Synthesis of Oligoribonucleotides by Use of 4,4′,4″-Tris(acyloxy)trityl Groups for Protection of the 6-Amino Group of Adenosine

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Two kinds of substituted trityls, i.e., 4,4′,4″-tris(benzoyloxy)trityl (TBTr) and 4,4′,4″-tris(*p*-anisoyloxy)-trityl (TAnTr), have been examined as protecting groups of the amino group of adenosine in oligoribonucleotide synthesis. The former was used for a 3′-terminal adenosine unit and the latter was introduced into an building block of internal adenosine. These protecting groups were successfully applied to the liquid-phase synthesis of pGUA and pGUAUUA which were the 5′-terminal oligoribonucleotide fragments of brome mosaic virus mRNA 4.

In the liquid-phase synthesis of oligoribonucleotides the phosphotriester approach has been most frequently adopted as a highly reproducible method to afford large quantities of target RNA oligomers required for physicochemical studies.[1] We have also employed this strategy to obtain synthetic mRNA fragments[2] which have been used as substrates for biological studies[3] of interaction with ribosomal RNAs in connection with the initiation mechanism of peptide synthesis in eukaryotic cells.[4] In order to facilitate purification of synthetic intermediates of fully protected building blocks, the 6-amino group of adenosine has been protected with the 4-methoxytrityl (MMTr)[2c,2e] or 4,4′-dimethoxytrityl[2d] (DMTr) group as an acid-labile protecting group. Especially, the former enabled us to use protic acids for selective removal of the DMTr group used for protection of the 5′-hydroxyl group so that rapid removal of the 5′-DMTr group was achieved. However, careful treatments were required for selective removal of the 5′-protecting group, since both the N^6-MMTr and 5′-*O*-DMTr groups were essentially removed under acidic conditions.[2c]

When the DMTr group was used for protection of the amino group of adenosine, it is necessary to use rather mild conditions such as 0.5 M $ZnBr_2/CH_2Cl_2$–2-propanol (85:15, v/v)[5] (1 M=1 mol dm^{-3}) for selective removal of the 5′-DMTr group.[2d] However, this Lewis acid cleaved rather slowly the 5′-terminal dimethoxytrityl ether bond when a 5′-terminal nucleoside was cytidine.[2d]

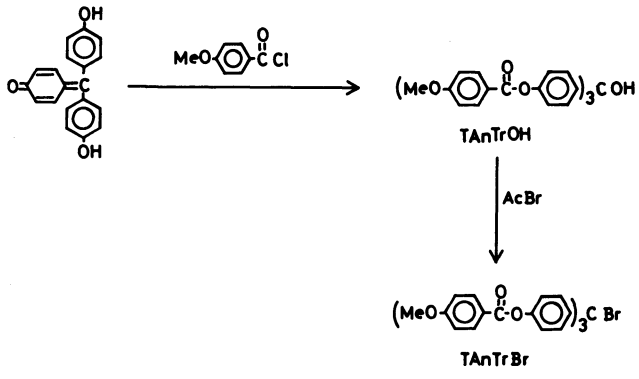
Recently, we have shown the utility of the 4,4′,4″-tris(benzoyloxy)trityl (TBTr) group as a protecting group of the amino group of deoxyadenosine.[6] The tritylating reagent TBTrBr can be prepared as crystals in a large scale from commercially available rosolic acid according to the facile procedure involving benzoylation and bromination.[7] This is one of advantageous points over the MMTr and DMTr groups. This protecting group has proved to have a unique property that it could be removed by dilute alkali via 1,6-elimination while it is rather resistant to acidic conditions.[7]

Based on these facts, we decided to apply the TBTr group to protect the amino group of adenosine to facilitate the selective removal of the 5′-DMTr group. We have chosen two 5′-terminal RNA fragments, pGUA and pGUAUUA, of brome mosaic virus mRNA 4 for synthetic targets during this study.

Results and Discussion

First, we prepared a 3′-terminal adenosine unit **4** bearing the TBTr group at the N^6 position in a manner similar to that described in the preparation of N^6-TBTr deoxyadenosine[6] as shown in Scheme 1. N^6-[4,4′,4″-Tris(benzoyloxy)trityl]adenosine (**2**) was obtained in 74% yield from adenosine (**1**). Reaction of **2** with 4,4′-dimethoxytrityl chloride gave the 5′-tritylated compound **3** in 94% yield. The successive benzoylation followed by detritylation afforded the 5′-hydroxy derivative **4** in 77% yield.

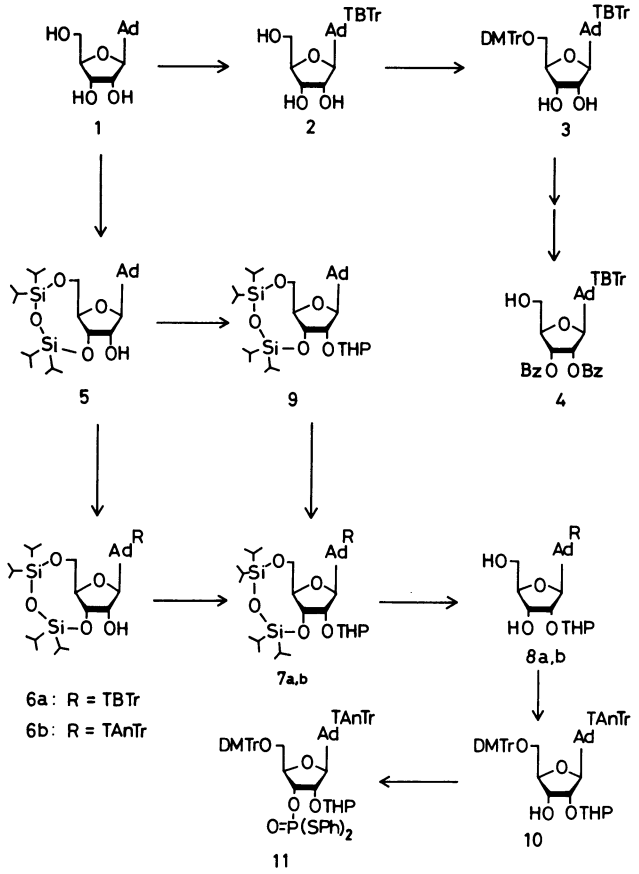
Next, in order to synthesize a key building block of adenosine unit **11**, 3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)adenosine (**5**)[9] was tritylated with TBTrBr in DMF in the presence of silver nitrate and lutidine. The tritylated product **6a** was obtained in 68% yield. Pyranylation of **6a** gave a fully protected adenosine derivative **7a** in 96% yield. However, when the cyclic silyl (TIPS) group was removed from **7a** by treatment with KF–Et_4NBr–H_2O/CH_3CN[2d] in the usual manner, the benzoyl group was partially eliminated from the TBTr group and the 3′,5′-diol **8a** was obtained in a low yield of 29%. This is probably because of the remaining basicity of the fluoride ion.



