Synthesis of a New Class of 2′-Phosphorylated Oligoribonucleotides Capable of Conversion to Oligoribonucleotides

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Eukaryotic nuclear RNAs transcribed from DNAs are enzymatically processed and modified at various sites involving base residues, 5′-terminal phosphates, and hydroxyl groups to give the mature functional molecules such as capped mRNAs and aminoacylated tRNAs. Among these modifications, Konarska first discovered a unique structure of 2′-phosphorylated circular RNA in ligation products derived from a fragment of tobacco mosaic virus RNA Ω.[1] Later, Abelson reported that a similar structure was found at the splice junction of yeast pre-tRNA^{Leu}.[2] These studies revealed that 2′-phosphorylated diribonucleotides [N(2′-p)pN] were considerably resistant to alkali and easily dephosphorylated by treatment with a phosphatase to give the usual 3′-5′ phosphodiester diribonucleotides [NpN].[1,2] Much attention has also been paid to such 2′-phosphorylated RNAs, since so-called "lariat" RNAs having 2′-phosphorylated 3′-5′ phosphodiester linkages have recently been found in circular introns formed by splicing of pre-mRNAs. However, no studies on the chemical synthesis of 2′-phosphorylated RNAs have been reported up to date in spite of their biological importance. These facts stimulated us to explore an approach to the chemical synthesis of 2′-phosphorylated RNAs. Since 2′-O-phosphorylated RNAs can be dephosphorylated by alkaline phosphatases as mentioned above, such a study means simultaneous new development of the chemical synthesis of RNAs. In the current RNA synthesis, the 2′-hydroxyl group of ribonucleotides has been protected by only three types of protecting groups, i.e., acetal,[3] benzyl,[4] and silyl[5] groups. Therefore, our RNA synthesis proposed here would give the fourth, unique strategy in which the 2′-hydroxyl group was protected with a "protected" phosphate.

Table I. Desilylation of Compounds 2 and 4 by Treatment with PPHF and TBAF

compound	desilylating agent	time	yield (%)	ratio of 5a:5b
2	HF/pyridine	10.5 h	83	96:4
2	TBAF	25 min	45	41:59
4	HF/pyridine	15 min	86	100:0
4	TBAF	2 h	75	49:51

In order to synthesize 2′-O-phosphorylated RNAs, a masked 2′-phosphate [$(RO)_2P(O)$] group must be introduced to the 2′-hydroxyl group of appropriately protected ribonucleosides. The most serious problem in our approach is how to prevent the 2′,3′ migration and 2′,3′-cyclization of the masked 2′-phosphate group upon removal of the 3′-hydroxyl protecting group, which is required to introduce a phosphate to the 3′-hydroxyl function for the preparation of monomer building units for chain elongation.[6] To overcome this problem, we considered the use of sterically hindered 2′-phosphate protecting groups such as *tert*-butyl. Consequently, it was found that a protected phosphate group, di-*tert*-butoxyphosphoryl, could meet the above criteria as the 2′-hydroxyl protecting group which did not cause 2′,3′ phosphoryl migration.

Results and Discussion

For the regioselective introduction of two different phosphoryl groups into the 2′,3′-*cis*-diol function of ribonucleosides, 3′,5′-protected ribonucleoside derivatives were required. As such a typical ribonucleoside, 6-*N*-benzoyl-3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-adenosine (1)[7] was chosen in the present study. Reaction of 1 with di-*tert*-butoxy(diethylamino)phosphine (DBDEP)[8] in the presence of 1*H*-tetrazole followed by oxidation with *m*-chloroperbenzoic acid (mCPBA) gave the 2′-phosphorylated product 2 in 86% yield. When 2 was treated with tetrabutylammonium fluoride in THF, considerable phosphoryl migration took place to give a nonselective mixture of the 3′,5′-unprotected and 2′,5′-unprotected species (**5a** and **5b**). The 3′-O-phosphorylated derivative **5b** was somewhat predominantly formed over **5a** as shown in Table I. It seemed that the isomerization occurred owing to the intramolecular attack of the 3′-oxide ion, which was generated by attack of the fluoride ion on the 3′-silicon atom, on the neighboring 2′-phosphoryl group. On the basis of this mechanism, we considered that, if the 3′-oxide ion could be neutralized as immediately as possible prior to the attack of the ion on the phosphorus, the phosphoryl migration would be avoided. Therefore, we next used a more acidic reagent, pyridinium poly(hydrogen fluoride) (PPHF), for the desilylation of **2**. As a result, the ratio of **5a**/**5b** was remarkably improved to 96/4.

