Chemical Synthesis of Branched Oligoribonucleotides

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This paper describes the full detail of the chemical synthesis of branched oligoribonucleotides by use of appropriately protected adenosine 2′,3′-diphosphate derivatives (**13** and **30**), as key intermediates, which were synthesized by a series of reactions involving phosphoryl rearrangement via a new phosphorylation process. Branched triribonucleotides A^{pG}_{pC} (**21**) and A^{pG}_{pU} (**27**) were obtained by using the synthetic unit **13**. Several problems during these syntheses were discussed. The use of **30** as an improved building unit enabled us to prepare synthetic intermediates as neutral, i.e., fully esterified species which allowed facile purification of them by means of silica-gel column thin-layer chromatography throughout the synthesis of a branched hexaribonucleotide derivative $CpUpGpA^{pG}_{pC}$.

Since Wallace and Edmonds[1] discovered branched RNAs, this unique structure has been found as part of the so-called lariat structure in splicing products of messenger RNAs in a variety of eukaryotic cells.[2]

Chemical synthesis of branched RNAs has been reported in several laboratories.[3-15] In this paper,# we report the detailed study of chemical synthesis of branched oligoribonucleotides, involving the regioselective introduction of two different phosphoryl groups into the 2′- and 3′-positions of adenosine at the branched point. Preliminary results of this study have already been reported.[3,13]

Results and Discussion

To introduce a protected phosphate group into the 2′-hydroxyl group of adenosine which is the ribonucleoside found in the target branched RNA, the following method for phosphorylation of the 2′-hydroxyl group of silylated adenosine derivative (**1**) was explored. Caruthers[16] has reported the P-N bond activation of deoxyribonucleoside phosphoramidite derivatives with tetrazole for rapid internucleotidic bond formation. On the other hand, we described previously the synthesis of deoxyribonucleoside phosphonates via phosphorodiamidite intermediates.[17]

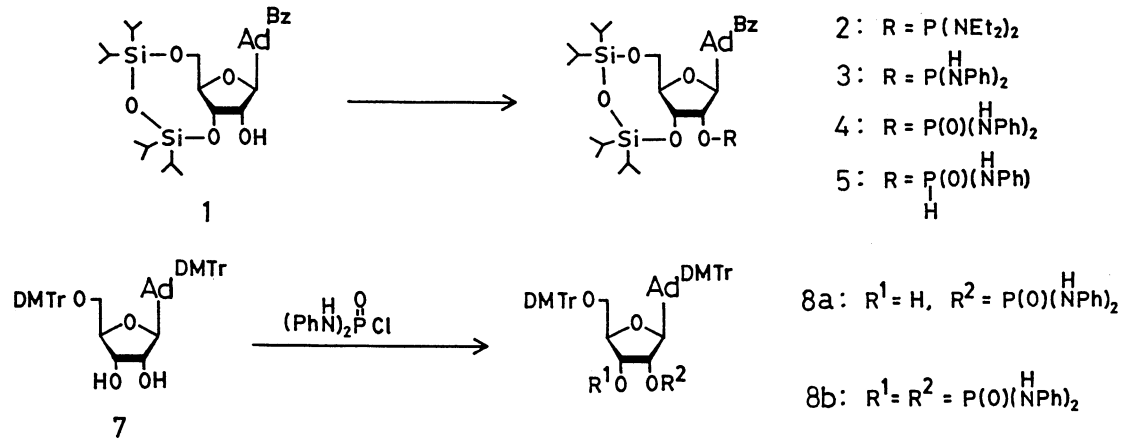
On the basis of these facts, **1** was allowed to react with 1.5 equiv of tris(diethylamino)phosphine (TEAP) in the presence of tetrazole in dichloromethane. The resulting phosphorodiamidite intermediate (**2**) was further treated with 4 equiv of aniline in the presence of 4 equiv of tetrazole for transamidation to give the phosphorodianilidite derivative (**3**). Addition of 1.5 equiv of iodine in pyridine to the mixture resulted in the phosphorylated species (**4**).

Desilylation of **4** with KF-Et_4NBr-H_2O in acetonitrile gave a mixture of the 2′,5′-diol derivative (**6a**) and 3′,5′-diol derivative (**6b**). Fortunately, the latter was easily crystallized from the mixture by evaporation of MeOH from its 1,2-dichloroethane-MeOH solution. However, the former did not crystallize from any solvents tested and decomposed upon silica-gel column chromatography. Therefore, we examined the possibility of isomerization of **6a** to **6b** in a variety of solvents. As a result, it was found that **6a** was gradually isomerized to **6b** in pyridine-water (9 : 1, v/v) at 40 °C for 1 h to attain equilibrium giving rise to a ca. 1 : 1 mixture of **6a** and **6b**. It is interesting that the above isomerization did not occur in anhydrous pyridine. Such effects of water on isomerization have also been reported in the case of the 2′-3′ silyl migration by Ogilvie and his co-workers.[18] Thus, compounds **6b** was ultimately obtained in 55% yield by repeated isomerization of the **6a**-rich filtrate followed by crystallization.

Attempts to obtain monophosphorylated adenosine derivative **8a** or **8b** by reaction of 5′-*O*: 6-*N*-bis(4,4′-dimethoxytrityl)adenosine (**7**) with *N*,*N*′-diphenylphosphorodiamidic chloride[19] failed since chromatographic separation of these compounds caused considerable decomposition.

With the hope that the isomerization could be suppressed by modification of the anilino group with an electron-donating *p*-substituent or by replacement of the P=O by the P=S bond, the corresponding 2′-[*N*,*N*′-bis(*p*-methoxyphenyl)phosphorodiamidate] (**9**) and 2′-(*N*,*N*′-diphenylphosphorodiamidothioate) (**10**) were synthesized in 84 and 75% yields from **1** via

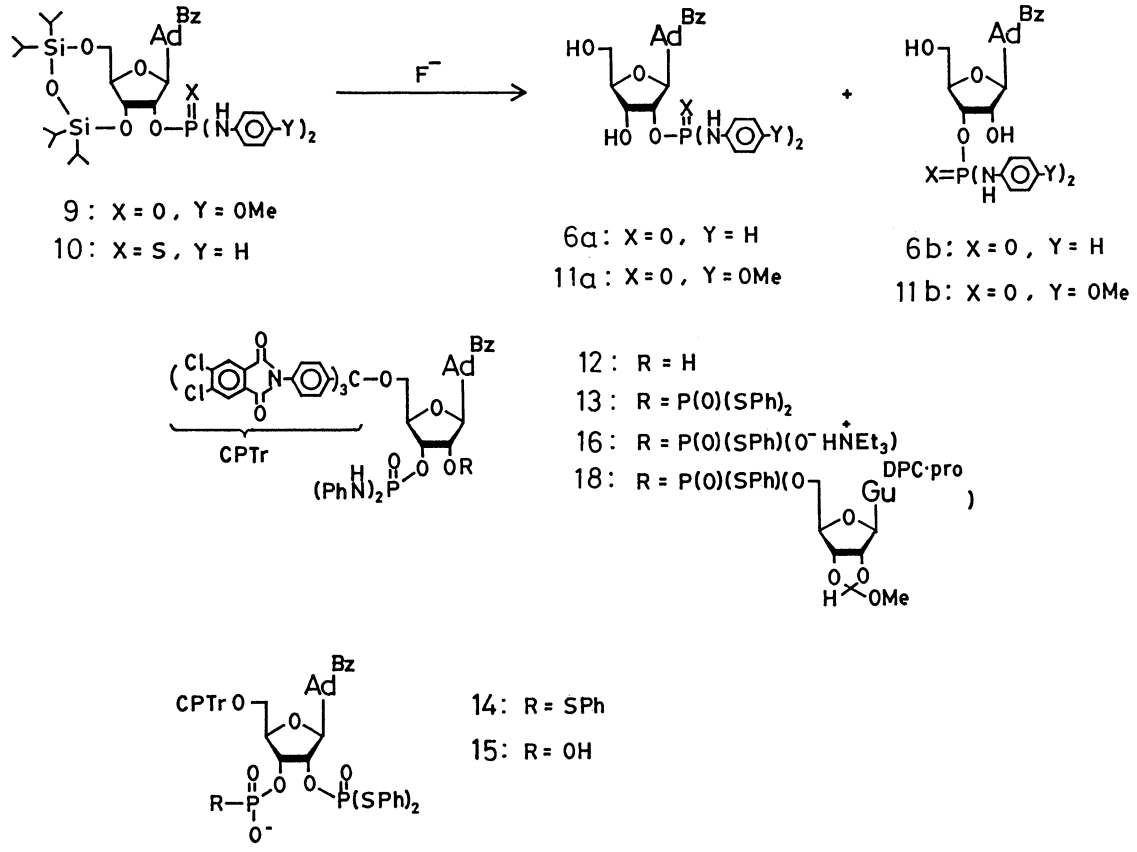
In this paper the following abbreviations are used: Tris(diethylamino)phosphine (TEAP); 4,4′,4″-tris(4,5-dichlorophthalimido)trityl (or tris[4(4,5-dichlorophthalimido)phenyl]methyl) (CPTr); 4,4′,4″-tris(4,5-dichlorophthalimido)trityl bromide (or tris[4-(4,5-dichlorophthalimido)phenyl]methyl bromide) (CPTrBr); bis(anilino)phosphinyl (PNN); cyclohexylammonium *S*,*S*-diphenyl phosphorodithioate (PSS); *N*,*N*′-dicyclohexylcarbodiimide (DCC); mesitylenedisulfonyl dichloride (MDS); isodurenedisulfonyl dichloride (or 1,2,3,5-tetramethylbenzenedisulfonyl dichloride) (DDS); 3-nitro-1*H*-1,2,4-triazole (NT); pyridinium *m*-nitrobenzenesulfonate (PNBS); 4-methoxytrityl (MMTr); 4,4′-dimethoxytrityl (DMTr); 2-cyanoethyl (CE); *N*,*N*-diphenylcarbamoyl (DPC); propionyl (pro); benzoyl (Bz); triethylammonium hydrogencarbonate (or triethylammonium bicarbonate) (TEAB); 2,2-dimethyl-2-silapentane-5-sulfonic acid sodium salt (DSS); snake venom phosphodiesterase (VSP); calf spleen phosphodiesterase (CSP).



similar iodine oxidation and sulfurization, respectively. However, desilylation of **9** resulted in a similar isomerization to give **11a** and **11b** in 18% and 42% yields, respectively. Unexpectedly, desilylation of **10** caused considerable decomposition.