Therefore, to increase the stability of the benzoate, the benzoyl group was replaced for the *p*-anisoyl group at the 4-positions of the trityl group. The corresponding tritylating reagent 4,4′,4″-tris(anisoyloxy)trityl bromide (TAnTrBr) was synthesized in a manner similar to that described in the preparation of TBTrBr.

Similarly, compound **6b** was obtained in 54% yield by treatment of **5** with TAnTrBr. The successive pyranylation of **6b** followed by desilylation gave **8b** in 56% yield. The yields of **6b** and **8b** were unexpectedly low but this was not caused by the instability of the TAnTr group. It is due mainly to difficult separation of **6b** or **8b** from the by-products. To avoid such problems, the tetrahydropyranyl group was introduced into the 2′-oxygen prior to the N^6-tritylation. Upon the TFA-catalyzed tetrahydropyranylation of **5**, the 2′-tetrahydropyranylated adenosine derivative **9** was obtained in 74% yield. Tritylation of **9** followed by desilylation of **7b** gave **8b** as pure material in 77% yield. Furthermore, the total yield of **8b** from **5** was improved from 57% to 80%, when all the reactions (**5–9–7b–8b**) were in situ carried out without chromatographic separation and the tritylation was carried out at 0 °C.

An internal synthetic unit **11** of adenosine was prepared as follows. The 5′-dimethoxytritylation of **8b** gave a 5′-*O*-tritylated product **10** in 66% yield.



Scheme 1.

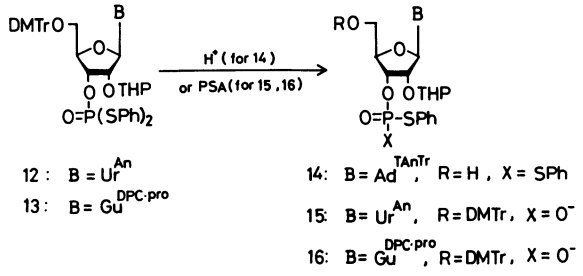
Treatment of **10** with cyclohexylammonium *S,S*-diphenyl phosphorodithioate (PPS)[10] in the presence of mesitylenedisulfonyl dichloride (MDS)[11] and tetrazole (Tet)[2f] gave the adenosine unit **11** in 85% yield.

For the synthesis of fully protected oligoribonucleotide blocks, 5′-hydroxyl and 3′-phosphodiester components **14—16** were prepared from the uridine and guanosine monomer units **12** and **13** by the literature method.[2d]

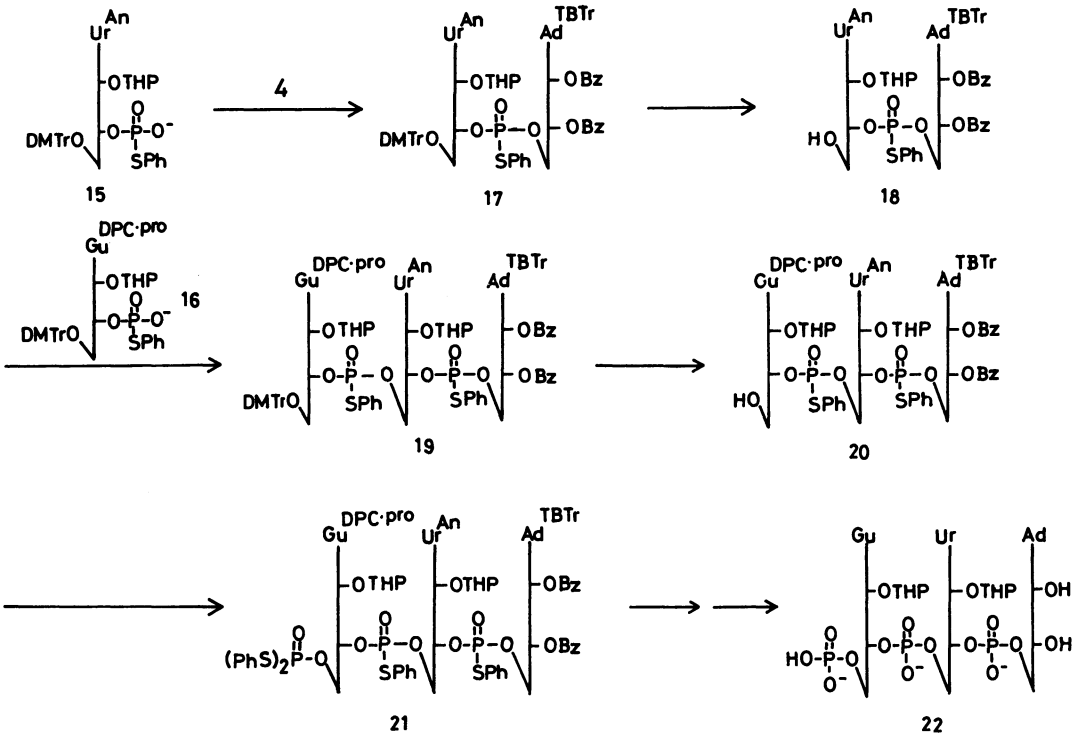
Next, to demonstrate the synthetic utility of **4** in oligoribonucleotide synthesis in the phosphorothioester approach[12] using *S,S*-diphenyl ribonucleoside 3′-phosphorodithioates, a fully protected UpA dimer **17** was synthesized by condensation of **4** with the phosphodiester unit **15** of uridine. A combined reagent of isodurenedisulfonyl dichloride (DDS)[11b] and 3-nitro-1,2,4-triazole (NT)[13] was employed as the condensing agent. Consequently, the dimer **17** was obtained in 88% yield. The dimer **17** could be detected as a distinct spot on TLC by spraying 10% H_2SO_4 followed by heating or by spraying dilute NaOH solution (be careful) owing to the dye character of the TBTr group. The purification was facilitated by its great lipophilicity. Treatment of **17** with TFA at 0°C for 33 min gave the 5′-hydroxyl component **18** in 82% yield. In a similar manner, a fully protected trimer **19** of GUA was obtained in 63% yield by condensation of **18** with the diester unit **16** of guanosine. In order to obtain a 5′-phosphorylated trimer **21** of pGUA, **19** was treated with TFA in CH_2Cl_2 to give the 5′-hydroxy derivative **20** in 82% yield, which was in turn phosphorylated with PSS–DDS–Tet. The fully protected pGUA (**21**) was synthesized in 88% yield.

A fully protected RNA fragment **23** of pGUAUUA was also synthesized according to the synthetic strategy as depicted in Scheme 4. The details of fragment condensation to construct this protected hexamer are shown in Table 1.

With the help of liphophilicity of the TBTr and TAnTr groups, these synthetic intermediates (fragments **1—6**) could be easily isolated in high yields without adsorption on silica gel. These tris(acyloxy)-trityl groups were apparently superior to the benzoyl or MMTr group in this point. At the hexamer level, the ease of its elution from silica gel was essentially



Scheme 2.



