Chemical Synthesis of Capped Oligoribonucleotides, $m^7G^{5'}pppAUG$ and $m^7G^{5'}pppAUGACC$

Mitsuo SEKINE, Shin-ichi NISHIYAMA, Takashi KAMIMURA, Yumi OSAKI, and Tsujiaki HATA*

Department of Life Chemistry, Tokyo Institute of Technology, Nagatsuta, Midoriku, Yokohama 227

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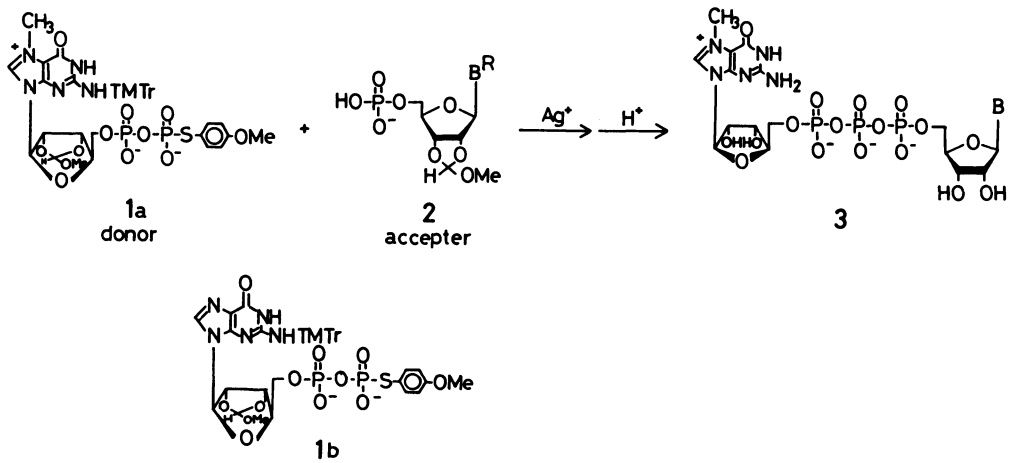
The fully-protected pAUG (**15**) and pAUGACC (**21**) were synthesized by the phosphotriester approach where the phenylthio group was employed as the internal and 5'-terminal phosphate-protecting groups. The partially unblocked oligomers (**32**) and (**33**), obtained by alkaline treatment of (**15**) and (**21**), were condensed with a capping reagent (**1a**) in the presence of imidazole by activation with silver nitrate to afford the capped trimer and hexamer protected with the acid-labile protecting groups, *i.e.*, 4,4,'4"-trimethoxytrityl (TMTr), 4-monomethoxytrityl (MMTr), tetrahydropyran-2-yl (THP), and methoxymethylene (mM) groups. The protected capped oligomers were unblocked by a dilute HCl solution to afford $m^7G^{5'}pppAUG$ and $m^7G^{5'}pppAUGACC$, which were purified by HPLC and characterized by enzyme assays.

Chemical synthesis of capped oligoribonucleotides, $m^7G^{5'}pppNupNupNu\cdots$[1] is one of the intriguing subjects in nucleic acid chemistry, since construction of the unique cap structure will be realized by exploration of new techniques. Previously, we have reported capping reactions utilizing the arylthio group as an activatable pyrophosphate protecting group.[2-6] These studies indicate that, because of the inherent insolubility of 7-methylguanosine derivatives in organic solvents, a capping agent should be protected with sufficiently lipophilic groups so that the capping reaction proceeds homogeneously to obtain reproducible results. Therefore, we recently reported a new capping agent (**1a**) that reacted with nucleotides in the presence of silver nitrate giving rise to the cap structure with high reproducibility.[4] This silver ion-catalyzed reaction required the use of an excess amount (2 equiv) of acceptor nucleotides (**2**) to obtain $m^7G^{5'}pppNu$ (**3**) in good yields (45—60%). This is because the reaction in the reverse ratio of **1** to **2** resulted in a very complicated mixture from which **3** was isolated only in a poor yield (<5%). Similar experiences have been encountered also in the synthesis of unmethylated cap structures, $G^{5'}pppNu$, from **1b** and **2**. The silver ion-mediated reaction can hardly be applied to the synthesis of capped oligoribonucleotides, because the excess use of oligomer components is undesirable. The above complicated reaction may be rationalized by the generation of an highly active metapyrophosphate intermediate (**4**), which can react again with the product so that the P(O)-O-P(O) bond was degraded. In the case of the reaction using the excess acceptor nucleotide, **4** might be trapped mainly with the excess **2** and a little with the product **3** to give a simpler result.