We tried to synthesize a branched triribonucleotide A^{pG}_{pC} by the use of the predominant product **6b** above-mentioned. The 5′-hydroxyl group of **6b** was protected with tris[4-(4,5-dichlorophthalimido)-phenyl]methyl (CPTr) which has recently been proposed as a hydrazine-labile protecting group of primary alcohols.[20] Reaction of **6b** with 2 equiv of tris[4-(4,5-dichlorophthalimido)phenyl]methyl bromide (CPTrBr) in the presence of 2 equiv of silver nitrate in *N*,*N*-dimethylformamide (DMF) gave the 5′-tritylated product (**12**) in 57% yield. Isomerization or decomposition of the 3′-bis(anilino)phosphinyl (PNN) group did not occur during the tritylation.

Phosphorylation of the 2′-hydroxyl group of **12** should be done in a manner where each protecting group of the 2′- and 3′-phosphate groups can be removed without damage of the other protecting group under different conditions in order to elongate the RNA chain in the 2′- or 3′-direction. If possible no chiral center should be introducted on the second phosphorus atom as well as the first as seen in the PNN group to avoid complexity on TLC or for analysis of further reactions. Therefore, we chose the phe-



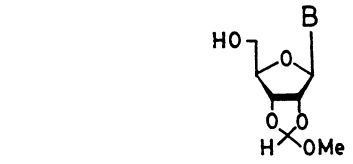
nylthio group for protection of both hydroxyl groups of the second phosphate function, since bis(phenylthio)phosphinyl group was known to be introduced rapidly into hydroxyl groups by reaction with cyclohexylammonium *S,S*-diphenyl phosphorodithioate (PSS)[21] in the presence of tetrazole[20] by use of a bifunctional condensing reagent, mesitylenedisulfonyl dichloride (MDS).[22] Application of this procedure gave a fully protected adenosine 2′,3′-diphosphate derivative (**13**) in 81% yield.

The ^{31}P NMR spectrum of **13** showed indeed two sharp resonance signals at 1.94 and 54.74 ppm. The latter signal was characteristic of *O*-alkyl *S,S*-diphenyl phosphorodithioates. For example, the resonance peak of *S,S*-diphenyl 5′-*O*-(4,4′-dimethoxytrityl)-thymidine 3′-phosphorodithioate[22] appeared at 49.63 ppm in $CDCl_3$. Thus, the homogenity of **13** could be readily ascertained by this spectroscopic analysis.

Next, we attempted to synthesize an appropriately protected adenosine 2′-phosphorothioate derivative (**14**), which seemed to be a fascinating intermediate for chain elongation in the 3′-direction since the same phosphate protecting group could be used commonly for both 2′- and 3′-side chains, from **13** by a two-step reaction. Treatment of **13** with isopentyl nitrite[23] in the presence of acetic anhydride[24] in pyridine-acetic acid for 15 h gave the 2′-unprotected adenylate derivative (**15**) in 50% yield. Under these conditions the phenylthio group was found to be stable as evidenced by ^{31}P NMR of the reaction mixture which showed a characteristic resonance peak at 51.11 ppm due to the 3′-bis(phenylthio)phosphinyl group.

However, conversion of **15** to **14** by treatment with 20 equiv each of tributylphosphine and diphenyl disulfide in acetonitrile[25] resulted in formation of an unexpected product without P-S bond formation.[26] Attempts to introduce the 2-chlorophenyl group into the 3′-phosphate of **15** have also failed. Dicyclohexylcarbodiimide (DCC) promoted reaction gave predominantly a symmetric pyrophosphate derivative of **15**.

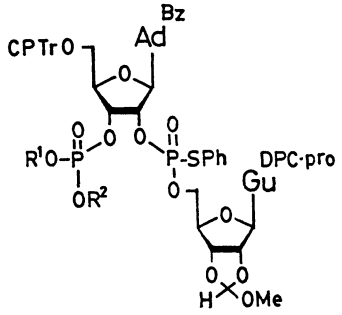
Therefore, our attention has been turned to the selective removal of one of the two phenylthio groups from the 2′-PSS group of **13** to change our original project of the first internucleotidic bond formation from the 3′-5′ to 2′-5′ direction. Treatment of **13** with phosphinic acid in pyridine gave the 2′-phosphorothioate derivative (**16**) in 93% yield. Under these conditions the other protecting groups were found to be stable. The ^{31}P NMR spectrum of **16** showed a set of two sharp resonance signals at 15.65 and −0.19 ppm. The former peak was assigned to the 2′-phosphorus of a diester-type of phosphoromonothioate from its chemical shift. Condensation of the resulting 2′-phosphorothioate **16** with 2′,3′-*O*-methoxymethylene-2-*N*-propionyl-6-*O*-diphenylcarbamoylguanosine (**17**) in the presence of 1,2,3,5-tetramethylbenzenedisulfonyl dichloride (DDS)[22] and 3-nitro-1,2,4-triazole (NT)[27] in pyridine for 80 min gave the 2′-5′ linked triester (**18**) in 90% yield. This



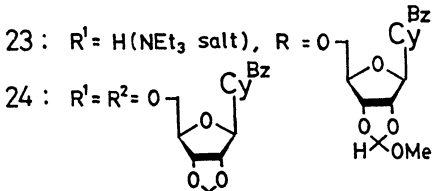
17 : B = Gu$^{DPC\text{-}pro}$

20 : B = Cy^{Bz}

28 : B = Ur^{Bz}

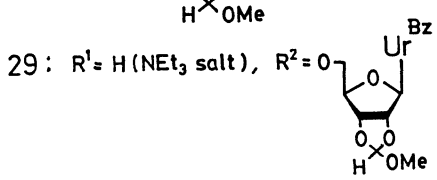


19 : R^1 = OH , R^2 = H (NEt_3 salt)

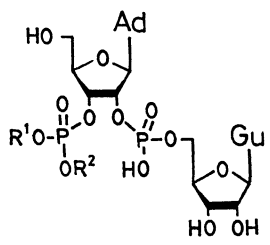


23 : R^1 = H(NEt_3 salt), R = O-Cy^{Bz}

24 : R^1 = R^2 = O-Cy^{Bz} , O-Cy^{Bz}



29 : R^1 = H(NEt_3 salt), R^2 = O-Ur^{Bz}



21 : R^1 = OH , R^2 = Cytidine

22 : R^1 = R^2 = Cytidine

25 : Symmetric pyrophosphate of 21

26 : R^1 = R^2 = OH

27 : R^1 = OH , R^2 = Uridine

dimer should have four diastereomers due to the chirality of the trisubstituted 2′-5′ linked phosphorus and the methoxymethylene group. However, the ^{31}P NNR spectrum of **18** showed two peaks at 26.76 and 26.59 ppm and a broad signal peak at 2.23 ppm, suggesting that the methoxymethylene group did not affect the chemical shift significantly since it was remote from the diasteromeric phosphorus atom.

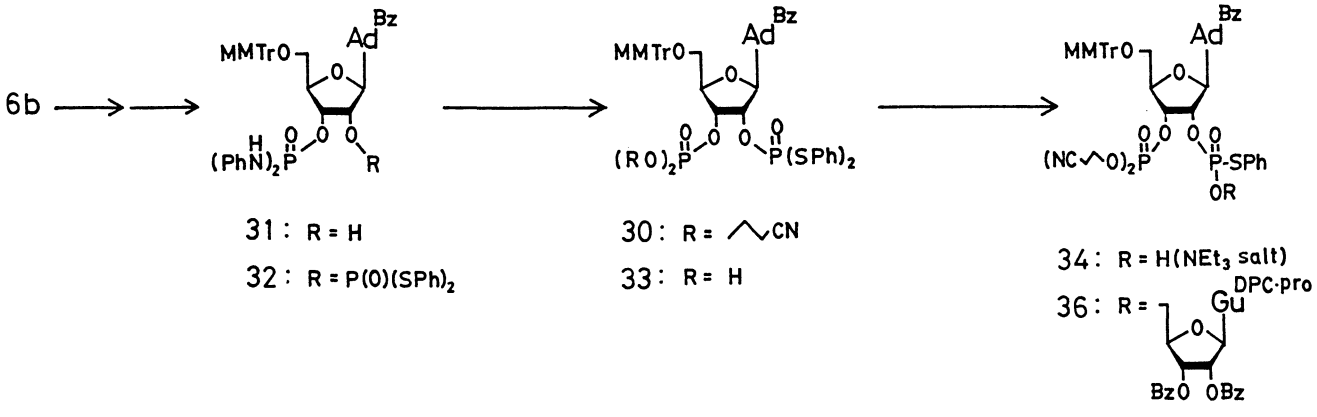
The anilino group was removed from **18** by the isopentyl nitrite treatment as described before to afford the 3′-unesterified phosphate (**19**) in 75% yield. An attempt to introduce the phenylthio group into the phosphate group by means of Bu_3P-PhSSPh has also failed as in the case of **15**. Therefore, direct condensation of **19** with 2′,3′-*O*-methoxymethylene-N^4-benzoylcytidine (**20**) in the phosphodiester method using DDS as the condensing reagent was performed. However, large excess amounts of **20** (8 equiv) and DDS (7 equiv) were required until **19** had disappeared (see Experimental). This reaction ultimately gave a mixture of the desired branched triribonucleotide (**21**) and a doubly esterified tetraribonucleoside diphosphate derivative (**22**) after deprotection.

Since DCC is known as the condensing agent that gives only phosphodiester products without formation of phosphotriesters,[28] the compound **22** should not be obtained when the condensing agent was changed from DDS to DCC. To ascertain this expectation, the condensation was performed in the presence of DCC. Consequently, the reaction became simpler and only two principal products were observed along with recovery of A_p^{pG}. It was indeed confirmed that compound **22** was not formed in the reaction. Since compound **19** was the triethylammonium salt which was difficult for activation with DCC, pyridinium 3-nitrobenzenesulfonate (PNBS)[29] was added as an acid catalyst. Without this salt the reaction was extremely slow to given **21** in only 2% yield after one day. With the improved condensation, the isolated yield of **21** was increased up to 29%. A significant amount of symmetrical pyrophosphate derivative (**25**) was formed as the by product. DCC-mediated condensation usually produced such relatively unreactive species.[28] In this case, it was difficult to activate the pyrophosphate intermediate **25** for condensation with **20**. Prolonged reaction for 4 days did not improve the yield of **21** although A_p^{pG} disappeared.