Scheme 3.

Table 1. Fragment Condensation of the Fully Protected Oligoribonucleotides **21** and **23**

Compd. No. or Frag. No.	Removal of 3′-terminal phenylthio group (40°C)			
	3′-Phosphodiester (equiv)	2 M H_3PO_2/Py	Et_3N	Time
		equiv	equiv	h
17 (=**F-1**)	U (1.3)	60	25	0.6
19	G (1.3)	60	25	2
21	PSS (1.5)			
F-2	U (1.3)	60	25	0.4
F-3	G (1.3)	60	25	2
F-4	U (1.3)	60	25	0.3
F-5	GUA (1.3)	60	25	0.5
F-6 (=**23**)	PSS (1.5)			

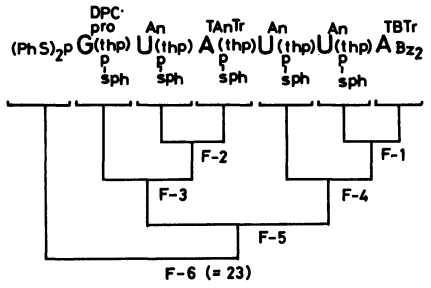
Compd. No. or Frag. No.	Detritylation (0°C)			Condensation	
	5′-Hydroxyl (mmol)	Time min	Yield %	Time min	Yield %
17 (=**F-1**)	A^{TBTr} (0.1)	17 (2% TFA)	77	60	88
19	UA	33	82	60	63
21	GUA	15	82	50	88
F-2	A^{TAnTr} (2.2)	10	81	50	91
F-3	UA (0.74)	15	78	90	79
F-4	UA (0.35)	17	95	80	87
F-5	UUA (0.283)	40	92	60	73
F-6 (=**23**)	GUAUUA	28	90	40	84

the same as that of dimers. The steric hindrance of the TBTr and TAnTr groups did not affect the condensation as shown in Table 1. This is because the TBTr group is in the remote position from the condensation site, i.e., on the adenine moiety. Expectedly, it was also confirmed that the TBTr and TAnTr groups on the adenine moiety were sufficiently stable during acid treatment for removal of the 5′-

DMTr group from fully protected oligoribonucleotide derivatives so that the loss of the THP group was essentially negligible to give 5′-hydroxyl products in high yields.

Deprotection of 21 and 23. Treatment of **21** with silver nitrate in aqueous pyridine gave an unesterified trimer as a sole product. The product was in situ treated with concentrated aqueous ammonia to remove the TBTr group of adenosine and the other base-labile protecting groups. Subsequent chromatography on Whatman 3 MM papers gave the THP containing trimer **22** in 64% yield. It was observed that the THP group of **22** was somewhat unstable during the workup after the ammonical treatment. When a mixture containing **22** was coevaporated with water to remove pyridine used as the solvent, a considerable loss of the THP group was observed so that paper chromatography often gave additional two bands of pG(thp)UA (or pGU(thp)A) and pGUA. Therefore, the partial deprotection was done in the presence of pyridine through all the process to avoid the loss of the THP group. Compound **22** appeared as a set of four peaks at ca. 17 min in reversed-phase HPLC as shown in Fig. 1-A, since it has two THP groups. Upon treatment with 0.01 M HCl, the quartet peaks were changed to one major peak at 9.2 min, which was corresponding to pGUA as shown in Fig. 1-B. Finally, the unprotected trimer was obtained in 20% overall yield from **21**.

It should be noted that, during treatment with ammonia, color of the mixture due to rosolic acid liberated became deep as the TBTr group was eliminated. This change in color is also a good visible indicator for judgement if the other acyl-type of protecting groups such as diphenylcarbamoyl (DPC), propionyl, anisoyl, and benzoyl could be removed completely since they were eliminated under similar or milder conditions. The structure of the trimer was confirmed by enzyme assay with snake venom phosphodiesterase and nuclease P_1 as shown in



Scheme 4. The strategy for synthesis of the fully protected hexaribonucleotide (**23**).

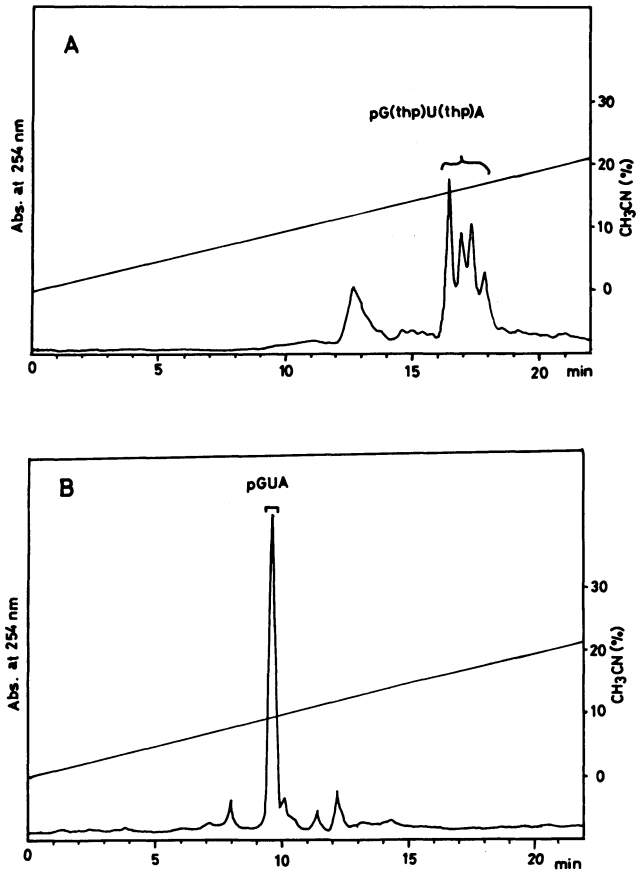


Fig. 1. A: The reverse-phase HPLC profile of pG(thp)U(thp)A (**22**) obtained by paper chromatography after partial deprotection of **21**.
B: The reverse-phase HPLC profile of crude pGUA obtained by paper chromatography after treatment of **22** with 0.01 M HCl (pH 2.0).

