBH_4$ (1.1 mg, 0.03 mmol) was added. The mixture was stirred at room temperature for 24 h, and acetone (0.5 mL) was added to decompose the excess reagent. The solvent was removed under reduced pressure, and the residue was dissolved in water (0.5 mL). To the mixture was added a solution of monobromobimane (1.2 mg, 4.4 μmol). The solution was stirred for 16 h and then chromatographed on Whatman 3MM papers developed with 2-propanol−concentrated ammonia−water (6:1:3, v/v/v) to give **33** (38A_{260}, 64%): UV(H_2O) λ_{max} 258 nm, λ_{min} 230 nm; ^{1}H NMR (270 MHz, D_2O, DSS) δ 1.74 (3H, S, CH3 of bimane), 1.81 (3H, s, CH3 of bimane), 2.39 (3H, s, CH3 of bimane), 2.88−2.95 (1H, m, CH_2 of bimane), 3.34−3.39 (1H, m, CH_2 of bimane), 3.81 (2H, d, J = 2.31 Hz, 5′-H and 5″-H of Up), 4.15−4.36 (6H, m, 2′-H, 3′-H, 4′-H, and 5′-H and 5″-H of pU, and 4′-H of Up), 4.75−4.90 (1H, m, 3′-H of Up), 4.91−5.01 (1H, m, 2′-H of Up), 5.80−5.87 (3H, m, 1′-H and 5-H of pU and 5-H of Up), 6.06 (1H, d, $J_{1'H-2'H}$ = 6.6 Hz, 1′-H of Up), 7.83−7.88 (2H, 2d, 6-H of Up and pU); ^{31}P NMR (109 MHz, D_2O, 85% H_3PO_4) δ 0.00, 17.16; MS (FAB-) calcd for $C_{28}H_{33}O_{18}N_6P_2S$ (M^+ − H) 835.1047, found 835.1049.

Enzymatic Assay of U(2′-p)pU. Treatment with Calf Intestinal Alkaline Phosphatase Followed by Treatment with Nuclease P1. Compound **29** (0.5A_{260}, 26 nmol) was dissolved in 50 mM Tris−HCl buffer (pH 8.5, 250 μL) containing 0.1 mM EDTA. To the mixture was added a solution (50 μL) of CIAP (1 unit/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 HPLC. The main peak which corresponds to UpU was pooled, lyophilized, and treated with a solution (10 μL) of nuclease P1 (1 unit/μL) in 20 mM AcOH−AcONa buffer (pH 5.3, 0.1 mL) at 50 °C for 1 h. After being heated at 100 °C for 1 min, the mixture was analyzed by HPLC.

Treatment with Nuclease P1. Compound **29** (0.5A_{260}, 26 nmol) was dissolved in 20 mM AcOH−AcONa buffer (pH 5.3, 0.25 mL). To the mixture was added a solution (25 μL) of nuclease P1 (1 unit/μL). The incubation was done at 50 °C for 40 h. After being heated at 100 °C for 1 min, the mixture was analyzed by HPLC.

Treatment with Calf Spleen Phosphodiesterase. Compound **29** (0.5A_{260}, 26 nmol) was dissolved in 0.1 M NH_4OAc buffer (pH 5.7, 90 μL). To the mixture was added a solution (20 μL) of CSP (2 mg/μL). The incubation was done at 37 °C for 23 h. After being heated at 100 °C for 1 min, the mixture was analyzed by HPLC.

Treatment with Snake Venom Phosphodiesterase. Compound **29** (0.5A_{260}, 26 nmol) was dissolved in Tris−HCl buffer (pH 7.5, 10 μL). To the mixture was added SVP (1 μL, 1 mg/μL). The incubation was done at 37 °C for 24 h. After being heated at 100 °C for 1 min, the mixture was analyzed by HPLC.

An Attempt to Synthesize [U(2′-p)p]$_6$U on Polymer Support. A CPG gel having U unit **4** (1 μmol, 26.4 μmol/g) as the 3′-terminus was used. Chain extension was performed according to the protocol described in Table 1. After all condensations were finished, a mixture of concentrated ammonia−pyridine (10 mL, 9:1, v/v) was added. The mixture was slowly rotated by a rotary evaporator at room temperature for 24 h, filtered, and washed with water. The filtrate and washing were collected, and the solution was evaporated under reduced pressure. The residue was treated with 20% trifluoroacetic acid in acetic acid (10 mL). Aliquots were taken after 3 and 5 h and analyzed by paper electrophoresis.

Solid Phase Synthesis of [U(2′-p)p]$_n$U (n = 1, 3, 5, 7, and 9). A CPG gel having a U unit (1 μmol, 26.4 μmol/g) as the 3′-terminus was used. The synthetic unit **22** was used for condensation. Chain extension was performed according to the protocol described in Table 1. After all operations were over, the CPG gel was evaporated with acetonitrile and suspended in BSA−pyridine (1:1, v/v, 0.4 mL). The suspension was slowly rotated in a vessel at room temperature for 1 h, and then DBU (12 μL, 0.08 mmol) was added. Rotation was continued at room temperature for 40 h. The CPG gel was then filtered and washed with pyridine (1 mL × 3). The CPG gel was treated with a 1 M solution of iodine in THF−pyridine−water (0.5 mL, 10:10:1, v/v/v) at room temperature for 48 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 (5 mL, 9:1, v/v) was added, and the suspension was slowly rotated by a rotary evaporator for 24 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 dissolved in water and subjected to polyacrylamide gel electrophoresis and HPLC. In the case of n = 1, 3, and 5, the main peaks were pooled and analyzed by HPLC.

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Supporting Information Available: Copies of ^{1}H NMR data for compounds **11**, **18a**,**b**, **33**, and **35**; ^{1}H and ^{13}C NMR data for compounds **5**, **7**, **8**, **10a**, **13**, **15**−**23**, **25**, **29**−**31**, **33**, and **35**; ^{31}P NMR data for compounds **5**, **6**, **15**−**20**, **33**, and **35**; FAB mass data of **29**, **30**, **33**, and **35**; ^{1}H−^{1}H COSY NMR data of **25**, **29**, **33**, and **35**; Figure 7 (stability of **11a** in pyridine), Figures 8 and 9 (HPLC profiles and ^{31}P NMR spectra of **29** and **30**), Figures 10 and 11 (HPLC profiles of the mixtures obtained by the reactions of **30** with **32** and **34**, respectively), Figure 12 (analysis of the polymer supported synthesis of U(2′-p)pU under various conditions), and Figures 13−15 (enzymatic analysis of [U(2′-p)p]$_n$U, n = 1, 3, and 5) (58 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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