The structural assignment of **21** was also supported by characterization of the deprotected material of the synthetic intermediate **18**. Incubation of the product with bacterial alkaline phosphatase gave a dephosphorylated material which was digested to A and pG with snake venom phosphodiesterase and resistant to nuclease P_1. Incubation of this material with other enzymes such as Ribonucleases T1, T2, and A, and spleen phosphodiesterase did not result in any change. These properties were in agreement with those of 2′-5′ linked ApG having an internal 3′-phosphate.

In a manner similar to that described in the synthesis of **21**, A_{pU}^{pG} (**27**) was also synthesized via compound **29** which was obtained in 21% yield by DCC-mediated condensation of **19** with 2′,3′-*O*-methoxymethylene-N^3-benzoyluridine (**28**), and characterized by the enzymatic assay. Finally, the structure of A_{pC}^{pG} and A_{pU}^{pG} was confirmed by 500 MHz ^{1}H NMR. As the reference materials, linear triribonucleotide diphosphates, GpApC and GpApU were synthesized according to our method reported previously.[20]

Synthesis of Branched Ribonucleotides via a New Fully Protected Synthetic Unit (30). As described above, the condensation of **23** with **20** or **28** in the phosphodiester approach gave charged products which were bothersome for isolation and further chain elongation. Therefore, we designed a more general approach to branched oligoribonucleotides. Since the CPTr group was rather stable under the conditions of removal of the anilino group which involved pyridine buffered by acetic acid, it was employed for the synthesis of the simplest branched triribonucleotide blocks in the previous method. However, this protecting group required restricted conditions for chain elongation, especially in the 5′-direction, where the pivaloyloxymethyl, 4-methoxytrityl (MMTr), and 4,4′-dimethoxytrityl (DMTr) groups should be used for protection of the



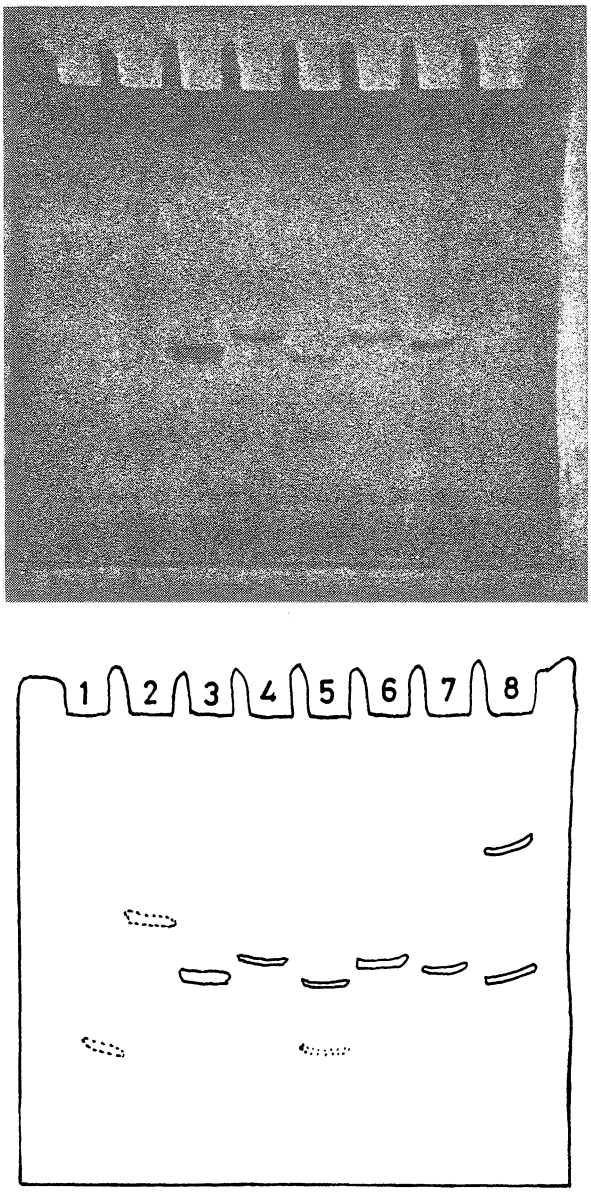


Fig. 1. 20% Polyacrylamide gel electrophoresis of branched and oligoribonucleotides and related compounds. 1: A_p^{pG} **26**; 2: $^{pG}_{(pC)_2}$ **22**; 3: A_{pC}^{pG}; 4: GpApC; 5: a ca. 4:1 mixture of A_{pU}^{pG} and A_p^{pG}; 6: GpApU; 7: $CpUpGpA_{pC}^{pG}$ 8: Bromphenol Blue and Xylene Cyanole FF as markers.

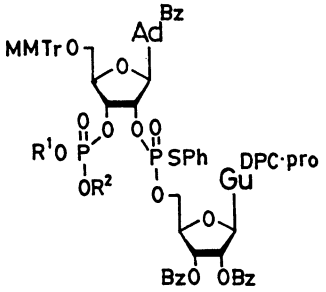
base residues of uridine, adenosine, and cytidine, respectively.[20] Since the DMTr group was somewhat too unstable during the isopentyl nitrite treatment, the MMTr group[30] was chosen as the 5′-hydroxyl protecting group. It was desirable to find more suitable protecting groups for the 3′-phosphate group since removal of the anilino group required a long time and tedious extractive workup. It was again considered that if possible no chiral centers should be produced when a new phosphate protecting group was designed.

On the basis of the above discussion, the anilino group should be changed to the 2-cyanoethyl (CE) group at the stage of the monomer building unit. The new synthetic unit at the branched point was a fully protected adenosine 2′,3′-diphosphate derivative (**30**). Reaction of **6b** with 4-methoxytrityl chloride followed by the successive phosphorylation of the resulting tritylated product (**31**) gave the fully protected adenosine derivative (**32**). Treatment of **32** with isopentyl nitrite in a manner similar to that described in the conversion of **13** to **15** gave the 3′-unesterified derivative (**33**) in 55% yield. Biscyanoethylation of **33** with excess 2-cyanoethanol in the presence of DDS in pyridine gave a fully protected adenosine 2′,3′-diphosphate derivative **30** in 67% yield. The ^{31}P NMR spectrum of **30** exhibited two sharp resonance signals at −3.10 and 51.98 ppm which were due to the 3′- and 2′-phosphoryl groups, respectively. The intermediates **32** and **33** also gave similar ^{31}P NMR spectra as summarized in Table 1.

The protection of each phosphoryl group with the same two protecting groups proved to be indeed useful for the structural assignment of synthetic interme-

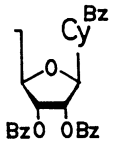
Table 1. ^{31}P NMR Spectra of Synthetic Intermediates and Branched Ribonucleotide Derivatives

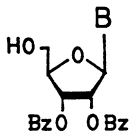
Compound	Solvent	Signals (ppm)		
3	$CDCl_3$	3.221		
6a	$CDCl_3$-Py (9:1, v/v)	3.003		
6b	$CDCl_3$-Py-MeOH (10:6:1, v/v/v)	2.857		
9	$CDCl_3$	5.933		
12	$CDCl_3$	2.518		
13	$CDCl_3$	1.937	54.737	
16	$CDCl_3$	−0.193	15.646	
18	$CDCl_3$	2.228	26.594 26.763	
23+24	$CDCl_3$	−3.003 −2.785 −2.640 −2.373 −2.276	24.292 24.583 24.777 25.189 25.261	25.382 25.649 25.746 25.915 26.012
30	$CDCl_3$	−3.100	51.977	
32	$CDCl_3$	3.100	55.756	
33	$CDCl_3$	−9.010	51.154	
34	$CDCl_3$	−3.234	13.374	
36	$CDCl_3$	−2.907 −3.270	24.822 25.694	
37	$CDCl_3$	−2.653	24.277 24.895	
39	$CDCl_3$	−2.216 −2.289 −2.471	24.786 24.931 25.258 25.367	



37 : R^1 = ∼CN, R^2 = H(NEt_3 salt)

39 : R^1 = ∼CN, R^2 =

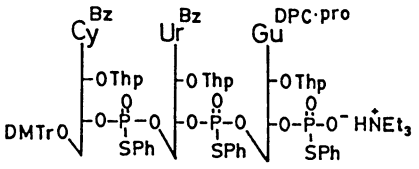




35 : B = $Gu^{DPC\cdot pro}$

38 : B = Cy^{Bz}

diates during the construction of the branched tribonucleotide block. Upon treatment of **30** with the phosphinate reagent gave quantitatively phosphodiester derivative (**34**). The high selectivity of this conversion was confirmed by ^{31}P NMR. Condensation of **34** with 2′,3′-di-*O*-benzoyl-6-*O*-diphenylcarbamoyl-2-*N*-propionylguanosine (**35**) gave the 2′-5′ linked dinucleotide derivative (**36**) in 87% yield. The decyanoethylation of **36** with *N*,*N*-diisopropylethylamine in pyridine at 40 °C for 4 h afforded the 3′-phosphodiester intermediate (**37**) which was purified by preparative TLC and obtained as triethylammonium salt in 82% yield. A similar condensation of **37** with 2′,3′-*O*: N^6-tribenzoylcytidine (**38**) gave the fully protected tribonucleotide unit (**39**) in 63% yield. This unit exhibited the ^{31}P NMR spectrum in which two kinds of resonance signals appeared at ca. −2.3 and 25.5 ppm. The former consisted of three peaks at −2.47, −2.29, and −2.22 ppm in a 2:1:1 ratio. The highest peak seemed to be imposed with



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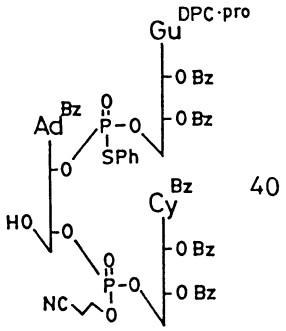

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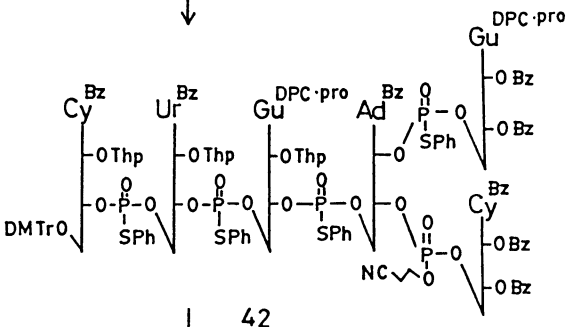