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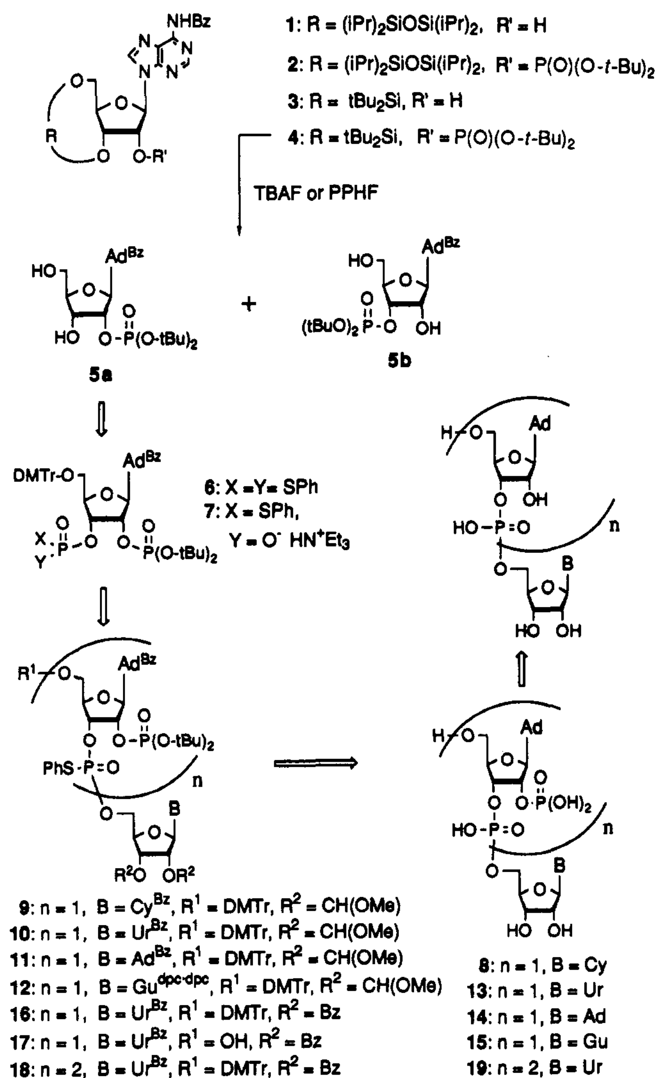

These results led us to examine the di-*tert*-butylsilanediyl (DTBS)[9] group in place of the 1,1,3,3-tetraisopropyldisiloxane-1,3-diyl (TIPS) group. This is because the former is promptly removed by treatment with PPHF within a few minutes while the latter requires 10–24 h for its complete removal under the same conditions. Thus, reaction of *N*-benzoyl-3',5'-*O*-(di-*tert*-butylsilanediyl)-adenosine (3) with 3.5 equiv of DBDEP[8] in the presence of 1*H*-tetrazole followed by successive oxidation with mCPBA gave the 2'-phosphorylated product **4** in 61% yield. When **4** was allowed to react with PPHF (20 equiv)–pyridine (36 equiv) in THF at rt, the DTBS group was removed cleanly and rapidly (essentially within 3 min) to afford exclusively the 2'-O-phosphorylated species **5a** in 84% yield, as shown in Table I. The ^{31}P NMR spectrum of **5a** exhibited only one signal at −10.71 ppm. It is noteworthy that this reaction gave the sole product **5a** without side reactions such as the phosphoryl migration and cyclization. Compared to this result, the use of TBAF for the desilylation of **4** resulted in a nearly 1:1 mixture of **5a** and **5b**, as shown in Table I. For isolation of **5a**, 0.5% pyridine was added to the developing solvent during silica gel column chromatography. Without addition of pyridine, **5a** was considerably decomposed with elimination of the *tert*-butyl group.

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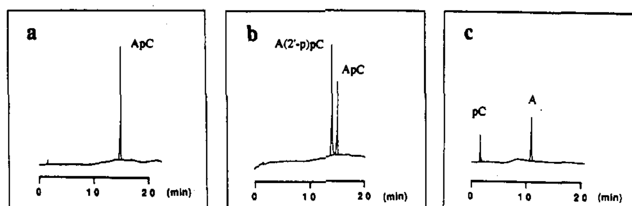

Figure 1. (a) The HPLC profile of the mixture obtained after treatment of A(2'-p)pC with calf intestinal alkaline phosphatase in 0.05 M Tris-HCl (pH 8.5) containing 0.1 mM EDTA at 50 °C for 30 min. (b) The HPLC profile obtained when an additional amount of A(2'-p)pC was added to the same mixture as described in a to ascertain the appearance of a new peak corresponding to ApC. (c) The HPLC profile obtained after treatment of ApC with nuclease P1.

Next, the stability of compound **5a** was examined by TLC analysis under various conditions. It was found that **5a** was sufficiently stable under both acidic and basic conditions. Even in 0.2 M NaOH–dioxane (1:1, v/v), **5a** survived considerably with a $t_{1/2}$ value of 6 h. As expected by the above experiment, **5a** remained unchanged upon treatment with 0.1 M PPHF in THF for 24 h. Compound **5a** was slowly decomposed to a base line material when dissolved in 80% CH_3COOH ($t_{1/2}$ = 1.5 h, at 25 °C) and in 1% CF_3CO_2H/CH_2Cl_2 ($t_{1/2}$ = 12 h, at 25 °C). These decompositions probably occurred via 2',3'-cyclization of the phosphoryl group owing to the neighboring 3'-hydroxyl group or via direct elimination of the *tert*-butyl group from **5a**. More interestingly, the 3'-regioisomer **5b** was not observed within the accuracy of TLC analysis in these experiments, suggesting no 2',3' isomerization of the phosphoryl group.