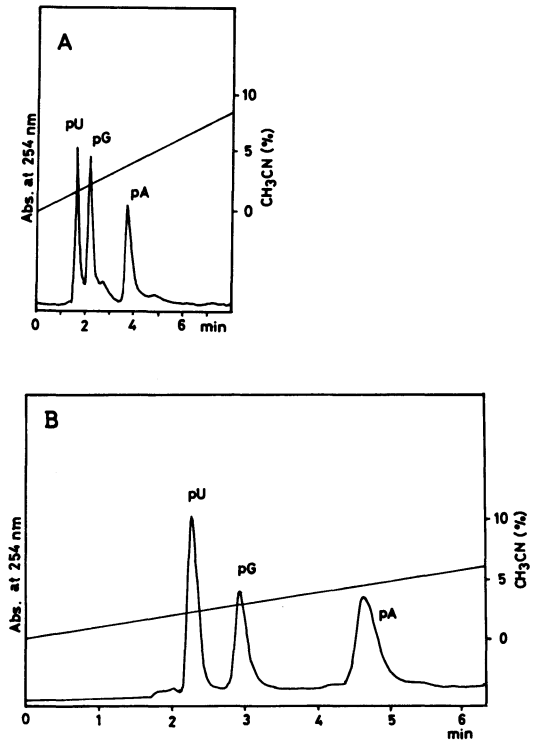


Fig. 2. The reverse-phase HPLC profile of the mixture obtained by enzyme digestion of pGUA (A) or pGUAUUA (B) with nuclease P_1.

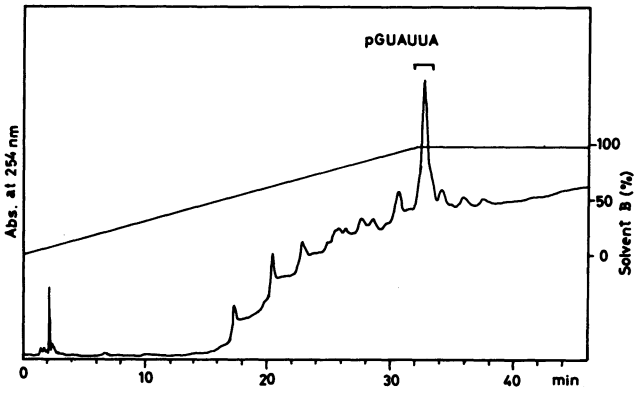


Fig. 3. The ion-exchage HPLC profile of crude pGUAUUA obtained by paper chromatography after full deprotection of **23**.

Fig. 2.

In a similar manner, pGUAUUA was obtained in 18% yield from **23** but the corresponding THP containing hexamer was not isolated and all the deprotection procedures were in situ done. In this case, the internal TAnTr group was completely removed by treatment with concentrated ammonia–pyridine (1:1, v/v) at room temperature for 24 h.

Purification of pGUAUUA was performed by paper chromatography followed by ion-exchange HPLC as shown in Fig. 3. The enzyme assay of the hexamer isolated is shown in Fig. 2-B.

The use of the TBTr and TAnTr groups in our approach to the synthesis of RNA fragments helped isolation of synthetic intermediates because of their great contribution to liphophilicity of the whole molecule. In addition to this merit, the choice of these protected-protecting groups also enabled us to make partially protected oligoribonucleotide derivatives which had the only THP group at each 2′-hydroxyl position of the RNA chain. Such THP containing oligoribonucleotides might be used for the synthesis of capped mRNAs if the capping reaction can be carried out in homogeneous media without protection of the exo-amino groups of A, G, and C. In this direction, we are now studying further application of partially protected oligoribonucleotides to the synthesis of capped mRNAs.

Experimental

General Remarks. ^{1}H NMR spectra were recorded at 100 MHz on a JNM-PS-100 spectrometer using ppm according to tetramethylsilane as an internal reference. UV spectra were obtained on a Hitachi 124 spectrophotometer. Column chromatography was performed with silica gel C-200 purchased from Wako Co., Ltd. Thin-layer chromatography was performed on precoated TLC plates, silica gel 60 F-254 (Merck). Reagent grade pyridine was distilled twice from *p*-toluenesulfonyl chloride and CaH_2 and then stored over Molecular Sieves 4A. Dichloromethane was dried over phosphorus pentoxide overnight, decanted, distilled from potassium carbonate and then stored over Molecular Sieves 4A.

For extraction of phosphodiester components after treatment of fully protected monomers or oligomers with phosphinic acid, two separatory funnels (A and B) were used to remove effectively the phosphinate reagent. First, the reaction mixture was diluted with dichloromethane and transferred to separatory funnel A, while CH_2Cl_2 was put in advance in separatory funnel B. The CH_2Cl_2 solution in separatory funnel A was washed with water, which was then transferred to separatory funnel B for back-extraction. After shaking, the aqueous layer was discarded from separatory funnel B. Further washing with water, 0.2 M triethylammonium hydrogencarbonate (TEAB) (×2), water (×2) was done in a similar manner when each aqueous layer was put first in separatory funnel A, transferred to separatory funnel B, and finally discarded. This extractive workup was used also for removal of excess phosphodiester components and arenedisulfonic acids after the condensation was completed but 5% sodium hydrogencarbonate was used in place of 0.2 M TEAB. The procedure was also effective for removal of DMF in the synthesis of **2**.

Ion-exchange HPLC was performed on a Partisil 10 SAX (Whatman) column using a linear gradient of 0.005 M KH_2PO_4 (pH 4.1), 20% CH_3CN to 0.5 M KH_2PO_4 (pH 4.5), 20% CH_3CN for 32 min at a flow rate of 1.5 ml ml^{-1}. Reversed-phase HPLC was performed on a μBondapak C_{18} (Waters) using a linear gradient of 0.1 M NH_4OAc to 30% CH_3CN in the same buffer for 30 min at a flow rate of 1.5 ml at 50 °C.

Elemental analysis was performed by the Microanalytical Laboratory, Tokyo Institute of Technology, at Nagatsuta.

N^6-[4,4′,4″-Tris(benzoyloxy)trityl]adenosine (2). To a solution of adenosine (2.68 g, 10 mmol) coevaporated twice with dry DMF were added 2,6-lutidine (4.86 ml, 72 mmol) and trimethylsilyl chloride (4,.56 ml, 36 mmol). After the mixture was stirred for 1 h, 2,6-lutidine (2.32 ml, 20 mmol), silver nitrate (3.40 g, 20 mmol), and TBTrBr (14.4 g, 20 mmol) were added successively to the mixture. After 1 h, 2,6-lutidine (4.17 ml, 36 mmol) and silver nitrate (6.12 g, 36 mmol) were added to complete the reaction. The mixture was stirred for 30 min, and then the resulting precipitate was removed by filtration and washed with DMF (100 ml). The filtrate and washing were combined and treated with water (32 ml). After being kept for 40 min, the solution was partitioned between dichloromethane and water for extraction, which was performed in the same manner as described in General Remarks to remove DMF completely. The organic layer was dried over sodium sulfate, filtered, and evaporated to dryness. The residue was coevaporated with six times with toluene and chromatographed on a column of silica gel (300 g) with dichloromethane–methanol. The fractions containing 2 were collected and evaporated. The residue was reprecipitated from dichloromethane into hexane to give **2** (6.41 g, 74%) as colorless powder; mp 165—170 °C; ^{1}H NMR ($CDCl_3$) δ=3.60—3.88 (2H, m, 5′-H), 4.16 (1H, m, 4′-H), 4.28 (1H, m, 3′-H), 4.82 (1H, m, 2′-H), 5.74 (d, 1H, 1′-H), 7.09 (6H, d, *J*=8 Hz, ArH), 7.46 (15H, m, ArH), 7.60 (1H, s, 2-H), 7.94 (1H, s, 8-H), 8.12 (6H, d, *J*=8 Hz).