Therefore, we have studied a more generally applicable method for the capping reaction. A reaction of **1b** with **2** (B=Cy, R=DMTr) was chosen to search for suitable conditions that enabled us to use excess amounts of **1b**. Consequently, it was found that coaddition of imidazole dramatically effected the silver ion-catalyzed reaction. When the reaction was carried out in the presence of 30 fold excess imidazole by using 1.7 equiv of **1b** to **2**, TLC exhibited a major spot corresponding to the protected $G^{5'}pppC$ and much less by-products. The workup gave a 61%



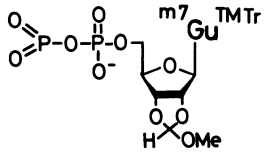
Scheme 1.

yield of G[5']pppC. This result is explained in terms of the *in situ* formation of the phosphorimidazolide (**5**)[7] capable of reacting gradually with the acceptor nucleotide and not with the product.

The protecting groups used for **2** have been reported in the previous paper.[4]

Since we could find the practical procedure for the construction of the cap structure, the fully-protected oligoribonucleotides of pApUpG and pApUpGpApCpC were synthesized by our phosphotriester method using the phenylthio group as the phosphate-protecting group. These synthetic strategies are shown in Scheme 3. All the nucleoside and nucleotide units (**6**—**11**) employed are illustrated in Scheme 4.

For the preparation of the uridine unit (**6**), an alternative to the previous route was employed. The new method involves introduction of the anisoyl group into the uracil ring[8] at an earier stage. This approach enabled us to extract the diol (**25**) easily with CH_2Cl_2, while the previous method required bothersome ion-exchange chromatography for removal of excess triethylammonium fluoride used in the conversion of the cyclic silyl ether (**28**) to 3'-*O*-(tetrahydropyran-2-yl)uridine (**29**). The uridine unit **6** was obtained *via* the six-step reaction in an overall yield of 45% from uridine. The 3'-terminal cytidine unit (**10**) was synthesized by benzoylation of the common intermediate (**30**) followed by detritylation of the resulting product (**31**). The 3'-terminal guanosine unit (**11**) was prepared by a new procedure for the methoxymethylenation catalyzed by trimethylsilyl chloride.[9]



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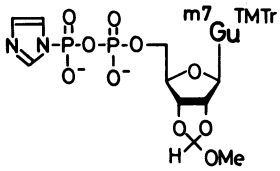

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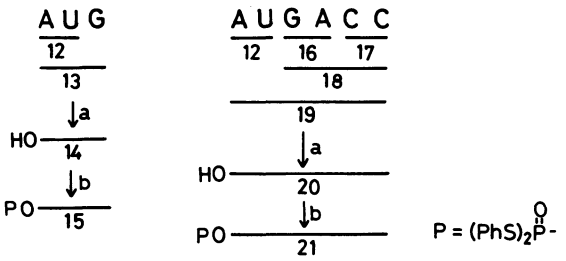
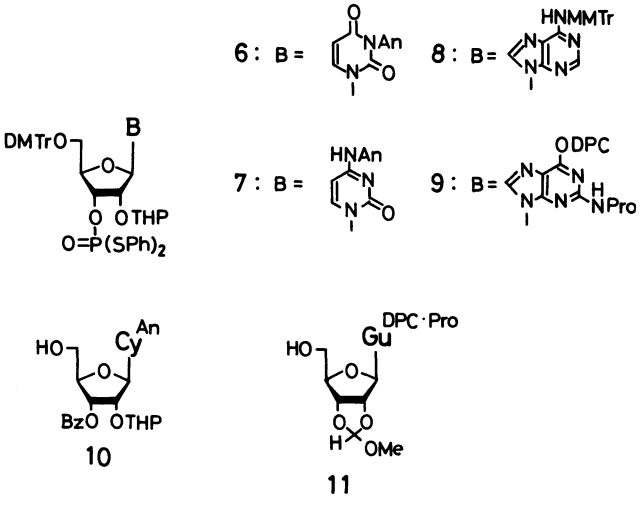
Scheme 2.

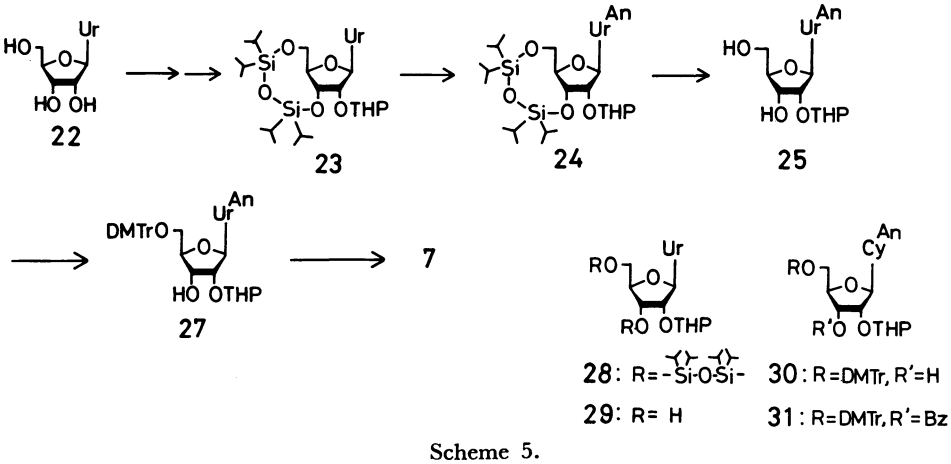


Strategy for the synthesis of the fully protected pAUG (15) and pAUGACC (21): The line (—) means fully protected oligonucleotide chains.