For the synthesis of 2'-O-phosphorylated adenylyl(3'-5')cytidine [A(2'-p)pC] (**8**), an appropriately protected building block **6** of adenosine was synthesized in 76% yield by the 5'-dimethoxytritylation of **5a** followed by the 3'-phosphorylation using cyclohexylammonium *S*,*S*-diphenyl phosphorodithioate/isodurenedisulfonyl dichloride (DDS)/1*H*-tetrazole.[10] During the phosphorylation no 2',3' phosphoryl migration was observed. The homogeneity of **6** was confirmed by ^{31}P NMR which exhibited a set of sharp resonance peaks at −9.93 and 51.35 ppm corresponding to the 2'- and 3'-phosphoryl groups, respectively. Treatment of **6** with Et_3N–pyridine–water (2:2:1, v/v/v)[11] at rt for 30 min gave quantitatively the *S*-phenyl 3'-phosphorothioate derivative **7**. Condensation of **7** with 2',3'-*O*-(methoxymethylene)-4-*N*-benzoylcytidine afforded the 3'-5' linked dimer (**9**) in 79% yield.

Deprotection of all protecting groups from **9** was simply performed by the following two-step procedure: (1) concd ammonia–MeOH (1:1, v/v) at rt for 7 h, (2) CF_3CO_2H–CH_3CO_2H (20:80, v/v) at rt for 7 h. The unprotected dimer A(2'-p)pC (**8**) was obtained as a single band in 56% yield by paper chromatography, purified finally by reverse-phase HPLC (14.0 min), and characterized by UV and ^{1}H, ^{13}C, and ^{31}P NMR spectroscopy. The 2'-phosphate group of **8** was removed completely by treatment with calf intestinal alkaline phosphatase (CIP) at pH 8.5 in Tris-HCl (50 mM)-EDTA (0.1 mM) at 50 °C for 3 h without affecting the internucleotidic bond to give ApC (HPLC, 15.0 min) as seen in Figure 1a,b. The dephosphorylated dimer ApC was further characterized by complete digestion with

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Table II. Synthesis of Fully Protected 2'-Phosphorylated Dimers (9–12)

B	NT (equiv)	DDS (equiv)	time	product	yield (%)
C^{bz}	3.0	3.0	40 m	9	79
Ur^{bz}	3.0	3.0	1 h	10	49
Ad^{bz}	3.0	3.0	2 h	11	62
$Gu^{dpc\ pro}$	3.0	3.0	2 h	12	50

Table III. Deprotection of Fully protected 2'-Phosphorylated Dimers A(2'-p)pX

base of X	step 1 time, h	step 2 time, h	retention time of hplc	yield, %	λ_{max}, nm	λ_{min}, nm
Cy	20	3	14 min, 32 s	56	259	226
Ur	36	4	14 min, 35 s	43	256.5	227
Ad	36	4	16 min, 24 s	68	256	226
Gu	33	4	14 min, 54 s	48	254	223.5

nuclease P1 (Yamasa) in the usual manner giving rise to A and pC in a ca. 1:1 ratio as shown in Figure 1c.

In a manner similar to that described in the synthesis of **9**, fully protected dimers (**10–12**) of A(2'-p)pX (X = U, A, and G) were synthesized in 49, 62, and 50% yields, respectively, by condensation of **7** with the corresponding N-protected 2',3'-*O*-(methoxymethylene)ribonucleosides. These results are summarized in Table II. Similar full deprotections of **10–12** gave the unprotected dimers A(2'-p)pX (X = U, A, and G) in 43, 68, and 48% yields, respectively. These results are shown in Table III. These 2'-phosphorylated dimers were also characterized by stepwise enzymatic treatments with CIP and nuclease P1.

Condensation of 2',3'-*O*,N^3-tribenzoyluridine with 1.2 equiv of **7** in the presence of 3 equiv each of DDS and 3-nitro-1*H*-1,2,4-triazole gave the dimer **16** in 70% yield. The selective removal of the 5'-terminal DMTr group of **16** by the action of $CF_3CO_2H–CH_2Cl_2$ (1:99, v/v)[12] afforded the 5'-hydroxyl component **17** in 92% yield without affecting the 2'-di-*tert*-butoxyphosphoryl group. The successive condensation of **17** with **7** gave the fully protected trimer **18** in 69% yield. Deprotection of **18** in a manner similar to that described above gave A(2'-p)pA(2'-p)U (**19**) in 56% yield. Finally, this trimer was purified by HPLC (retention time 15.9 min). Enzymatic treatment of this purified material with CIP gave quantitatively ApApU (see Figure 2a,b), which was completely digested with nuclease P1 to A, pA, and pU in a ca. 1:1:1 ratio as shown in Figure 2c. Further characterization of **19** was conducted by ^1H, ^{13}C, and ^{31}P NMR spectroscopy.