Found: C, 68.07; H, 4.52; N, 7.62%. Calcd for $C_{50}H_{39}O_{10}N_5 \cdot H_2O$: C, 67.64; H, 4.43; N, 7.89%.

5′-*O*-(4,4′-Dimethoxytrityl)-N^6-[4,4′,4″-tris(benzoyloxy)trityl]adenosine (3). To a solution of **2** (5.90 g, 6.79 mmol), coevaporated several times with dry pyridine, in dry pyridine (19.5 ml) was added 4,4′-dimethoxytrityl chloride (2.64 g, 7.8 mmol). After being kept for 9 h, the mixture was quenched by addition of ice. The usual workup involving extraction with dichloromethane and coevaporation with toluene followed by silica-gel column chromatography (eluent: dichloromethane–methanol) gave **3**. The compound was reprecipitated from its dichloromethane solution into hexane gave pure **3** (7.45 g, 94%); mp 143.5—147 °C; ^{1}H NMR ($CDCl_3$) δ=3.72 (3H, s, OCH_3), 3.76 (3H, s, OCH_3), 4.22—4.34 (2H, m, 5′-H), 4.36 (1H, m, 4′-H), 4.64—5.00 (2H, m, 2′-H and 3′-H), 5.84 (1H, m, 1′-H), 6.77 (4H, m, ArH), 7.12 (9H, m, ArH), 7.45 (15H, m, ArH), 7.70 (s, 1H, 2-H), 8.00 (1H, s, 8-H), 8.14 (6H, d, J=8 Hz, ArH).

Found. C, 72.65; H, 5.20; N, 5.56%. Calcd for $C_{71}H_{57}O_{14}N_5 \cdot 1/2H_2O$: C, 72.19; H, 4.95; N, 5.93%.

2′,3′-*O*-Dibenzoyl-N^6-[4,4′,4″-tris(benzoyloxy)trityl]adenosine (4). Compound **3** (2.35 g, 2 mmol) was coevaporated three times with dry pyridine and finally dissolved in dry pyridine (20 ml). Benzoyl chloride (6.96 μl, 6 mmol) was added to the solution. After the mixture was stirred for 1 h, benzoyl chloride (232 μl, 2 mmol) was added. The mixture was stirred for an additional 1 h. The usual workup followed by chromatography gave the dibenzoylated product (2.96 g, quant.) as foam. This material was treated with 2% trifluoroacetic acid in dichloromethane (200 ml) at 0 °C for 17 min. After the reaction was quenched by addition of 5% sodium hydrogencarbonate (200 ml), the organic phase was collected, washed with 5% sodium hydrogencarbonate, dried over sodium sulfate, and filtered. The filtrate was evaporated and the last traces of pyridine were removed by repeated coevaporation with toluene. The residue was chromatographed on a column of silica gel with dichloromethane–methanol to give **4**. This material was reprecipitated from its dichloromethane solution into hexane to give **4** (1.65 g, 77%); mp 146.5 °C; ^{1}H NMR ($CDCl_3$) δ=4.01 (3H, m, 4′-H and 5′-H), 4.57 (1H, m, 3′-H), 6.04 (1H, m, 2′-H), 6.27 (1H, m, 1′-H), 7.12 (6H, m, ArH), 7.48 (21H, m, ArH), 7.80 (1H, s, 2-H), 7.91 (4H, m, ArH), 8.02 (1H, s, 8-H), 8.16 (6H, m, ArH).

Found: C, 70.40; H, 4.39; N, 6.31%. Calcd for $C_{64}H_{47}O_{12}N_5 \cdot H_2O$: C, 70.13; H, 4.51; N, 6.39%.

3′,5′-*O*-(1,1,3,3-Tetraisopropyldisiloxane-1,3-diyl)-N^6-[4,4′,4″-tris(benzoyloxy)trityl]adenosine (6a). Compound **5**[9] (1.02 g, 2 mmol) was rendered anhydrous by repeated coevaporation with dry DMF and dissolved finally in dry DMF (20 ml). To the solution were added successively 2,6-lutidine (463 μl, 4 mmol), silver nitrate (683 mg, 4 mmol), and TBTrBr (2.89 g, 4 mmol). After being stirred at room temperature for 25 min, the mixture was quenched with water. The resulting precipitate was removed by filtration and the filtrate was transferred to a separatory funnel. The extractive workup was done to remove DMF as described in General Marks. The combined dichloromethane layer was dried over sodium sulfate, filtered, and evaporated in vacuo. The resulting residue was chromatographed on a column of silica gel with dichloromethane–methanol. The product thus obtained was reprecipitated from dichloromethane into hexane to give a pure material (1.51 g, 68%); ^{1}H NMR ($CDCl_3$) δ=1.04 (28H, m, $C(CH_3)_2$), 3.36 (br, 1H, 2′-OH), 4.05 (3H, m, 4′- and 5′-H), 4.58 (1H, m, 3′-H), 5.08 (1H, m, 2′-H), 5.89 (1H, m, 1′-H), 6.96 (1H, s, NH), 7.14 (6H, m, ArH), 7.48 (15H, m, ArH), 7.96 (1H, s, 2-H), 8.00 (1H, s, 8-H), 8.20 (6H, m, ArH).

Found: C, 66.37; H, 5.81; N, 6.09%. Calcd for $C_{62}H_{65}O_{11}N_5Si_2 \cdot 1/2H_2O$: C, 66.41; H, 5.93; N, 6.25%.

3′,5′-*O*-(1,1,3,3-Tetraisopropyldisiloxane-1,3-diyl)-N^6-[4,4′,4″-tris(*p*-anisoyloxy)trityl]adenosine (6b). The same procedure as described in the case of **6a** was performed except for the reaction time (6 h). The yield was 54%; ^{1}H NMR ($CDCl_3$) δ=1.09 (24H, m, $C(CH_3)_2$), 1.59 (4H, m, SiCH), 3.21 (2H, m, 5′-H), 3.92 (s, 6H, OCH_3), 4.12 (1H, m, 4′-H), 4.68 (1H, m, 3′-H), 5.20 (1H, m, 2′-H), 5.94 (1H, m, 1′-H), 7.09 (6H, d, J=8.8 Hz, ArH), 7.22 (6H, d, J=8.8 Hz, ArH), 7.50 (6H, d, J=8.8 Hz, ArH), 7.92 (1H, s, 2-H), 8.04 (1H, s, 8-H), 8.20 (6H, d, J=8.8 Hz, ArH).

Found: C, 64.82; H, 5.96; N, 5.73%. Calcd for $C_{65}H_{71}O_{14}N_5Si_2$: C, 64.93; H, 5.95; N, 5.82%.