42

CpUpGpA$^{pG}_{pC}$ 43

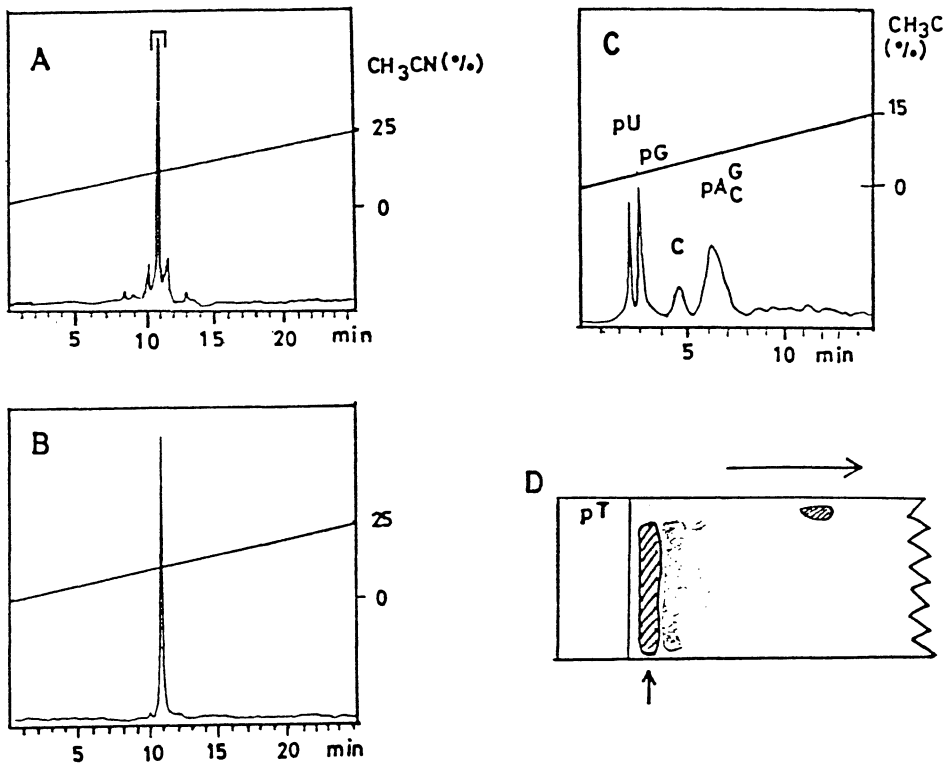

Fig. 2. A: HPLC profile of the crude branched hexaribonucleotide obtained after paper chromatography as shown in Fig. 2D. B: HPLC profile of the purified branched hexaribonucleotide. C: Enzyme analysis of the purified branched hexaribonucleotide with nuclease P_1. D: Paper chromatography of the mixture obtained by deprotection of the fully protected branched hexaribonucleotide (**42**).

two different peaks. The latter involved two sets of doublets as shown in Table 1.

Since compound **39** was neutral species unlike **23**, it was possible to extend the chain in the 5′-direction after removal of the MMTr group. Acid treatment of **39** with 1% trifluoroacetic acid gave the 5′-hydroxyl compound (**40**), which was further condensed with a triribonucleotide block (**41**) to give the fully protected hexaribonucleotide derivative (**42**) in 56% yield. The trimer **41** was synthesized according to our standard method.[31,32]

Deprotection of **42** was achieved by the following procedure: 1) 0.5 M (M=mol dm^{-3}) bis(tributyltin) oxide in pyridine r.t. 28 h.[33] 2) trimethylsilyl chloride in pyridine, r.t., 15 min. 3) concd aqueous ammonia-pyridine, r.t., 19 h. 4) 0.02 M HCl-dioxane, r.t., 48 h. 5) paper chromatography, *i*-PrOH-concd ammonia-water. Thus, the unprotected hexamer CUGA_{pC}^{pG} (**43**) was isolated in 32% yield. The HPLC profile of the mixture obtained after deprotection is shown in Fig. 2A. The peak at 10.8 min was collected and characterized by enzyme analysis as the desired branched hexaribonucleotide. As shown in Fig. 2C, digestion of this material with nuclease P1 gave four products which were identified with pU,pG, C, and pA_{pC}^{pG}. The latter was further dephosphorylated with alkaline phosphatase to give A_{pC}^{pG} which was also identified with authentic material of **21**.

The branched trimers A_{pC}^{pG} and A_{pC}^{pG} were not phosphorylated with kinase as reported by Caruthers.[5] On the other hand, the branched hexamer could be expectedly labelled with this enzyme in the presence of [γ-^{32}p]ATP to give ^{32}pCpUpGpA_{pC}^{pG} (data not shown).[34]

Finally, the synthetic branched trimers and hexamer were tested for inhibition experiments of splicing reaction using the extract from Hela cell. However, our preliminary results revealed that significant inhibitory effects were not observed under the standard conditions.[34] This is probably because the lariat structure was the ultimate deletion product and did not interact with splicing intermediates.

Conclusion

There have been reported several strategies for construction of branched ribonucleotide fragments. They are mainly classified in two types. One is the use of monomer building blocks having 2′,3′-protected phosphoryl or phosphoramidite groups represented by this research and Ogilvie's original approach.[4] The other involves condensation of 2′- or 3′-phosphorylated species with ribonucleoside 5′-

phosphoramidite units. In the former case, it is possible to add stepwise the oligonucleotide chain in any direction, as shown by the present study. However, multistep reactions for synthesis of such building units are required. Indeed, our approach involves a series of seven reactions to obtain the unit **30** from 6-*N*-benzoyladenosine. Although some improvements should be done to shorten the number of the step, the unit **30** is easy to handle and can be stored for long periods of time without decomposition since this material is a neutral species.

As one of the representative approaches for the latter classification, Kierzek and Caruthers[5] reported the regioselective condensation of 2′-side chains with 3′-5′ linked dimer derivatives in which the internal phosphate and 2′-hydroxyl groups were unprotected. In this case, the number of reactions was less than that of ours but synthetic intermediates and final products were charged molecules. Chattopadhyaya[8] has used a combination of the *p*-toluoyl and 9-phenylxanthene-9-yl groups as the 5′- and 2′-protecting groups, respectively, for his synthesis of branched trimers using a similar amidite approach. Chattopadhyaya[9] also applied the 5′-selective cleavage of Markiewicz's protecting group[35] with dilute HCl to the synthesis of branched tetramers. This method still involved a series of charged intermediates. More recently, his research group reported an improved synthesis of neutral protected branched trimer blocks from a 2′-phosphorylated 6-*N*-benzoyladenosine derivative.[15] The latter intermediate, however, was prepared via 7 step-reactions from 6-*N*-benzoyladenosine. The 3′-5′ phosphate bond formation required large excess amounts (ca. 3.5—9 equiv) of 3′-components bearing the phosphoramidite group. The other approaches reported up to date have encountered similar drawbacks. In our own approach, new methods for regioselective phosphorylation in a more straightfoward manner should be explored. We are now studying improved procedures in this direction.

Experimental

Melting points were determined on a Mitamura MELT-POINTER apparatus and are uncorrected. ^{1}H NMR spectra were recorded at 60 MHz on a Hitachi 24B spectrometer, at 100 MHz on a JEOL UNM PS-100 spectrometer, and at 270 MHz on a JEOL GX270 with Me_4Si as the internal standard and at 500 MHz on a JEOL GX500 with DDS as the internal reference. UV spectra were obtained on a Hitachi 220A spectrophotometer. Paper chromatography was performed by use of a descending technique with Whatman 3MM papers and Toyo Roshi 51A papers using the following solvent systems: Solvent I (2-propanol-concd aq ammonia-water. 7:1:2, v/v/v); Solvent II (2-propanol-concd aq ammonia-water, 6:1:3, v/v/v); Solvent III (1-propanol-concd aq ammonia-water, 55:10:35, 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. TLC was performed on precoated TLC plates of silica gel 60 F-254 (Merck). Reversed-phase HPLC was performed on a Waters Model A25 using a μ Bondapack 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 1.5 ml min^{-1} for 30 min (System A). Ribonucleosides were purchased from Yamasa Co., Ltd. Pyridine was distilled two times from *p*-toluenesulfonyl chloride and from calcium hydride and then stored over molecular sieves 3A. The linear trimers of GpApC and GpApU were prepared by condensation of $C^{P}TrG^{DPC\cdot pro}$(thp)p(SPh)A^{MMTr}(thp)($O^-HN^+Et_3$) with $C^{bz}(bz)_2$ or $U^{bz}(bz)_2$, respectively, followed by the usual deprotection procedure. The detail of these trimers will be reported elsewhere. The yields of branched oligoribonucleotides were estimated by using following ε values at λ_{max}: A_p^{pG} 29.9×10^3 at 257 nm, A_{pC}^{pG} 27.5×10^3 at 258 nm, A_{pU}^{pG} 33.5×10^3 at 256.5 nm, CpUpGpA_{pC}^{pG} 48.3×10^3 at 257 nm. Elemental analyses were performed by the Microanalytical Laboratory, Tokyo Institute of Technology, at Nagatsuta.

5′-*O*: 6-*N*-Bis(4,4′-dimethoxytrityl)adenosine (7). Adenosine (5.34 g, 20 mmol) was rendered anhydrous by repeated coevaporation with dry pyridine (50 mL×3) and finally dissolved in dry pyridine (180 mL). 4,4′-Dimethoxytrityl chloride (15.25 g, 45 mmol) was added to the pyridine solution. After being stirred for 5 h, the mixture was partitioned between CH_2Cl_2 and water. The organic phase was collected and the aqueous layer was extracted two times with CH_2Cl_2. The CH_2Cl_2 extracts were combined, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was chromatographed on a column of silica gel (300 g) with CH_2Cl_2–MeOH to give the foammy material of **7**, which was reprecipitated from its CH_2Cl_2 solution into hexane to give **7** as the powder (14.5 g, 83%).

7: ^{1}H NMR ($CDCl_3$–D_2O) δ=3.31 (2H, m, 5′-H), 3.74 (6H, s, OCH_3), 3.74 (1H, m, 4-H), 4.33 (1H, m, 3′-H), 4.57 (1H, m, 2′-H), 5.83 (1H, d, J=5.83 Hz, 1′-H), 6.70 (8H, d, J=9 Hz, ArH), 7.16 (18H, m, ArH), 7.95 (1H, s, 2-H), 7.89 (1H, s, 8-H). Anal. Calcd for $C_{52}H_{49}N_5O_8$: C, 71.63, H, 5.66; N, 8.03%. Found: C, 71.40; H, 5.94; N, 7.77%.