These results clearly showed that the 2'-protected or unprotected phosphate group could serve as a kind of 2'-hydroxyl protecting group which would provide a new tool to oligoribonucleotide synthesis. The successful introduction of the di-*tert*-butoxyphosphoryl group to the 2'-position described here allowed us not only to use stronger acidic and basic conditions in oligoribonucleotide synthesis but also to produce a new class of 2'-phosphomonoester-containing oligoribonucleotides which would be utilized as antisense RNAs as well as precursors of naturally occurring RNAs.

Experimental Section

Melting points were obtained on a Mitamura Melt-Temp apparatus and are uncorrected. ^1H NMR spectra were measured on a JEOL-EX 270-MHz spectrometer, JEOL-FX90Q spectrometer, and JEOL-GX 500 spectrometer with TMS (for $CDCl_3$)

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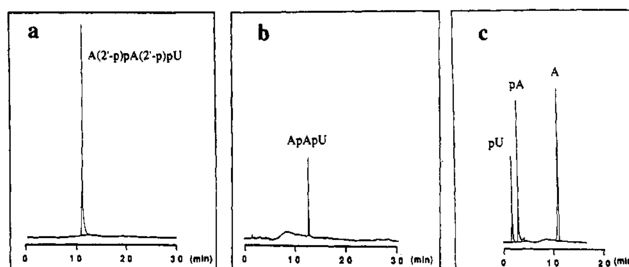

Figure 2. (a) The HPLC profile of purified A(2'-p)pA(2'-p)pU. (b) The HPLC profile obtained after treatment of A(2'-p)pA-(2'-p)pU with calf intestinal alkaline phosphatase. (c) The HPLC profile of the mixture of A, pA, and pU which was obtained by treatment of dephosphorylated ApApU with nuclease P1.

or DSS (for D_2O) as the internal standard. ^{13}C NMR spectra were recorded on a JEOL-EX 270-MHz spectrometer and a JEOL-GX 500 spectrometer at 67.8 and 125.65 MHz, respectively, with $CHCl_3$ (for $CDCl_3$) as the internal standard or DSS (for D_2O) as the external standard. ^{31}P NMR spectra were recorded on a JEOL-EX 270-MHz spectrometer and a JEOL-FX100 spectrometer at 109 and 40.5 MHz, respectively, with an external reference of 85% H_3PO_4. UV spectra were taken on a Hitachi U-2000 spectrophotometer. HPLC was performed on a Waters LC module apparatus with a μBondasphere 5-μm column (Waters, C_{18}-100A, 3.9 × 150 mm) using a linear gradient of 0–30% acetonitrile in 0.1 M NH_4OAc (pH 7.0) for 30 min at a flow rate of 1.0 mL/min at 50 °C. Paper chromatography was done by use of a descending technique with Whatman 3MM papers using iPrOH–concentrated ammonia–H_2O (6:1:3, v/v/v). Thin-layer chromatography was performed by the use of Merck Kieselgel 60 F-254 (0.25 mm) with a developing solvent of CH_2Cl_2–MeOH (9:1, v/v) or iPrOH–concentrated ammonia–H_2O (7:1:2, v/v/v). Column chromatography was performed with silica gel C-200 purchased from Waco Co. Ltd., and a minipump for a goldfish bowl was conveniently used to attain sufficient pressure for rapid chromatographic separation.

2'-*O*-(Di-*tert*-butoxyphosphoryl)-3',5'-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-6-*N*-benzoyladenosine (2). Compound 1 (307 mg, 0.5 mmol) was rendered anhydrous by repeated coevaporation with dry pyridine and finally dissolved in CH_2Cl_2 (3 mL). To the mixture were added di-*tert*-butoxy(*N*,*N*-diethylamino)phosphine (1.11 mL, 4.5 mmol) and tetrazole (946 mg, 13.5 mmol). After being stirred for 2 h, the mixture was treated with mCPBA (0.74 g, 4.5 mmol) at 0 °C. The solution was stirred for an additional 1 h. The usual workup was followed by column chromatography to give crude **2** (354 mg, 88%, more than 95% pure) which contained a small amount of impurity that could not be removed by repeated chromatography: ^1H NMR (100 MHz, $CDCl_3$–CD_3OD, 99:1, v/v) δ 1.10 (9H, s, CH_3), 1.14 (9H, s, CH_3), 4.22–3.98 (2H, m, 5'-H), 4.46 (1H, m, 4'-H), 4.66-4.82 (1H, m, 3'-H), 4.82–4.95 (1H, m, 2'-H), 5.90 (1H, s, 1'-H), 7.43 (3H, m, ArH), 7.46 (2H, m, ArH), 8.10 (1H, s, 2-H), 8.75 (1H, s, 8-H); ^{31}P NMR (40.5 MHz, $CDCl_3$, 85% H_3PO_4) δ -9.39.