2′-*O*-(Tetrahydro-2-pyranyl)-3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-N^6-[4,4′,4″-tris(benzoyloxy)trityl]adenosine (7a). To a solution of **6a** (1.17 g, 1.05 mmol) in dry dichloromethane (4.5 ml) were added Molecular Sieves 4A (1 g), 3,5-dihydro-2*H*-pyran (2.30 ml, 22.5 mmol), and trifluoroacetic acid (174 μl, 2.26 mmol). After the mixture was stirred at room temperature for 5 h, pyridine (0.5 ml) was added. The Molecular Sieves were removed by filtration and the filtrate was evaporated in vacuo. The residue was partitioned with dichloromethane and water. The organic phase was collected, dried over sodium sulfate, filtered, and evaporated to dryness. The residue was chromatographed on a silica-gel column with dichloromethane–methanol to give **7a** as foam (1.21 g, 96%); ^{1}H NMR ($CDCl_3$) δ=1.07 (24H, m, $C(CH_3)_2$), 1.56 (6H, m, THP), 1.72 (4H, m, SiCH), 3.46 (2H, m, THP), 4.18 (3H, m, 4′-H and 5′-H), 4.70 (1H, m, 3′-H), 5.00 (1H, m, 2′-H), 5.23 (1H, m, THP), 6.00 (1H, m, 1′-H), 6.93 (1H, s, NH), 7.22 (6H, m, ArH), 7.54 (15H, m, ArH), 7.95 (1H, s, 2-H), 8.04 (1H, s, 8-H), 8.20 (6H, m, ArH).

Found: C, 66.61; H, 6.12; N, 5.45%. Calcd for $C_{67}H_{73}O_{12}N_5Si_2 \cdot 1/2H_2O$: C, 66.75; H, 6.19; N, 5.81%.

2′-*O*-(Tetrahydro-2-pyranyl)-3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-N^6-[4,4′,4″-tris(*p*-anisoyloxy)trityl]adenosine (7b). This compound was prepared in the same way described in the case of **7a** starting from **6b** (536 mg, 0.45 mmol) except for the reaction time (26 h). The yield was 581 mg (100%); ^{1}H NMR ($CDCl_3$) δ=1.08 (24H, m, $C(CH_3)_2$), 1.56 (6H, m, THP), 1.82 (1H, m, SiCH), 3.48 (2H, m, THP), 3.90 (9H, s, OCH_3), 4.16 (3H, m, 4′-H and 5′-H), 4.52—4.83 (2H, m, 2′-H and 3′-H), 5.24 (1H, m, THP), 6.01 (1H, m, 1′-H), 6.98 (6H, d, J=8.4 Hz, ArH), 7.14 (d, J=9 Hz), 7.48 (6H, d, J=8.4 Hz, ArH), 7.97 (1H, s, 2-H), 8.07 (1H, s, 8-H), 8.16 (6H, d, J=9 Hz, ArH).

Found: C, 65.98; H, 7.05; N, 3.92%. Calcd for $C_{70}H_{89}O_{15}N_5Si_2 \cdot 1/2H_2O$: C, 64.89; H, 7.00; N, 5.40%.

2′-*O*-(Tetrahydro-2-pyranyl)-N^6-[4,4′,4″-tris(benzoyloxy)trityl]adenosine (8a). To a solution of **7a** (506 mg, 423 μmol) in acetonitrile (2.2 ml) were added potassium fluoride (155 mg, 2.67 mmol), tetraethylammonium bromide (565 mg, 2.69 mmol), and water (48.2 μl, 2.67 mmol). The mixture was stirred at 50 °C for 3 h and then the precipitate was removed by filtration. The filtrate was evaporated and the residue was partitioned with dichloromethane and water. The organic phase was collected, dried

over sodium sulfate, filtered, and evaporated in vacuo to dryness. The residue was chromatographed on a column of silica gel with dichloromethane–methanol to give **8a**. Reprecipitation from dichloromethane into hexane gave a pure material (124 mg, 29%); ^{1}H NMR ($CDCl_3$) δ=1.60 (8H, m, THP), 3.40 (2H, m, THP), 3.82 (2H, m, 5′-H), 4.44 (2H, m, 3′-H and 4′-H), 4.88 (1H, m, 2′-H), 4.90 (1H, m, THP), 5.96 (1H, m, 1′-H), 7.22 (6H, m, ArH), 7.54 (15H, m, ArH), 7.87 (1H, s, 2-H), 8.10 (1H, s, 8-H), 8.22 (6H, d, J=8 Hz, ArH).

Found: C, 67.47; H, 5.06; N, 6.67%. Calcd for $C_{55}H_{47}O_{11}N_5 \cdot 1.5H_2O$: C, 67.34; H, 5.14; N, 7.14%.

Tris[4-(*p*-anisoyloxy)phenyl]methanol (TAnTrOH). To a solution of rosolic acid (14.5 g, 50 mmol) in dry pyridine (200 ml) was added *p*-anisoyl chloride (42.7 g, 250 mmol). The mixture was heated at 80—90 °C for 1.5 h. After being cooled to room temperature, the mixture was quenched with ice-water and extracted with CH_2Cl_2. The organic phase was washed several times with 5% $NaHCO_3$, dried over sodium sulfate, filtered, and evaporated under reduced pressure to a gum. The gummy residue was coevaporated several times with toluene to remove the last traces of pyridine. The solid material was recrystalized from benzene to give TAnTrOH (15.3 g, 44%); mp 175.5—176.5 °C; ^{1}H NMR ($CDCl_3$) δ=3.05 (1H, s, OH), 3.86 (9H, s, OCH_3), 6.88—7.38 (18H, m, ArH), 8.05 (3H, s, ArH), 8.13 (3H, s, ArH).

Found: C, 73.41; H, 4.86%. Calcd for $C_{43}H_{34}O_{10}$: C, 72.67; H, 4.82%.

4,4′,4″-Tris(*p*-anisoyloxy)trityl Bromide (TAnTrBr). To a suspension of TAnTrOH (4 g, 5.6 mmol) in dry benzene (10 ml) was added acetyl bromide (1 ml, 13.5 mmol). The mixture was refluxed for 1.5 h and the hot solution was filtered to remove a small amount of insoluble material. Dry hexane was added portionwise to the hot filtrate. After cooling, the resulting precipitate was collected by filtration and washed with dry hexane–benzene (2:1, v/v) and then with dry hexane to give TAnTrBr (3.9 g, 90%): mp 96—97 °C; ^{1}H NMR ($CDCl_3$) δ=3.88 (9H, s, OCH_3), 6.90—7.39 (18H, m, ArH), 8.02 (3H, s, ArH), 8.13 (3H, s, ArH).

Found: C, 66.59; H, 4.34; Br, 10.57%. Calcd for $C_{43}H_{33}O_9Br$: C, 66.76; H, 4.30; Br, 10.33%.