3′,5′-*O*-(1,1,3,3-Tetraisopropyldisiloxane-1,3-diyl)-6-*N*-benzoyladenosine 2′-Phosphorodianilidate (4). A mixture of compound **1** (614 mg, 1 mmol) and tetrazole (105 mg, 1.5 mmol) was coevaporated three times with dry toluene (10 mL) and dissolved in dry 1,2-dichloroethane (10 mL). TEAP (411 μL, 1.5 mmol) was added with vigorous stirring to the solution. After the mixture was stirred for 10 min, aniline (365 μL, 4 mmol) and tetrazole (280 mg, 4 mmol) were successively added and stirring was continued for 10 min. Then a solution of iodine (381 mg, 1.5 mmol) in dry pyridine (2 mL) was added to the mixture. After being stirred for 10 min, the mixture was diluted with CH_2Cl_2 and 5% aqueous Na_2SO_3 was added to remove the excess iodine. 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 (30 g) with CH_2Cl_2–MeOH to give **4** (703 mg, 83%). **4:** ^{1}H NMR ($CDCl_3$) δ=1.03 (24H, m, $(C\underline{H}_3)_2CH$), 4.10 (3H, m, 4′- and 5′-H), 5.19 (1H, m, 3′-H), 5.63 (1H, dd, $J_{2'\text{-}3'}$=5.3 Hz, $J_{P\text{-}H}$=9.3 Hz, 2′-H), 6.43 (1H, s, 1′-H), 6.80—7.27 (10H, m, ArH), 7.53 (2H, m, ArH), 8.18 (3H, m, ArH), 8.18 (1H, s, 2-H), 8.62 (1H,

s, 8-H), 8.75 (1H, s, NH).

6-*N*-Benzoyladenosine 2'-Phosphorodianilidate (6a) and Its 3'-Regioisomer (6b). Small Scale Synthesis: To a solution of **8** (591 mg, 0.7 mmol) in acetonitrile (8.4 mL) were added KF (236 mg, 4.2 mmol), Et_4NBr (883 mg, 4.2 mmol), and water (0.076 mL, 4.2 mmol). The mixture was stirred at 45 °C for 20 min and diluted with CH_2Cl_2–pyridine (2 : 1, v/v, 30 mL). The resulting solution was washed six times with water (30 mL) to remove quarternary ammonium salts. Each aqueous washing was reextracted with another CH_2Cl_2–pyridine (2 : 1, v/v, 30 mL) solution which was used commonly for six times extraction in another separatory funnel. The two extracts were combined, dried over Na_2SO_4, filtered, concentrated under reduced pressure, and coevaporated four times with toluene. The residue was dissolved in CH_2Cl_2 (15 mL). Upon standing of this solution for a few minutes a powder-like precipitate appeared. Collection of this material by filtration took ca. 1 h to give **6b** (182 mg, 43%). Concentration of the filtrate followed by preparative thin-layer chromatography with CH_2Cl_2–MeOH (8 : 1, v/v) gave **6a** (156 mg, 37%) and **6b** (56 mg, 13%).

6a: ^{1}H NMR ($CDCl_3$–CD_3OD, 5 : 1, v/v) δ=3.72 (2H, m, 5'-H), 4.21 (1H, m, 4'-H), 4.57 (1H, m, 3'-H), 5.43 (1H, m, 2'-H), 6.10 (1H, d, J=7 Hz, 1'-H), 6.37—7.20 (10H, m, ArH), 7.40 (3H, m, ArH), 7.91 (2H, m, ArH), 8.05 (1H, s, 2-H), 8.38 (1H, s, 8-H). Anal. Calcd for $C_{29}H_{28}N_7O_6P \cdot 0.5H_2O$: C, 57.05, H, 4.79; N, 16.06%. Found: C, 56.98; H, 4.76; N, 15.95%.

6b: ^{1}H NMR ($CDCl_3$–CD_3OD, 2 : 1, v/v) δ=3.72 (2H, m, 5'-H), 5.00 (2H, m, 2'- and 3'-H), 6.03 (1H, d, J=6.4 Hz, 1'-H), 6.60—7.30 (10H, m, ArH), 6.43 (3H, m, ArH), 7.91 (2H, m, ArH), 8.32 (1H, s, 2-H), 8.53 (1H, s, 8-H). Anal. Calcd for $C_{29}H_{28}N_7O_6P \cdot 0.5H_2O$: C, 57.05, H, 4.79; N, 16.06%. Found: C, 57.04; H, 4.65; N, 15.98%.

Large Scale Synthesis of 6b: The same procedure described in the small scale synthesis of **6a** and **6b** before crystallization of **6b** was done except that the scale was increased to 16 mmol. The residue containing **6a** and **6b** was dissolved in MeOH (40 mL) and 1,2-dichloroethane (360 mL). Concentration of the solution under reduced pressure to ca 200 mL followed by standing at 0 °C for 15 min resulted in crystallization of **6b**. The crystals were collected by filtration and washed with minimum amounts of 1,2-dichloroethane to give 3.67 g (38.3%) of **6b**. The filtrate, which contained a ca. 5 : 1 mixture of **6a** and **6b**, was concentrated under reduced pressure and the residue was dissolved in pyridine (60 mL) and water (6 mL). The solution was kept at 60 °C for 2 h, at which time the ratio of **6a** to **6b** was changed to ca. 1 : 1. The mixture was then evaporated and coevaporated several times with toluene. The residue was tritulated with petroleum ether (3×20 mL) to remove the remaining silyl derivative derived from the 1,1,3,3-tetraisopropyldisiloxane-1,3-diyl group, dissolved in a mixture of 1,2-dichloroethane (150 mL)–MeOH (30 mL), and the solution was passed through a column of silica gel (100 g). The column was further eluted with a mixture of 1,2-dichloroethane–MeOH (300 mL–60 mL). The eluate was concentrated and the residue was dissolved in a mixture of 1,2-dichloroethane–MeOH (250 mL–25 mL). Concentration to half the volume followed by standing for 15 min and successive collection gave the second crop of **6b** (0.75 g, 7.8%). More two times repetition of the same procedure gave additional crops (0.44 g and 0.31 g) of **6b** from each filtrate. The total yield of **6b** was 5.17 g (55%). Each crop was confirmed to be homogeneous on TLC.

3',5'-*O*-(1,1,3,3-Tetraisopropyldisiloxane-1,3-diyl)-6-*N*-benzoyladenosine 3'-[*N*,*N*'-Bis(*p*-methoxyphenyl)phosphorodiamidate] (9). Under conditions similar to those described in the case of **4**, 759 mg (84%) of **9** was obtained from 0.4 mmol of **1**.

9: ^{1}H NMR ($CDCl_3$) δ=1.04 (24H, m, $(CH_3)_2CH$), 3.67 (6H, s, CH_3O), 4.05 (3H, m, 4'- and 5'-H), 4.80—6.00 (2H, m, 2'- and 3'-H), 6.11 (1H, s, 1'-H), 6.59 (4H, d, J=8.5 Hz, ArH), 6.90 (4H, d, J=8.5 Hz, ArH), 7.42 (3H, m, ArH), 7.88 (2H, m, ArH), 8.00 (1H, s, 2-H), 8.49 (1H, s, 8-H). Anal. Calcd for $C_{43}H_{58}N_7O_9PSi_2$: C, 57.13; H, 6.47; N, 10.84%. Found: C, 56.93; H, 6.61; N, 10.94%.

3',5'-*O*-(1,1,3,3-Tetraisopropyldisiloxane-1,3-diyl)-6-*N*-benzoyladenosine 3'-[*N*,*N*'-Diphenylphosphorodiamidothioate] (10). This compound was synthesized by using 0.4 mmol of **1** under the same conditions as described in the case of **4** except that at the final stage the sulfurization (10 min) was undertaken by addition of elemental sulfur (38 mg, 1.2 mmol) in dry pyridine (2.5 mL) to the mixture containing **3** in place of iodine oxidation. The yield was 256 mg (75%).

10: ^{1}H NMR ($CDCl_3$) δ=1.06 (24H, m, $(CH_3)_2CH$), 4.07 (3H, m, 4'- and 5'-H), 5.15 (1H, m, 3'-H), 5.70 (1H, dd, J=5 Hz, J=13 Hz, 2'-H), 6.08 (1H, m, 1'-H), 7.05 (10H, m, ArH), 7.37 (3H, m, ArH), 7.88 (2H, m, ArH), 7.92 (1H, s, 2-H), 8.58 (1H, m, 8-H). Anal. Calcd for $C_{41}H_{54}N_7O_6PSSi_2$: C, 57.25; H, 6.33; N, 11.40; S, 3.73%. Found: C, 57.05; H, 6.42; N, 11.39%.

6-*N*-Benzoyladenosine 2'-[*N*,*N*'-Bis(*p*-methoxyphenyl)-phosphorodiamidate (11a) and Its Regioisomer (11b). These compounds were obtained by the same procedure as described in the small scale synthesis of **6a** and **6b** starting from 0.79 mmol of **10** except that the reaction was carried out at 45 °C for 45 min. The yields of **11a** and **11b** were 86 mg (18%) and 295 mg (42%), respectively.

11a: ^{1}H NNR ($CDCl_3$) δ=3.54 (6H, s, CH_3O), 3.59 (2H, m, 5'-H), 4.26 (1H, m, 4'-H), 4.73 (1H, m, 3'-H), 5.59 (1H, m, 2'-H), 6.00—7.15 (9H, m, 1'-H and ArH), 7.57 (3H, m, ArH), 8.05 (2H, m, ArH), 8.24 (1H, s, 2-H), 8.57 (1H, s, 8-H). Anal. Calcd for $C_{31}H_{32}N_7O_8P \cdot 0.5H_2O$: C, 55.52; H, 4.96; N, 14.62%. Found: C, 55.10; H, 4.92; N, 14.66%.

11b: ^{1}H NMR ($CDCl_3$–CD_3OD, 2 : 1, v/v) δ=3.73 (2H, m, 5'-H), 4.38 (1H, m, 4'-H), 5.03 (2H, m, 2'- and 3'-H), 6.02 (1H, d, J=6.5 Hz, 1'-H), 6.67 (4H, d, J=8.5 Hz, ArH), 6.95 (4H, d, J=8.5 Hz, ArH), 7.44 (3H, m, ArH), 7.95 (2H, m, ArH), 8.30 (1H, s, 2-H), 8.57 (1H, s, 8-H). Anal. Calcd for $C_{31}H_{32}N_7O_8P \cdot 0.5H_2O$: C, 55.52; H, 4.96; N, 14.62%. Found: C, 55.88; H, 5.08; N, 14.36%.