Scheme 3.

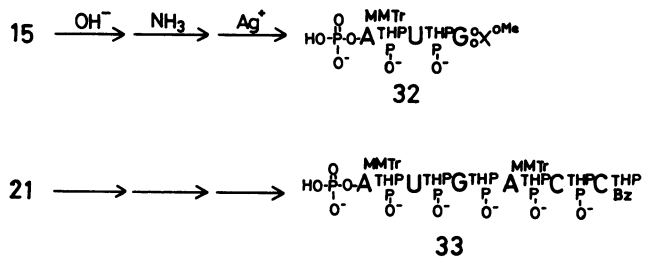


Scheme 4.



Scheme 5.

The conditions employed for the fragment condensations of oligomers were essentially the same as those reported previously.[10,11] For the construction of the triphosphate bridge, the 5′-terminal hydroxyls of compounds **14** and **20** obtained from **13** and **19** were phosphorylated with cyclohexylammonium *S,S*-diphenyl phosphorodithioate (**PSS**)[12] in the presence of mesitylene-1,3-disulfonyl dichloride (**MDS**)[13] to afford compounds (**15**) and (**21**), respectively. The phosphorylations proceeded smoothly without any side reactions. Since the cap structure was extremely labile to basic media, all the phenylthio- and acyl-protecting groups were removed by the successive treatments with 0.2 M NaOH (1 M=1 mol dm^{-3}), concentrated aqueous ammonia, and silver acetate prior to the capping reactions. This partial deprotection gave the oligomers (**32**) and (**33**) having the 2′-*O*-tetrahydropyran-2-yl and adenosine N^6-MMTr groups. The former is preserved to avoid side reactions associated with the internal phosphate groups upon the capping reaction. The latter serves as the marker for monitoring the reaction on TLC. Before the capping reactions, the roughly purified trimer **32** and hexamer **33** were treated with 0.01 M HCl to obtain the unprotected trimer pApUpG and hexamer pApUpGpApCpC, respectively. Isolation of the two oligomers was performed by HPLC as shown in Figs. 1 and 2. The yields of pApUpG and pApUpGpApCpC were 20 and 10% yields, respectively. Since these oligomers were prepared as the references in the capping reactions, no attempts to increase the yields have not been made. The enzyme treatments of the trimer and hexamer using venom phosphodiesterase resulted in mixtures of the parent 5′-mononucleotides in the correct ratios as shown in Fig. 3. Next, we examined the triphosphate bridge formation between the oligomer **32** or **33** and the unmethylated precursor (**1b**) of the capping agent **1a**. In these reactions, 5 equiv of **1b**, 20 equiv of imidazole, and 30 equiv of silver nitrate were employed. After the reactions were completed, the remaining acid-labile protecting groups were removed by treatment with 0.01 M HCl. The reaction mixtures were chromatographed on Whatman 3 MM papers and then separated by HPLC. The HPLC elution patterns are shown in Figs. 4 and 5. In the case of G^5′pppApUpG, a main peak, which appeared after pApUpG, was collected and analyzed with venom phosphodiesterase. The enzyme analysis suggested that the main peak was the desired product of G^5′pppApUpG (see Fig. 6A). This capped trimer was isolated in an overall yield of 9% from **15**. Since pApUpG was obtained in 20% yield from **15** by a similar unblocking procedure, it was estimated that the triphosphate bond formation was attained to a degree of *ca.* 45%. This means that the phos-



Scheme 6.