2′-*O*-(Tetrahydro-2-pyranyl)-*N*^{6}-[4,4′,4″-tris(*p*-anisoyloxy)trityl]adenosine (8b). This compound was synthesized in the same way as described in the above experiment except for the reaction time (1 h). The yield was 56%; ^{1}H NMR ($CDCl_3$) δ=1.52 (6H, m, THP), 3.90 (9H, s, OCH_3), 4.25—4.64 (4H, m, 3′,4′, and 5-H), 4.90 (1H, m, 2′-H), 5.10 (1H, m, THP), 5.92 (1H, m, 1′-H), 6.98 (6H, d, J=9 Hz, ArH), 7.16 (6H, d, J=9 Hz, ArH), 7.48 (6H, d, J=9 Hz, ArH), 7.83 (1H, s, 2-H), 8.08 (1H, s, 8-H), 8.16 (6H, d, J=9 Hz).

Found: C, 66.59; H, 5.33; N, 6.56%. Calcd for $C_{58}H_{53}O_{16}N_5$: C, 66.72; H, 5.12; N, 6.71%.

Synthesis of 8b from 5 without Purification of the Synthetic Intermediates 7b and 9. To a solution of **5**[9] (5.61 g, 11 mmol) in dry dichloromethane (110 ml) were added 3,4-dihydro-2*H*-pyran (22.5 ml, 220 mmol) and trifluoroacetic acid (1.7 ml, 22 mmol). The mixture was stirred at room temperature for 17 h. Then, additional trifluoroacetic acid (0.85 ml, 11 mmol) was added and the mixture was further stirred for 4 h. Five per cent sodium hydrogencarbonate (100 ml) was added to the mixture and the solution was extracted with dichloromethane (100 ml). The organic phase was collected, dried over sodium sulfate, filtered, and evaporated in vacuo to dryness. The residue was coevaporated several times successively with ethanol and dry DMF, and finally dissolved in dry DMF (110 ml). To the solution were added 2,6-lutidine (2.55 ml, 22 mmol), silver nitrate (3.74 g, 22 mmol), and TAnTrBr (17 g, 22 mmol) with vigorous stirring at 0 °C. The precipitate was removed by filtration and washed with dichloromethane. The filtrate and washing were combined and washed with water to remove DMF in a manner similar to that described in General Remarks. The dichloromethane layer was dried over sodium sulfate, filtered, and evaporated in vacuo to dryness. The residue was coevaporated several times with toluene and dissolved in acetonitrile (55 ml). To the solution were added potassium fluoride (3.83 g, 66 mmol), tetraethylammonium bromide (13.9 g, 66 mmol), and water (1.19 ml). The mixture was stirred at 50 °C for 1 h. The precipitate was removed by filtration. The filtrate was transferred to a separatory funnel and diluted with water. Extraction was performed with dichloromethane, in a manner similar to that described for extractive removal of DMF in General Remarks, to remove the quarternary ammonium salt. The dichloromethane phase was dried over sodium sulfate, filtered, and evaporated in vacuo to dryness. The usual workup followed by chromatography on silica gel with dichloromethane–methanol and reprecipitation gave **8b** (9.24 g, 80%).

***S,S*-Diphenyl 2′-*O*-(Tetrahydro-2-pyranyl)-5′-*O*-(4,4′-dimethoxytrityl)-*N*^{6}-[4,4′,4″-tris(*p*-anisoyloxy)trityl]adenosine 3′-Phosphorodithioate (11).** Compound **8b** (7.76 g, 7.43 mmol) was rendered anhydrous by repeated coevaporation with dry pyridine and finally dissolved in dry pyridine (22 ml). To the solution was added 4,4′-dimethoxytrityl chloride (3.03 g, 8.92 mmol). The mixture was stirred at room temperature for 1.5 h. After the usual extractive workup, chromatography on a column of silica gel with dichloromethane–methanol gave **10** (6.60 g, 66%). This compound was used in the next reaction without further characterization. Compound **10** (6.60 g, 4.9 mmol) was mixed with cyclohexylammonium *S,S*-diphenyl phosphorodithioate (2.80 g, 7.35 mmol) and 1-*H*-tetrazole (1.37 g, 19.6 mmol), and the mixture was coevaporated several times with dry pyridine and finally dissolved in dry pyridine (30 ml). To the mixture was added MDS (3.11 g, 9.8 mmol). After being stirred at room temperature for 1 h, the mixture was quenched by diluted with dichloromethane (100 ml). The solution was washed with water and 5% sodium hydrogencarbonate as described in General Remarks. The usual workup followed by chromatography (silica gel, eluent: dichloromethane–methanol) and reprecipitation from dichloromethane into hexane gave **11** (6.66 g, 85%); ^{1}H NMR ($CDCl_3$) δ=8.11 (6H, d, J=8 Hz, ArH), 8.01 (1H, s, 8-H), 7.80 (1H, s, 2-H), 6.72—7.68 (41H, m, ArH, 2H), 5.77 (d, 1H, 1′-H), 5.07—5.28 (2H, m, 2′-H and THP), 4.56 (1H, m, 3′-H), 4.18 (1H, m, 4′-H), 3.85 (9H, s, OCH_3 of TArTr), 3.75 (6H, s, OCH_3 of DMTr), 3.72 (2H, m, 5′-H), 2.85 (2H, m, THP), 1.24—1.80 (6H, m, THP).

Found: C, 69.48; H, 6.56; N, 3.88; S, 3.78%. Calcd for $C_{91}H_{80}O_{17}N_5S_2P \cdot 2.5C_6H_{14}$: C, 69.72; H, 6.35; N, 3.84; S, 3.51%.

General Procedure for Fragment Condensation. Preparation of Phosphodiester Components: An appropriately

protected monomer unit or oligomer block was rendered anhydrous by repeated coevaporation with dry pyridine and dissolved in a solution of 2 M phosphinic acid (60 equiv) in pyridine containing triethylamine as shown in Table 1. After the reaction was completed, 0.2 M TEAB was added and extracted with dichloromethane. The excess phosphinate reagent was removed by the extractive workup as described in General Remarks. In this case, washing was performed with water (×2) and 0.2 M TEAB (×2). The organic phase was dried over sodium sulfate, filtered, and evaporated in vacuo to dryness. The residue containing a mixture of the phosphodiester component and benzenethiol was used in the successive condensation without further purification.

Preparation of Hydroxyl Components: A fully protected monomer or oligomer block was dissolved in 0.5% or 0.75% (v/v) trifluoroacetic acid in dichloromethane at 0 °C. After the reaction was completed, 5% sodium hydrogencarbonate was added for neutralization. The solution was extracted with dichloromethane. The organic phase was dried over sodium sulfate, filtered, evaporated, and chromatographed on a column of silica gel for isolation of the hydroxyl component. The details of the results are listed in Table 1.