3',5'-*O*-(Di-*tert*-butylsilanediyl)-6-*N*-benzoyladenosine (3). 6-*N*-Benzoyladenosine (1.86 g, 5 mmol) was rendered anhydrous by repeated coevaporation with dry pyridine and finally dissolved in DMF (30 mL). Silver nitrate (2.55 g, 15 mmol) was added and di-*tert*-butyldichlorosilane (1.59 mL, 7.5 mmol) was added dropwise with vigorous stirring at 0 °C to the mixture. After being stirred at room temperature for 15 min, the mixture was treated with triethylamine (2.1 mL, 15 mmol). The mixture was further stirred for 5 min and was evaporated under reduced pressure. The residue was partitioned between CH_2Cl_2–H_2O and the organic layer was dried over Na_2SO_4. The solution was filtrated and evaporated under reduced pressure. The residue was chromatographed on a column of silica gel with CH_2Cl_2–MeOH to give **3** (2.12 g, 99%): mp 240–245 °C (EtOH); ^1H NMR (100 MHz, $CDCl_3$–CD_3OD, 99:1, v/v) δ 1.10 (9H, s, CH_3), 1.14 (9H, s, CH_3), 4.22–3.98 (2H, m, 5'-H), 4.46 (1H, m, 4'-H), 4.66–4.82 (1H, m, 3'-H), 4.82–4.95 (1H, m, 2'-H), 5.90 (1H, s, 1'-H), 7.43 (3H, m, ArH), 7.46 (2H, m, ArH), 8.10 (1H, s, 2-H), 8.75 (1H, s, 8-H); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 164.88, 152.57, 150.94,

149.76, 141.67, 133.39, 132.69, 128.64, 127.96, 123.36, 90.96, 75.51, 74.97, 73.64, 67.24, 27.23, 27.13, 27.06, 22.61, 20.24, 19.70. Anal. Calcd for $C_{25}H_{33}N_5O_5Si\cdot{}^1/_4H_2O$: C, 58.18; H, 6.54; N, 13.57. Found: C, 58.13; H, 6.41; N, 13.56.

2′-*O*-(Di-*tert*-butoxyphosphoryl)-3′,5′-*O*-(di-*tert*-butylsilanediyl)-6-*N*-benzoyladenosine (4). Compound 3 (512 mg, 1 mmol) was rendered anhydrous by repeated coevaporation with dry pyridine and finally dissolved in CH_2Cl_2 (5 mL). To the mixture were added di-*tert*-butoxy(*N*,*N*-diethylamino)phosphine (2.22 mL, 9 mmol) and tetrazole (946 mg, 13.5 mmol). After being stirred for 3 h, the mixture was treated with mCPBA (1.53 g, 9 mmol) at 0 °C. The solution was stirred for an additional 1 h. The usual workup was followed by column chromatography to give crude 4 (476 mg, 68%, more than 95% pure) which contained a small amount of impurity that could not be removed by repeated chromatography: ^{1}H NMR (100 MHz, $CDCl_3$–CD_3OD, 99:1, v/v) δ 1.10 (9H, s, CH_3), 1.14 (9H, s, CH_3), 4.22–3.98 (2H, m, 5′-H), 4.46 (1H, m, 4′-H), 4.66–4.82 (1H, m, 3′-H), 4.82–4.95 (1H, m, 2′-H), 5.90 (1H, s, 1′-H), 7.43 (3H, m, ArH), 7.46 (2H, m, ArH), 8.10 (1H, s, 2-H), 8.75 (1H, s, 8-H); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 164.42, 152.24, 150.71, 149.51, 142.44, 133.19, 132.42, 128.45, 127.58, 123.31, 89.83, 83.54, 74.74, 74.14, 67.01, 29.62, 29.54, 29.47, 29.40, 29.29, 29.13, 27.26, 27.03, 26.92, 26.85, 26.65, 22.37, 19.93; ^{31}P NMR (40.5 MHz, $CDCl_3$, 85% H_3PO_4) δ −9.83; MS (FAB+) Calcd for $C_{33}H_{51}N_5O_8SiP$ (M^+ + H) 704.3245, found 704.3229.

2′-*O*-(Di-*tert*-butoxyphosphoryl)-6-*N*-benzoyladenosine (5). Commercially available HF·pyridine (Merck, 1.20 mL, 46 mmol) was mixed with pyridine (5.94 mL, 82.8 mmol). This mixture was added dropwise at 0 °C to a THF solution (7 mL) of 2 (1.62 g, 2.3 mmol). The mixture was stirred at room temperature for 10 min and then diluted with pyridine (20 mL). Extraction was performed by use of CH_2Cl_2 (60 mL)–H_2O (80 mL). The organic layer was collected, washed with 5% $NaHCO_3$, and dried over Na_2SO_4. The solution was filtrated 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 5 (1.11 g, 86%): **5a**: ^{1}H NMR (500 MHz, $CDCl_3$, TMS) δ 1.33 (9, s, *t*-Bu), 1.39 (9, s, *t*-Bu), 3.76 (1, d, $J_{5'Ha-5'Hb}$ = 13.1 Hz, 5′-Ha), 3.97 (1, dd, *J* = 1.53 Hz, $J_{5'Ha-5'Hb}$ = 13.1 Hz, 5′-Hb), 4.60 (1, br, OH), 4.37 (1, s, 4′-H), 4.68 (1, d, $J_{2'H-3'H}$ = 4.58 Hz, 3′-H), 5.43 (1, m, $J_{1'H-2'H}$ = 7.32 Hz, $J_{2'H-3'H}$ = 4.58 Hz, $J_{P-2'H}$ = 4.88 Hz, 2′-H), 5.89 (1, br, OH), 6.10 (1, d, $J_{1'H-2'H}$ = 7.32 Hz, 1′-H), 7.53 (2, t, *J* = 7.63 Hz, *m*-ArH), 7.63 (1, t, *J* = 7.48 Hz, *p*-ArH), 8.04 (12, d, *J* = 7.62 Hz, *o*-ArH), 8.15 (1, s, 2-H), 8.81 (1, s, 8-H), 9.31 (1, br, NH); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 164.47, 152.09, 150.31, 142.55, 133.41, 132.94, 128.91, 128.30, 127.87, 124.62, 113.12, 87.96, 87.57, 83.34, 83.24, 78.53, 65.82, 63.24, 29.98, 29.90; ^{31}P NMR (40.5 MHz, $CDCl_3$, 85% H_3PO_4) δ −10.71; MS (FAB+) Calcd for $C_{25}H_{35}N_5O_8P$ (M^+ + H) 564.2223, found 564.2258.