5'-*O*-[Tris[4-(4,5-dichlorophthalimido)phenyl]methyl]-6-*N*-benzoyladenosine 3'-Phosphorodianilidate (12). Compound **6b** (60 mg, 0.1 mmol) was coevaporated two times each with dry toluene and DMF and finally dissolved in dry DMF (1 mL). To the solution were added silver nitrate (34 mg, 0.2 mmol) and tris[4-(4,5-dichlorophthalimido)-phenyl]methyl bromide (193 mg, 0.2 mmol). The mixture was stirred for 45 min and then quenched with 2,6-lutidine (23 μL, 0.2 mmol). The resulting mixture was diluted with dichloromethane, filtered, transferred to a separatory funnel, and washed six times with water. Each washing was reextracted with another CH_2Cl_2. The two CH_2Cl_2 extracts were combined, dried over Na_2SO_4, and filtered. Removal of the solvent by evaporation followed by thin-layer chroma-

tography using two plates of Merck 5717 with CH_2Cl_2–MeOH gave **12** (86 mg, 57%).

12: ^{1}H NMR ($CDCl_3$) δ=3.35 (2H, m, 5′-H), 4.38 (1-H, m, 4′-H), 5.15 (2H, m, 2′,3′-H), 5.99 (1H, m, 1′-H), 6.50—8.00 (27H, m, ArH), 7.83 (6H, s, ArH), 8.01 (qH, s, 2-H), 8.47 (1H, s, 8-H). Anal. Calcd for $C_{72}H_{45}N_{10}O_{12}PCl_6 \cdot H_2O$: C, 57.50; H, 3.15; N, 9.31%. Found: C, 57.52; H, 3.03; N, 9.30%.

2′-*O*-Bis(phenylthio)phosphinyl-5′-*O*-[tris[4-(4,5-dichlorophthalimido)phenyl]methyl]-6-*N*-benzoyladenosine 3′-Phosphorodianilidate (13). To a pyridine solution (3 mL) of **12** (446 mg, 0.3 mmol), cyclohexylammonium *S*,*S*-dipheyl phosphorodithioate (171 mg, 0.45 mmol) and tetrazole (84 mg, 1.2 mmol) dried by repeated coevaporation with dry pyridine was added mesitylenedisulfonyl dichloride (MDS) (190 mg, 0.6 mmol). After being stirred for 15 min, the mixture was partitioned between CH_2Cl_2 and water. The organic layer was collected, washed with 5% $NaHCO_3$, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was chromatographed on a column of silica gel (20 g) with CH_2Cl_2–MeOH to give **13** (423 mg, 81%).

13: ^{1}H NMR (270 MHz, $CDCl_3$) δ=3.48 (1H, dd, J=11 Hz, J=3.0 Hz, 5′-Ha), 3.65 (1H, d, J=11.0 Hz, 5′-b), 4.53 (1H, m, 4′-H), 5.38 (1H, m, 3′-H), 6.13 (1H, d, J=5.3 Hz, 1′-H), 6.18 (1H, m, 2′-H), 6.50—7.65 (37H, m, ArH), 7.93 (6H, s, ArH), 8.09 (1H, s, 2-H), 8.63 (1H, s, 8-H), 9.31 (1H, br, NH). Anal. Calcd for $C_{84}H_{54}N_{10}O_{13}P_2S_2Cl_6$: C, 57.65; H, 3.11; N, 8.00%. Found: C, 57.44; H. 3.16; N, 7.96%.

Triethylammonium 2′-*O*-Bis(phenylthio)phosphinyl-5′-*O*-[tris[4-(4,5-dichlorophthalimido)phenyl]methyl]-6-*N*-benzoyladenosine 3′-Phosphate (15). Compoud **13** (175 mg, 0.1 mmol) was dissolved in a mixture of pyridine–acetic acid–acetic anhydride (1 : 1 : 1, v/v/v, 3 mL) and isopentyl nitrite (100 μL) was added. After the mixture being stirred for 11 h, the mixture was diluted with CH_2Cl_2 and water was added. The CH_2Cl_2 layer was collected, washed successively with water and 0.2 M TEAB, and each aqueous layer was backextracted with another CH_2Cl_2. The combined organic phase was dried over Na_2SO_4, filtrated, concentrated under reduced pressure and coevaporated with toluene. The residue was subjected to a preparative TLC developed with CH_2Cl_2–MeOH (9 : 1, v/v) to give **15** (85 mg, 50%).

15: ^{1}H NMR ($CDCl_3$) δ=1.30 (9H, t, J=7 Hz, $C\underline{H}_3CH_2N$), 3.00 (6H, q, J=7 Hz, $CH_3C\underline{H}_2N$), 3.68 (2H, m, 5′-H), 4.62 (1H, m, 4′-H), 5.58 (1H, m, 3′-H), 6.28 (1H, m, 1′-H), 6.43 (1H, m, 2′-H). 6.90—8.10 (27H, m, ArH), 7.93 (6H, s, ArH), 8.24 (1H, s, 2-H), 8.69 (1H, s, 8-H).

Triethylammonium *S*-Phenyl 3′-*O*-Dianilinophosphinyl-5′-*O*-[tris[4-(4,5-dichlorophthalimido)phenyl]-methyl]-6-*N*-benzoyladenosine 2′-Phosphorothioate (16). Compound **13** (1.75 g, 1 mmol) was treated with a solution of 3 M pyridinium phosphinate (65 mmol) in dry pyridine (21.7 mL) at room temperature for 1 h. The resulting mixture was diluted with CH_2Cl_2, which was washed subsequently with water, 0.2 M TEAB (×2), and water. Each washing was backextracted with another CH_2Cl_2. The two CH_2Cl_2 extracts were combined, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. Coevaporation of the residue with toluene followed by precipitation from its CH_2Cl_2 solution into hexane gave **16** as white powder (1.744 g, 99%). This material was used without further purification for the next condensation with **17**.

Synthesis of the Fully Protected 2′–5′ Linked ApG Dimer (18). A mixture of **16** (1.58 g, 0.9 mmol), **17** (778 mg, 1.35 mmol), and 3-nitro-1*H*-1,2,4-triazole (308 mg, 2.7 mmol) was rendered anhydrous by three times coevaporation with dry pyridine and finally dissolved in dry pyridine (10 ml). To the mixture was added DDS (894 mg, 2.7 mmol). After being stirred for 2 h, the extractive workup followed by column chromatography gave **18** (1.83 g, 92%).

Removal of the Anilino Group from 18 by Isopentyl Nitrite Treatment. Compound **18** (130 mg, 58.6 μmol) was treated with isopentyl nitrite (58.6 μL) in pyridine–acetic acid–acetic anhydride (1 : 1 : 1, v/v/v, 586 μL) for 16 h. Then the mixture was diluted with CH_2Cl_2, which was washed subsequently with water (×4), 0.2 M TEAB (×2), and water. Each washing was backextracted with another CH_2Cl_2. The two CH_2Cl_2 extracts were combined, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was coevaporated with toluene and chromatographed on two preparative TLC plates developed with CH_2Cl_2–MeOH (6 : 1, v/v). Elution was performed with CH_2Cl_2–MeOH (4 : 1, v/v). The eluate was evaporated and the residue was partitioned between CH_2Cl_2 and 0.2 M TEAB. The CH_2Cl_2 extract was collected, dried over Na_2SO_4, and concentrated to give **19** free of silica gel as triethylammonium salt (95.3 mg, 75%).

Synthesis of the Partially Protected Branched Triribonucleotide (23). Method A: A mixture of **19** (152 mg, 70 μmol) and **20** (54.5 mg, 0.14 mmol) was rendered anhydrous by repeated coevapoartion with dry pyridine and finally dissolved in dry pyridine (1 mL), and DDS (70 mg, 0.21 mmol) was added. After 9 h, TLC showed that a product which appeared higher than **19** was formed in a considerable amount and ca. 40% of **19** remained unchanged. This faster moving material seemed to be a tetrasubstituted pyrophosphate intermediate. To make the condensation complete, **20** (54.5 mg, 0.14 mmol) and DDS (46 mg, 0.14 mmol) were added. After being stirred for an additional 2 h, DDS (46 mg, 0.14 mmol) was added to convert excess **20** to the corresponding 5′-sulfonated derivative which was hydrolyzed to a more polar sulfonic acid derivative upon addition of water. After the mixture was kept for 3 h, water (0.2 mL) was added, and the resulting mixture was stirred for 15 min and then extracted in the usual manner as described in the synthesis of **16**. Preparative TLC developed with CH_2Cl_2–MeOH (9 : 1, v/v) gave a mixture of **23** and **24** (143 mg).

Method B: A mixture of **19** (28 mg, 13 μmol), **20** (20.1 mg, 52 μmol), and pyridinium 3-nitrobenzenesulfonate (11 mg, 39 μmol) was rendered anhydrous by repeated coevapoartion with dry pyridine and finally dissolved in dry pyridine (0.2 mL). DCC (20 mg, 97 μmol) was added to the mixture. After being stirred for 36 h, the mixture was diluted with CH_2Cl_2 which was washed with 0.2 M TEAB. The organic phase was dried over Na_2SO_4, filtered, concentrated, and chromatographed on a preparative TLC with CH_2Cl_2–MeOH (7 : 1, v/v) to give the crude material of **23** (49.3 mg).

Deprotection of 23 Giving Rise to Branched Triribonucleotide (21). Part (88 mg) of the mixture of **23** and **24** obtained in Method A described in the above experiment was dissolved in pyridine (5 mL) and 0.2 M NaOH (5 mL) was added at 0 °C to the solution. The solution was stirred at 0 °C for 15 min and then neutralized with Dowex 50W X2 (pyridinium form, 5 mL). The supernatant and washing

with pyridine–water (1 : 1, 40 mL) were combined, concentrated, and the residue was dissolved in pyridine (5 mL)–concd ammonia (45 mL). After being stirred at 50 °C for 6 h, the mixture was concentrated and treated with 80% acetic acid (50 mL) for 6h. The solvent was then evaporated and the residue was chromatographed on Whatman 3MM papers developed with Solvent II to give **21** (237 A_{258}, 20%) and **22** (193 $A_{256.5}$, 14%).