Fragment Condensation: The hydroxyl component obtained by the above procedure was mixed with the diester component and 3-nitro-1,2,4-triazole, and the mixture was rendered anhydrous by repeated coevaporation with dry pyridine and finally dissolved in dry pyridine (10 ml mmol^{-1} of hydroxyl component). DDS was added to the solution and stirring was continued until the hydroxyl component had disappeared. Then, extractive workup was performed with dichloromethane as described in General Remarks. Washing was performed each twice with water, 5% sodium hydrogencarbonate, and water. After the usual workup chromatographic separation (silica gel, eluent: dichloromethane–methanol) gave an condensation product as summarized in Table 1.

Deprotection of the Fully Protected Trimer 21. To a solution of the trimer **21** (232 mg, 0.088 mmol) in pyridine (2.25 ml) were added successively water (2.25 ml) and silver acetate (2.20 g, 13.2 mmol). The mixture was stirred vigorously at 50 °C for 5 h. After cooling to room temperature, the solution was diluted with pyridine–water (2:1, v/v, 45 ml) and treated with hydrogen sulfide gas at 0 °C for 15 min. The excess gas was removed under reduced pressure by an aspirator for 30 min. The resulting precipitate was removed by centrifugation. The supernatant was collected and passed through Dowex 50 W×2 (pyridinium form, 5 ml). The resin was washed with pyridine–water (2:1, v/v, 50 ml). The eluant and washings were combined and evaporated in vacuo after addition of pyridine (70 ml). The residue was coevaporated with pyridine (5×20 ml) to remove the last traces of acetic acid and then in dissolved pyridine (5 ml). The solution was mixed with concentrated aqueous ammonia (45 ml). The mixture was warmed at 50 °C for 5 h. After cooling, pyridine (20 ml) was added and the solution was evaporated under reduced pressure to its half volume. The concentrated solution was extracted with CH_2Cl_2 (5×20 ml). Each organic layer was washed with the same aqueous solution put in another separatory funnel. The two aqueous layers were combined and three hold of pyridine was added. The solution was evaporated in vacuo, the residue being coevaporated three times with pyridine and chromatographed on Whatman 3 MM papers with 2-propanol–concentrated aqueous ammonia–water (6:1:3, v/v/v). A broad band which moved around AMP was eluted with water to give pG(thp)pU(thp)pA (1752 OD at 260 nm, 64%). This compound was stored as its pyridine solution (5.6 μmol ml^{-1}). An aliquot of this solution (5.0 μmol) was taken and evaporated under reduced pressure. The residue was coevaporated with MeOH–toluene (1:1, v/v, 3×5 ml) and treated with 0.01 M HCl in dioxane–water which was finally adjusted to pH 2.0 by addition of 0.1 M HCl. After being stirred for 16 h, the solution was neutralized with concentrated aqueous ammonia and concentrated under reduced pressure to half the volume. The concentrate was partitioned between ether and water. The aqueous layer was collected and washed twice with ether. The aqueous solution was concentrated and applied to a column of Sephadex A-25 (triethylammonium salt, 1 ml). The column was first washed with water (50 ml) and eluted with 1 M TEAB buffer (50 ml). Fractions containing UV were combined, concentrated under reduced pressure, and the residue was covaporated several times with water to give pGUA (153 OD at 260 nm, 4.95 μmol, 98%). This compound (20 OD) was finally purified by reversed-phase HPLC (Fig. 1-B). The peak eluted at 9.2 min was collected and evaporated under reduced pressure. The residue was dissolved in water so as to adjust the concentration of ammonium acetate to less than 0.05 M and applied to a column of Sephadex A-25, 2 ml). After washing with water (100 ml), the trimer was eluted with 1 M TEAB buffer. The eluant was concentrated under reduced pressure and coevaporated repeatedly with water to give pGUA (6.4 OD at 260 nm, 0.207 μmol, 32%): UV: λ_{max}=257 nm, λ_{min}=234 nm.

Enzymatic Assay of pGUA. The trimer (1.0 OD) was incubated with shake venom phosphodiesterase (1 mg ml^{-1}, 10 μl) in 0.05 Tris–HCl buffer (pH 8.0, 50 μl) containing 1 M $MgCl_2$ (2.5 μl) at 37 °C for 3 h. The mixture was analyzed by reversed-phase HPLC, which showed three peaks of pU, pG, and pA in a ratio of 1.1:1.0:0.9.

The trimer (1.0 OD) was incubated with nuclease P_1 (2 mg ml^{-1}, 15 μl) in 0.05 M sodium acetate buffer (pH 5.4, 35 μl) at 37 °C for 30 min. A similar HPLC analysis gave a mixture of pU, pG, and pA in a ratio of 1.1:1.0:0.8.

Deprotection of the Fully Protected Hexamer 23. The fully protected hexamer **23** (25.9 mg, 5 μmol) was dissolved in pyridine (450 μl), and water (150 μl) and silver acetate (251 mg, 1.5 mmol) were successively added. The resulting mixture was vigorously stirred at 50 °C for 5 h and bubbled with hydrogen sulfide gas at 0 °C until a clear supernatant had been obtained (ca. 15 min). The excess gas was removed under reduced pressure by an aspirator. The precipitate was removed by centrifugation and the supernatant was passed through a column of Dowex 50 W×2 (Py form, 3 ml). The resin was eluted with pyridine–water (2:1, v/v, 20 ml). The eluant was evaporated under reduced pressure and coevaporated several times with pyridine to remove the last traces of acetic acid. The residue was dissolved in pyridine (25 ml) and concentrated aqueous ammonia (25 ml) was added. The mixture was stirred at room temperature for 24 h. The precipitate was removed by filtration and washed with pyridine (20 ml). The filtrate and washing were com-

bined, evaporated under reduced pressure, and coevaporated with water–dioxane (1:2, v/v, 10 ml). The residue was treated with 0.01 M HCl in water–dioxane (1:1, v/v, 10 ml) which was adjusted to pH 2.0 by addition of 0.1 M HCl. The mixture was stirred at room temperature for 1 day and then neutralized by addition of 1 M aqueous ammonia. After being evaporated under reduced pressure, the mixture was chromatographed on Whatman 3 MM papers with 2-propanol-concentrated aqueous ammonia–water (55:10:35, v/v/v) to give crude material of pGUAUUA (178 OD at 260 nm, 59%). This crude material (30 OD) was purified by anion-exchange HPLC as described in the case of the synthesis of pGUA to give pure hexamer (5.5 OD at 260 nm, 18.3%); λ_{max}=257 nm, λ_{min}=232 nm.

Enzymatic Assay of pGUAUUA. The hexaribonucleotide (0.2 OD) was incubated with nuclease P_1 (2 mg ml^{-1}, 6 μl) in 0.05 M sodium acetate (pH 5.4, 14 μl) at 37 °C for 80 min. The HPLC analysis showed a mixture of pU, pG, and pA in a ratio of 3.0:1.3:2.2.

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