When compound 4 was treated with TBAF, the 3′-regioisomer **5b** was obtained along with **5a**. The ^{1}H NMR spectrum of **5b** was analyzed by 500-MHz NMR using ^{1}H–^{1}H COSY technique. **5b**: ^{1}H NMR (500 MHz, $CDCl_3$, TMS) δ 1.55, (9, s, *t*-Bu), 1.56 (9, s, *t*-Bu), 3.79 (1, d, $J_{5'Ha-5'Hb}$ = 13 Hz, 5′-Ha), 3.99 (1, d, $J_{5'Ha-5'Hb}$ = 13 Hz, 5′-Hb), 4.53 (1, s, 4′-H), 5.02 (1, m, $J_{2'H-3'H}$ = 5.0 Hz, $J_{P-3'H}$ = 7.8 Hz, 3′-H), 5.12 (1, m, $J_{1'H-2'H}$ = 7.63 Hz, $J_{2'H-3'H}$ = 5.0 Hz, 2′–H), 5.95 (1, d, $J_{1'H-2'H}$ = 7.63 Hz, 1′-H), 7.47 (2, m, *m*-ArH), 7.56 (1, m, *p*-ArH), 8.00 (12, m, *o*-ArH), 8.28 (1, s, 2-H), 8.75 (1, s, 8-H), 9.39 (1, br, NH); ^{31}P NMR (40.5 MHz, $CDCl_3$, 85% H_3PO_4) δ −9.49.

2′-*O*-(Di-*tert*-butoxyphosphoryl)-3′-*O*-bis[(phenylthio)phosphoryl]-5′-*O*-(4,4′-dimethoxytrityl)-6-*N*-benzoyladenosine (6). Compound **5a** (224 mg, 0.4 mmol) was rendered anhydrous by repeated coevaporation with dry pyridine and finally dissolved in pyridine (5 mL). To the mixture was added 4,4′-dimethoxytrityl chloride (219 mg, 0.45 mmol). After being stirred, the mixture was treated with cyclohexylammonium *S*,*S*-diphenyl phosphorodithioate (229 mg, 0.6 mmol) and 1*H*-tetrazole (112 mg, 1.6 mmol). The mixture was stirred for 45 min and then extracted with CH_2Cl_2–H_2O. The organic layer was collected, washed with 5% $NaHCO_3$, and dried over Na_2SO_4. The solution was 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 6 (345 mg, 76%): ^{1}H NMR (500 MHz, $CDCl_3$, TMS) δ 1.27 (9H, s, *t*-Bu), 1.36 (9H, s, *t*-Bu), 3.38 (2H, dq, $J_{5'Ha-Hb}$ = 10.8 Hz, $J_{4'H-5'H}$ = 5.9 Hz, 5′-H), 3.79 (6H, s, OCH_3), 4.33 (1H, m, 4′-H), 5.53–5.56 (1H, m, 3′-H), 5.82 (1H, d, *J* = 4.88 Hz, 2′-H), 6.36 (1H, d, *J* = 7.3 Hz, 1′-H), 6.82 (6H, m, ArH), 7.15–7.45 (17H, m, ArH), 7.55 (3H, m, ArH), 7.61 (2H, m, ArH), 8.04 (1H, d, *J* = 7.3 Hz, 2-H), 8.29 (1H, s, 8-H), 8.77 (1H, s, 6-NH); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 164.27, 158.34, 152.59, 151.77, 149.36, 143.99, 141.81, 139.26, 135.43, 135.36, 135.18, 135.11, 135.06, 133.46, 132.50, 129.85, 129.288, 128.88, 128.61, 127.89, 127.78, 127.58, 127.55, 126.75, 122.71, 113.06, 112.88, 86.81, 84.78, 83.664, 83.053, 81.17, 74.82, 62.55, 54.95, 29.52, 29.47, 29.43, 29.38; ^{31}P NMR (40.5 MHz, $CDCl_3$, 85% H_3PO_4) δ 51.35 [P(SPh)], −9.93 [P(O-*t*-Bu)]. Anal. Calcd for $C_{58}H_{61}N_5O_{11}P_2S_2\cdot H_2O$: C, 60.67; H, 5.53; N, 6.10. Found: C, 60.68; H, 5.59; N, 6.48.