21: λ_{max} 258 nm, λ_{min} 227 nm; R_f 0.61 (relative to pU) (Solvent II, R_f 0.74 (relative to pU) (Solvent III); R_m 0.47 (relative to pU) (0.03 M phosphate buffer, pH 7.1); Retension time 11.8 min (HPLC system A); ^{1}H NMR (500 MHz, D_2O/DSS) δ=3.70 (1H, dm, J=11.7 Hz), 3.79 (1H, dd, J=12.8 Hz, J=2.2 Hz, 5′-Ha of A), 3.86 (1H, d, J=12.8 Hz, J=1.3 Hz, 5′-Hb of A), 3.95 (1H, tm, J=11.7 Hz), 4.04 (1H, br. s), 4.20 (1H, m), 4.28 (2H, q, J=5.2 Hz), 4.33 (2H, m), 4.47 (1H, t, J=8.2 Hz), 4.51 (1H, d, J=2.6 Hz), 5.32 (1H, quintet, J=9.6 Hz, J=4.8 Hz, 3′-H of A), 5.64 (1H, d, J=4.9 Hz, 1′-H of G), 5.91 (1H, d, J=4.0 Hz, 1′-H of C), 5.95 (1H, d, J=7.3 Hz, 5-H of C), 6.17 (1H, d, J=5.5 Hz, 1′-H of A), 7.73 (1H, s, 8-H of G), 7.84 (1H, d, J=7.3 Hz, 6-H of C), 7.87 (1H, s, 2-H of A), 8.22 (1H, s, 8-H of A). **22:** λ_{max} 256.5 nm, λ_{min} 225 nm R_f 0.34 (relative to pU) (Solvent II), R_f 0.37 (relative to pU) (Solvent III); R_m 0.12 (relative to pU) (0.03 M phosphate buffer, pH 7.1).

In a similar manner, the partially protected branched trimer **23** (49.3 mg, 13 μmol) obtained in Method B was deprotected except that the ammonia and acid treatments were performed at room temperature for 26 h and for 11 h, respectively. The yield of **21** was 109 A_{258} (29%).

Synthesis of Branched Triribonucleotide A^{pG}_{pU} (27). A mixture of **19** (43.3 mg, 20 μmol), **28** (15.6 mg, 40 μmol), and pyridinium *m*-nitrobenzenesulfonate (16.9 mg, 60 μmol) was coevaporated three times with dry pyridine and then dissolved in dry pyridine (0.5 mL). DCC (29 mg, 0.14 mmol) was added and the solution was stirred for 4 d. The resulting mixture was diluted with pyridine (3 mL) and cooled to 0 °C. To the solution was added 0.2 M NaOH (3 mL), and the mixture was kept at 0 °C for 10 min. Then, cation-exchange DIAION resin (sulfonic acid form, 3 mL) was put to neutralize the solution, and the resin was removed by filtration and washed with pyridine–water (2 : 1, v/v, 36 mL). The eluate was evaporated and the residue was dissolved with pyridine (5 mL). Concentrated aqueous ammonia (50 mL) was added and this solution was kept for 3 d. Then the mixture was concentrated and the residue was treated with 80% acetic acid (50 mL) for 1 d. After the solvent was removed by evaporation, the residue was chromatographed on a Whatman 3MM paper developed with Solvent III to give **27** (122 A_{257}, 20%).

27: λ_{max} 257 nm; λ_{min} 227 nm; R_f 0.82 (relative to pU) (Solvent III) (55 : 10 : 35); R_m 0.71 (relative to pU) (0.03 M phosphate buffer, pH 7.1). ^{1}H NMR (500 MHz, D_2O/DSS) δ=3.60 (1H, dm, J=12.3), 3.80 (1H, d, J=12.3 Hz, 5′-Ha of A), 3.85 (1H, d, J=12.3 Hz, 5′-Hb of A), 3.92 (1H, m), 3.95 (1H, br. s), 4.20 (1H, m), 4.27 (1H, m), 4.35 (1H, quintet, J=5.0 Hz), 4.41 (1H, t, J=5.0 Hz), 4.51 (1H, br. s), 5.32 (1H, m, 2′-H of A), 5.63 (1H, d, J=5.2 Hz, 1′-H of G), 5.84 (1H, d, J=7.6 Hz, 5-H of U), 5.95 (1H, d, J=4.9 Hz, 1′-H of U), 6.20 (1H, d, J=5.8 Hz, 1′-H of A), 7.69 (1H, s, H-8 of G), 7.84 (1H, d, J=7.6 Hz, 6-H of U), 7.85 (1H, s, 2-H of A), 8.23 (1H, s, 8-H of A).

Enzymatic Analysis of 21 and 27. The branched triribonucleotide **21** (10 A_{258} unit) was incubated with snake venom phosphodiesterase (10 μL, 1 mg mL^{-1}) in 50 mM Tris-HCl (pH 7.5, 100 μL). Aliquots of the incubation mixture were analyzed after 1.5 h, 5 h, and 12 h by paper electropheresis using 0.05 M phosphate buffer (pH 8.0, 1250 V, 1 h 15 min). In paper electrophoresis done after 1.5 h and 5 h, two digestion intermediates of A(3′–5′)pC and A(2′–5′)pG were detected at R_m values 0.59 and 0.20, respectively. The ultimate ratio of pG, pC, and A, which was obtained after 12 h, was 1.0 : 1.0 : 1.1.

In a similar manner, **27** was digested under the same conditions to give pG, pU, and A in a ratio of 0.8 : 1.0 : 1.2.

Deprotection of 19 Giving Rise to 3′-*O*-Phosphinyladenylyl(2′-5′)guanylate. To a solution of compound **19** (152 mg, 70 μmol) in pyridine (10 mL) was added with stirring 0.2 M NaOH–pyridine (10 mL) at 0 °C. After being stirred at 0 °C for 15 min, the mixture was neutralized with Dowex 50W X2 (pyridinium form, 5 mL), and the resin was removed by filtration and washed with pyridine–water (1 : 1, v/v, 10 mL and 2 : 1, v/v, 36 mL). The filtrate and washing were combined, concentrated, dissolved in pyridine (10 mL), and mixed with concentrated aqueous ammonia (90 mL). The solution was kept for 24 h and evaporated. The residue was allowed to react with 80% acetic acid (50 mL) for 6 h. The solvent was removed under reduced pressure and the residue was chromatographed on Whatman 3MM papers with Solvent III. A band of R_f 0.66 relative to pU was eluted with water and lyophilized to give **26** (1464 A_{256}): R_m 0.85 (pH 7.1, 0.03 M phosphate buffer, 1 h 40 min, 1200 V).

Enzymatic Assay of 26. Compound **26** (70.4 OD at 256 nm) lyophilized was incubated with bacterial alkaline phosphatase (35.2 U/802 μL) in 50 mM Tris-HCl (pH 7.5, 704 μL) at 55 °C for 3 h. Then the incubation mixture was directly applied to a Whatman 3MM paper developed with Solvent II to give A(2′–5′)pG (62.4 $A_{255.5}$) as the sole product (λ_{max} 255.5 nm, λ_{min} 226.5 nm); R_m 0.32 (pH 7.1, 0.03 M phosphate buffer, relative to pU). The dinucleotide (5 $A_{255.5}$ each) was further incubated with RNase T_1, RNase T_2, RNase A, Nuclease P_1, snake venom phosphodiesterase (SVP), and calf spleen phosphodiesterase (CSP) at 37 °C for at least 13 h under the following conditions: 1) RNase T_1 (30 U, 6 μL), 10 mM Tris-HCl (100 μL) containing 1 mM EDTA, pH 7.5; 2) RNase T_2 (30 U, 150 μL), 20 mM NaOAc (100 μL), pH 5.5; 3) RNase A (150 μg, 15 μL), 10 mM Tis-HCl (30 μL) containing 1 mM EDTA, pH 7.5; 4) Nuclease P_1 (30 μg, 15 μL), 20 mM NaOAc (100 μL), pH 5.5; 5) SVP (30 μg, 30 μL), 50 mM Tris-HCl (100 μL), pH 7.5; 6) CSP (10 μg, 5 μL), 0.01 M pyrophosphate (50 μL) and 0.05 M ammonium acetate (100 μL), pH 6.5.

These enzyme reactions were analyzed after 1.5 h and 13 h by paper electrophoresis (0.03 M phosphate buffer, pH 7.1, 1200 V, 1 h 10 min). Among these enzymes, only SVP degraded the dinucleotide to A and pG in a ratio of 1.03 : 1.00 after 2 h. The digetion products were identified as the authentic samples by comparison of their R_f and R_m values as well as UV spectra.

***S,S*-Diphenyl 3′-*O*-Dianilinophosphinyl-5′-*O*-(4-methoxytrityl)-6-*N*-benzoyladenosine 2′-Phosphorodithioate (32).** Compound **6b** (1.21 g, 2 mmol) was rendered anhydrous by repeated coevaporation with dry pyridine and finally dissolved in dry pyridine (5 mL). 4-Methoxytrityl chloride (1.85 g, 6 mmol) was added and the mixture was stirred for 3

h. To the mixture were added successively cyclohexylammonium *S,S*-diphenyl phosphorodithioate (1.14 g, 3 mmol), tetrazole (560 mg, 8 mmol), and MDS (1.27 g, 4 mmol). The resulting mixture was stirred for 15 min and then diluted with CH_2Cl_2. After the usual workup as described in the synthesis of **13**, chromatography on a column of silica gel (100 g) with CH_2Cl_2–MeOH gave **32** (1.89 g, 83%).

32: ^{1}H NMR (500 MHz, $CDCl_3$) δ=3.43 (2H, br s, 5′-H), 3.61 (3H, s, OCH_3), 4.72 (1H, br s, 4′-H), 5.41 (1H, t, J=6.0, 3′-H), 5.98 (1H, q, J=6.0 Hz, J=6.8 Hz, 2′-H), 6.34 (1H, d, J=6.8 Hz, 1′-H), 6.75 (2H, d, J=8.79 Hz, ArH), 6.83 (2H, q, J=7.33 Hz, J=6.83 Hz, ArH), 6.88 (2H, t, J=7.32 Hz, ArH), 6.98—7.60 (31H, m, ArH), 8.07 (2H, d, J=7.8 Hz, ArH), 8.17 (1H, s, 2-H), 8.46 (1H, s, 8-H), 9.58 (1H, br, NH).

Removal of the Anilino Groups from 32. Compound **32** (1.85 g, 1.6 mmol) was allowed to react with isopentyl nitrite (1.6 mL) in pyridine–acetic acid–acetic anhydride (1 : 1 : 1, v/v/v) for 15 h. Then pyridine (5 mL) was added and the mixture was partitioned between CH_2Cl and water. The organic phase was collected and washed with water, 0.2 M TEAB (×5), water. Each washing was backextracted with another CH_2Cl_2. The two CH_2Cl_2 extracts were combined, concentrated, and the residue was chromatographed on a column of silica gel (64 g) with CH_2Cl_2–MeOH in the presence of 0.5% pyridine to give **33** as glassy material. As described in the case of **19**, further extractive workup followed by precipitation of its CH_2Cl_2 solution into hexane gave **33** as white powder (969 mg, 55%).