General Procedure for the Synthesis of Fully Protected 2′-Phosphorylated Dimers. A Typical Example. Compound 6 (136 mg, 0.12 mmol) was treated with pyridine–triethylamine–water (2:2:1, v/v/v, 5 mL) at room temperature for 2 h. The solvent was evaporated under reduced pressure. The residue was mixed with 3-nitro-1*H*-1,2,4-triazole (34.2 mg, 0.3 mmol) and 2′,3′-*O*-(methoxymethylene)-4-*N*-benzoylcytidine (38.9 mg, 0.1 mmol) and the mixture was rendered anhydrous by repeated coevaporation with dry pyridine. The dried mixture was dissolved in dry pyridine (3 mL) and isodurenedisulfonyl dichloride (DDS, 99.4 mg, 0.3 mmol) was added. After being stirred for 40 min, the mixture was quenched by addition of pyridine–water (9:1, v/v, 1 mL). The usual extraction with CH_2Cl_2 followed by column chromatography with 0.5% pyridine-containing eluent of CH_2Cl_2–MeOH to give fully protected dimer 9 (111 mg, 79%).

General Procedure for the Deprotection of Fully Protected 2′-Phosphorylated Dimers. A Typical Example. Compound 9 (14.1 mg, 10 μmol) was dissolved in methanol (20 mL), and concentrated ammonia (20 mL) was added. The mixture was kept at room temperature for 24 h and then evaporated under reduced pressure. The residue was dissolved in acetic acid (3.2 mL), and trifluoroacetic acid (0.8 mL) was added at 0 °C to the mixture. After being kept at room temperature for 4 h, the solution was evaporated and the residue was chromatographed on 3MM papers with iPrOH–concd NH_3–H_2O (7:1:2, v/v/v) to give A(2′-p)pC (8) (132 A_{262} nm, 56%): ^{1}H NMR (500 MHz, D_2O, DSS) δ 3.86 (1H, dd, $J_{5'Ha-4'H}$ = 2.44 Hz, $J_{5'Ha-5'Hb}$ = 13.13 Hz, A/5′-Ha), 3.90 (1H, dd, $J_{5'Hb-4'H}$ = 3.05 Hz, $J_{5'Ha-5'Hb}$ = 13.13 Hz, A/5′-Hb), 4.21 (1H, br d, $J_{5'Ha-5'Hb}$ = 12.21 Hz, C/5′-Ha), 4.31 (5H, m, C/2′-H, 3′-H, 4′-H, and 5′-Hb), 4.50 (1H, s, A/4′-H), 4.89 (1H, m, A/3′-H), 5.31 (1H, m, A/2′-H), 5.91 (1H, d, *J* = 3.87, 1′-H), 6.02 (1H, d, *J* = 7.63 Hz, C/5-H), 6.17 (1H, d, *J* = 6.40, 1′-H), 7.93 (1H, *J* = 7.63, C/6-H), 8.21 (1H, s, A/2-H), 8.33 (1H, s, A/8-H); ^{13}C NMR (125.65 MHz, D_2O) δ 168.46, 158.35, 155.22, 153.50, 151.34, 143.79, 143.45, 121.89, 98.90, 92.12, 89.33, 87.81, 85.25, 76.85, 71.77, 67.64, 64.05, 62.03; ^{31}P NMR (202 MHz, D_2O, 85% H_3PO_4) δ 0.60, −0.39; MS (FAB^-) calcd for $C_{19}H_{25}N_8O_{14}P_2$ (M − H)$^-$ 651.0965, found 651.0984; UV (H_2O) λ_{max} 262 nm, λ_{min} 228 nm; R_f 0.34 (solvent system: iPrOH–concd NH_3–H_2O, 6:1:3, v/v/v); HPLC retention time 14.0 min.

Synthesis of Fully Protected 2′-Phosphorylated A(2′-p)pU Derivative (16). As described in General Procedure for the Synthesis of Fully Protected 2′-Phosphorylated Dimers, compound 16 was synthesized in 70% yield by using 2′,3′,N^3-tribenzoyluridine as the 3′-component.

Synthesis of Fully Protected 2′-Phosphorylated A(2′-p)pA(2′-p)pU Derivative (18). Compound 16 (110 mg, 69 μmol) was treated with trifluoroacetic acid (30 μL) in CH_2Cl_2 (3 mL) at 0 °C for 30 min. Pyridine (5 mL) was added and the mixture was partitioned between CH_2Cl_2–5% $NaHCO_3$. The organic layer was collected and dried over Na_2SO_4. The solution was 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 17 (82 mg, 92%). This material (80 mg, 74 μmol) was mixed with 7, which was prepared as previously described, and 3-nitro-1*H*-1,2,4-triazole (34 mg, 0.3 mmol) and the resulting mixture was rendered anhydrous by repeated coevaporation with dry pyridine. The dried mixture was dissolved in dry pyridine (3 mL) and DDS (99.4 mg, 0.3 mmol) was added. Stirring was continued for 90 min and then the reaction was stopped by addition of pyridine–water (9:1, v/v).