***S,S*-Diphenyl 3′-*O*-Bis(2-cyanoethoxy)phosphinyl-5′-*O*-(4-methoxytrityl)-6-*N*-benzoyladenosine 2′-Phosphorodithioate (30).** Compound **33** (219 mg, 0.2 mmol) was rendered anhydrous by repeated coevaporation with dry pyridine and finally dissolved in dry pyridine (4 mL). To the solution were added 2-cyanoethanol (272 μL, 4 mmol) and DDS (662 mg, 2 mmol). The resulting mixture was sitirred for 1 h and then diluted with CH_2Cl_2. The CH_2Cl_2 solution was washed with water (×2) and 5% $NaHCO_3$ (×3) and each washing was backextracted with another CH_2Cl_2. The two CH_2Cl_2 extracts were combined, dried over Na_2SO_4, filtered, concentrated, and chromatographed on a column of silica gel (20 g) with CH_2Cl_2–MeOH in the presence of pyridine to give **30** as foam (147 mg, 67%).

30: ^{1}H NMR ($CDCl_3$) δ=2.67 (4H, t, J=6 Hz, CH_2CN), 3.55 (2H, m, 5′-H). 3.74 (3H, s, CH_3O), 4.24 (4H, q, J=6 Hz, CH_2O-P), 4.45 (1H, m, 4′-H), 5.36 (1H, m, 3′-H), 6.08 (1H, m, 2′-H), 6.17 (1H, m, 1′-H), 6.73 (2H, d, J=8 Hz, 6.92—7.80 (25H, m, ArH), 7.91 (2H, m, ArH), 8.01 (1H, s, 2-H), 8.58 (1H, s, 8-H). Anal. Calcd for $C_{54}H_{49}N_7O_{10}P_2$: C, 59.94; H, 4.56; N, 9.06; S, 5.93%. Found: C, 60.31; H, 4.81; N, 8.85; S, 5.89%.

The Fully Protected 2′-5′ Linked ApG Derivative (36). A 5 M solution of pyridinium phosphinate in pyridine (1.1 mL) was mixed with triethylamine (0.55 mL) and **30** (100 mg, 91 μmol) was dissolved in this solution. After the mixture was stirred at room temperature for 30 min, the resulting mixture was diluted with CH_2Cl_2, which was washed subsequently with water, 0.2 M triethylammonium hydrogencarbonate (×2), and water. Each washing was backextracted with another CH_2Cl_2. The two CH_2Cl_2 extracts were combined, dried over Na_2SO_4, Filtered, and concentrated under reduced pressure. The residue was mixed with **35** (81 mg, 109 μmol) and 3-nitro-1*H*-1,2,4-triazole (31 mg, 0.27 mmol). The mixture was coevaporated several times with dry pyridine, dissolved in dry pyridine (1 mL), and DDS (90 mg, 0.27 mmol) was added. After the mixture was stirred for 1 h, the usual extractive workup followed by chromatography on a column of silica gel (20 g) with CH_2Cl_2–MeOH gave **36** (137 mg, 87%).

Synthesis of the Fully Protected Branched Trimer (39). The 2′-5′ linked dimer **36** (52 mg, 30 μmol) was treated with *N,N*-diisopropylethylamine (1 mL) in pyridine (2 mL) at 40 °C for 4 h. Evaporation followed by preparative TLC chromatography (CH_2Cl_2–MeOH 9 : 1, v/v) gave **37** as triethylammonium salt (44 mg, 82%).

A mixture of **37** (42 mg, 23 μmol), **38** (19.5 mg, 35 μmol), and 3-nitro-1*H*-1,2,4-triazole (24 mg, 0.21 mmol) was rendered anhydrous by repeated coevaporation with dry pyridine and finally dissolved in dry pyridine (0.5 mL). DDS (23 mg, 71 μmol) was added and the resulting mixture was stirred for 1 h. At this time the condensation was almost complete but an additional amount of DDS (23 mg, 71 μmol) was added to convert the excess hydroxy component to a sulfonate derivative. After stirring was continued for 1 h, the mixture was diluted with CH_2Cl_2, which was washed successively with water, 5% $NaHCO_3$, and water. Each washing was backextracted with another CH_2Cl_2. The two extracts were combined, dired over Na_2SO_4, filtered, concentrated and chromatographed on a preparative TLC plate developed with CH_2Cl_2–MeOH (9 : 1, v/v) to give **39** (33 mg, 63%).

Removal of the Methoxytrityl Group from 39. Compound **39** (33 mg, 15 μmol) was treated with trifluoroacetic acid (20 μL) in CH_2Cl_2 (2 mL) for 5 min. Then 5% $NaHCO_3$ was added and the mixture was extracted with CH_2Cl_2 (×3). The extracts were combined, dried over Na_2SO_4, filtered, concentrated, and chromatographed on a preparative TLC plate developed with CH_2Cl_2–MeOH (9 : 1, v/v) to give **40** (17.5 mg, 60%).

Synthesis of Fully Protected UpG Dimer. A mixture of triethylammonium *S*-phenyl 5′-*O*-(4,4′-dimethoxytiryl)-3′-*O*-(tetrahydro-2-pyranyl)-N^3-(*p*-anisoyl)uridine 3′-phosphorothioate (343 mg, 0.33 mmol) and *S,S*-diphenyl 3′-*O*-(tetrahydro-2-pyranyl)-2-*N*-propionyl-6-*O*-(diphenylcarbamoyl)guanosine 3′-phosphorodithioate (170 mg, 0.275 mmol), which were obtained from the corresponding monomer units[31,32] by treatments with phosphinate and trifluoroacetic acid, respectively, was rendered anhydrous by repeated coevaporation with dry pyridine and finally dissolved in dry pyridine (5 mL). To the solution were added 3-nitoro-1*H*-1,2,4-triazole (114 mg, 1 mmol) and DDS (331 mg, 1 mmol). After being stirred for 40 min, the mixture was quenched by the addition of water and extracted four times with CH_2Cl_2. Each CH_2Cl_2 extract was washed with 5% $NaHCO_3$. The CH_2Cl_2 extracts were combined, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was chromatographed on a column of silica gel (15 g) with CH_2Cl_2–MeOH to give the fully protected UpG dimer (329 mg, 78%).

Removal of the DMTr Group of the Fully Protected UpG Dimer. The Fully protected UpG dimer (310 mg, 0.21 mmol) was dissolved in CH_2Cl_2 (20 mL) and the solution was cooled at 0 °C. Trifluoroacetic acid (100 μL) was added and the resulting mixture was kept at the same temperature for 10 min. Then 5% $NaHCO_3$ was added to neutralize the solution. The usual extract with CH_2Cl_2 followed by preparative TLC developed with CH_2Cl_2–MeOH gave the 5′-

hydroxy component of the fully protected UpG dimer (194 mg, 76%).

Synthesis of the Fully Protected CpUpG Trimer. A mixture of *S*-phenyl 5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-(tetrahydro-2-pyranyl)-N^4-benzoylcytidine 3′-phosphorothioate (174 mg, 0.173 mmol) and the 5′-hydroxy component (186 mg, 0.15 mmol) obtained in the above experiment was rendered anhydrous by repeated coevaporation with dry pyridine and finally dissolved in dry pyridine (3 mL). To the mixture were added 3-nitro-1*H*-1,2,4-triazole (51.4 mg, 0.45 mmol) and DDS (149 mg, 0.45 mmol). After being stirred for 1 h, the mixture was quenched by the addition of water. The usual workup followed by chromatography on a column of silica gel gave the fully protected CpUpG trimer (282 mg, 88%).

Removal of the 3′-Terminal Phenylthio Group from the Fully Protected CpUpG Trimer. The fully protected CpUpG trimer (106 mg, 0.05 mmol) was dissolved in a mixture of 5 M phosphinic acid (pyridine solution) and triethylamine (2 : 1, v/v, 0.91 mL). The mixture was kept at room temperature for 1 h. Extraction with CH_2Cl_2 as described in the synthesis of **16** was performed. Chromatography on preparative TLC plates (Merck 5717) with CH_2Cl_2–MeOH gave **41** (65 mg, 60%) as triethylammonium salt.

Synthesis of the Fully Protected Branched Hexamer (42). A mixture of **40** (10.0 mg, 5.2 μmol), **41** (14.3 mg, 6.7 μmol), and 3-nitro-1*H*-1,2,4-triazole (3.8 mg, 34 μmol) was rendered an hydrous by repeated coevaporation with dry pyridine, finally dissolved in dry pyridine (0.2 mL), and DDS (11.1 mg, 34 μmol) was added. After being stirred for 1 h, the mixture was diluted with CH_2Cl_2. Extraction followed by preparative TLC gave **42** (8.7 mg, 56%).

Full Deprotection of 42. The fully protected branched hexamer **42** (8.5 mg, 2.1 μmol) was treated with bis(tributyl)tin oxide (0.15 mL, 0.23 mmol) in pyridine (0.25 mL) for 28 h. Then trimethylsilyl chloride (70 μL, 0.46 mmol) was added and the mixture was kept for 15 min. Pyridine (20 mL) and concentrated aqueous ammonia (20 mL) were added to the solution. After being stirred for 19 h, the mixture was evaporated and coevaporated two times with dioxane to remove the last traces of pyridine. The residue was dissolved in dioxane (20 mL) and 0.02 M HCl (20 mL) was added. The resulting solution was further arranged to pH 2.0 by addition of 0.1 M HCl and stirred for 48 h. Then the solution was neutralized by addition of concentrated aqueous ammonia, evaporated, and the residue was chromatographed on a Toyo Roshi 51A paper developed with Solvent II to give **43** (36 A_{257}, 32%): λ_{max} 257 nm, λ_{min} 226 nm; Retension time 11.0 min (HPLC System A); R_f 0.09 (Solvent II, relative to pT).

Enzyme Analysis of 43. The sample of **43** (0.1 A_{257}) purified by HPLC was incubated with nuclease P_1 (10 μL) in 10 mM sodium acetate (pH 5.5, 200 μL) at 37 °C for 15 min. The result of this enzyme reaction is depicted in Fig. 2C. The peak of pA^{pG}_{pC} was collected, desalted by repeated lyophilization from water, and incubated with bacterial alkaline phosphatase (0.3 μg/μL, 4 μL) in 50 mM Tris-HCl (pH 7.5, 30 μL) for 15 min. The peak of pA^{pG}_{pC} was completely shifted to an authentic sample of A^{pG}_{pC}.

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