The usual workup was followed by silica gel column chromatography to give the title compound **18** (99 mg, 69%).

Deprotection of Fully Protected 2′-Phosphorylated A(2′-p)pA(2′-p)pU Derivative (18). Compound **18** (14.1 mg, 10 μmol) was dissolved in methanol (2 mL) and concentrated ammonia (2 mL) was added. The resulting mixture was kept with stirring at 40 °C for 4 h, and the mixture was evaporated under reduced pressure. The residue was dissolved in acetic acid (3.2 mL), and trifluoroacetic acid (0.8 mL) was added at 0 °C. The mixture was stirred at room temperature for 3 h and then evaporated under reduced pressure. The residue was washed with ether (10 mL) and chromatographed on Whatman 3MM papers with iPrOH–concd NH_3–H_2O (7:1:2, v/v/v) to give A(2′-p)pA(2′-p)pU (**19**) (160 A_{257} nm, 56%). This compound was further purified by reverse-phase HPLC to give pure material: ^{1}H NMR (500 MHz, D_2O, DSS) δ 3.71 (1, dd, $J_{5'Ha-4'H}$ = 12 Hz, $J_{5'Ha-5'Hb}$ = 42.5 Hz, A/5′-Ha), 3.79 (1, dd, $J_{5'Hb-4'H}$ = 13 Hz, $J_{5'Ha-5'Hb}$ = 42.5 Hz, A/5′-Hb), 4.20–5.20 (11, m, A/3′, 4′-H, A′/3′,4′,5′-H, and U/2′,3′,4′,5′-H), 5.27 and 5.31 (2, br, A/2′-H and A′/2′-H), 5.85 (1, d, J = 8.0 Hz, U/5-H), 5.97 (1, d, J = 4.5 Hz, U/1′-H), 6.02 and 6.30 (2, br s, A/1′-H and A′/1′-H), 7.88 (1, d, J = 8.0 Hz, U/6-H), 8.21, 8.27, 8.32 (3, s, 2-H and 8-H of A and A′), 8.49 (1, br, 2-H or 8-H of A and A′); ^{13}C NMR (125.65 MHz, D_2O) δ 158.26, 157.94, 155.34, 154.84, 154.21, 144.09, 105.02, 91.07, 89.91, 88.25, 87.28, 84.34, 77.68, 76.37, 76.36, 72.11, 67.90, 64.13; ^{31}P NMR (D_2O, 85% H_3PO_4) δ -0.40 (broad); MS (FAB$^-$) calcd for $C_{29}H_{37}N_{12}O_{24}P_4$ $(M - H)^-$ 1061.0994, found 1061.1017; UV (H_2O) λ_{max} 257 nm, λ_{min} 227 nm; R_f 0.08 (solvent system: iPrOH–concd NH_3–H_2O, 6:1:3, v/v/v); HPLC retention time 15.9 min.

Enzymatic Treatment of A(2′-p)pX (X = C, U, A, and G) with Calf Intestinal Alkaline Phosphatase. An appropriate A(2′-p)pX (0.2 A at its λ_{max}) was dissolved in 0.05 M Tris-HCl (pH 8.5, 100 μL) containing 0.1 M EDTA and 20 μL of calf intestinal alkaline phosphatase (2 units, 0.1 units/μL) in glycerin–water (1:1, v/v) was added. The resulting mixture was incubated at 50 °C for 30 min and then heated at 100 °C for 30 s. The mixture was analyzed by reverse-phase HPLC and the main peak was collected. The fractions collected were freeze-dried two times and used in the next enzyme reaction.

Enzymatic Treatment of ApX (X = C, U, A, and G) with Nuclease P1. An appropriate ApX (0.2 A at its λ_{max}) collected in the above experiment was dissolved in 0.05 M AcOH–AcONa (pH 5.3, 100 μL) containing 0.1 mM $ZnCl_2$, and 10 μL of nuclease P1 (10 units, 1 units/μL) in glycerine–water (1:1, v/v) was added. The resulting mixture was incubated at 50 °C for 30 min and then heated at 100 °C for 30 s. The mixture was analyzed by reverse-phase HPLC.

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Supplementary Material Available: ^{1}H NMR (500 MHz) spectra of 2′-phosphorylated oligoribonucleotides **8**, **13–15**, and **19**, ^{1}H–^{1}H COSY NMR spectrum of **19**, ^{13}C NMR (67.8 and 125.65 MHz) spectra of **3**, **4**, **5a**, **6**, **8**, and **19**, the ^{31}P NMR spectrum of **8** and **19**, FAB mass spectral data of **4** and **5b**, the ^{31}P NMR spectrum of a mixture of **5a** and **5b** obtained by treatment of **2** with TBAF in THF (Figure 3a), and the ^{31}P NMR spectrum of a mixture of **5a** and **5b** obtained by treatment of **4** with PPHF in THF (Figure 3b) (16 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.