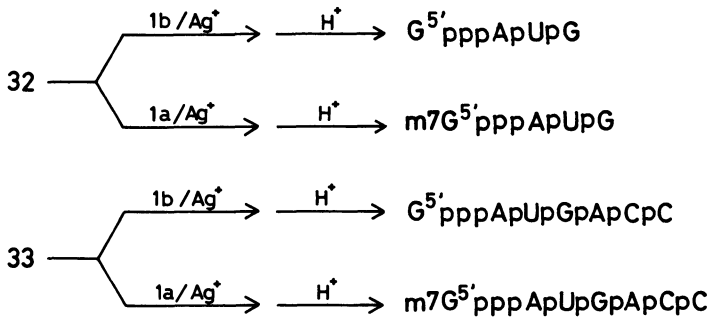
TABLE 1. CONDITIONS AND RESULTS OF FRAGMENT CONDENSATIONS OF OLIGORIBONUCLEOTIDE BUILDING BLOCKS

3′-Phosphonate component (equiv)	Removal of PhS group 4 M TEPS[a]	Time min	5′-Hydroxyl component	Removal of DMTr group Time min	Yield %	Condensation NT equiv	MDS equiv	Time min	Product	Yield %
A (1.2)	20	60	U	—	—	6	3	60	**12**	82
G (1.2)	20	40	A	—	—	6	3	45	**16**	91
AU(1.2)	20	40	G	45	86	6	3	75	**13**	69
C (1.1)	20	30	C	30	84	6(MeIm)[b]	3	30	**17**	54
GA(1.5)	20	75	CC	—	—	4	2	75	**18**	86
AU(1.6)	20	45	GACC	—	—	3	2	180	**19**	54

a) TEPS refers to triethylammonium phosphinate. b) In this reaction methylimidazole (MeIm) was employed in place of **NT**.

TABLE 2. CONDITIONS AND RESULTS OF PHOSPHORYLATION OF **14** AND **20**

Oligomer mg (μmol)	PSS mg (μmol)	MDS mg (μmol)	Pyridine mL	Time min	Yield %,	Yield mg (μmol)
14 127 (62)	28 (74)	25 (74)	2	40	73	104 (45)
15 318 (74)	39 (89)	31 (89)	0.2	70	91	305 (67)



Scheme 7.

phorylation itself proceeded successfully.

In the case of the hexamer, several peaks eluted after pApUpGpApCpC were similarly collected and analyzed. As a consequence, an eminent peak was found to be composed of pA, pC, pG, and pU in the expected ratio and thereby it was determined as G5'-pppApUpGpApCpC. The isolated yield of G5'-ppppApUpGpApCpC was 5% on the basis of the fully protected hexamer **21**. The other peaks of 38 min and 48 min were derived from pG and did not contain the other nucleotides.

Finally, we tried to synthesize the capped oligoribonucleotides, m7G5'pppApUpG and m7G5'-pppApUpGpApCpC, by using G5'pppApUpG and G5'pppApUpGpApCpC as the references for HPLC analysis. In a similar manner, the capping reactions were carried out. The HPLC patterns obtained after the acid treatment are shown in Figs. 7 and 8. We expected that the capped oligomers would appear at positions near or a little behind the starting oligomers owing to the positive charge of the m7G residue. In order to detect the m7G residue, the mixtures were analyzed simultaneously by a HPLC apparatus equipped with a fluorescene detector. The detector was set up by use of the ex and em values of 279 nm and 393 nm, respectively. This double check technique has proved to be very useful for detection of the capped oligomers. On the basis of our expectation mentioned above, some prominent peaks with both UV and fluorescence were collected (Figs. 7 and 8) and characterized by enzyme assay with venom phosphodiesterase (Fig. 9). Consequently, the peaks at 27 min (Fig. 7A) and 35 min (Fig. 8A) were finally identified as the capped oligomers, m7G5'-pppApUpG and m7G5'pppApUpGpApCpC, which were isolated in 8 and 5% overall yields from the fully protected oligomers **15** and **21**, respectively. In these cases, the capping reactions were estimated to proceed to a degree of 40—50%. The enzyme assays of the capped trimer and hexamer with venom phosphodiesterase exhibited the peaks corresponding to pm7G, pA, pU, pG, and pC in the ratios of approximately 1:1:1:1:0 and 1:2:1:1:2, respectively as shown in Fig. 9. Gel electrophoresis of the isolated capped oligomers also showed the expected mobilities as shown in Fig. 10. The structures of the capped oligomers were also more clearly confirmed by digestion with nuclease P_1, which gave m7G5'-pppA and the other nucleotide monomers in the correct ratios as shown in Fig. 11. The cap structure, m7G5'pppA, formed by these enzyme degradations was completely consistent with an authentic sample by comparison with their retention times.

In these reactions the HPLC patterns detected with fluorescence were not so complicated, showing 6—7 eminent peaks. Since the two peaks were assigned as pm7G and ppm7G, the other three or four peaks contained both pm7G and oligomers. Compared with the simple fluorescence HPLC patterns, the UV HPLC patterns were very complicated. Since the UV HPLC patterns obtained in the case of G5'pppApUpG and G5'pppApUpGpApCpC were also complicated, the main reason for the relatively low isolated yields of the capped oligoribonucleotides might be ascribed to the deprotection procedure used for the preparation of **32** and **33**, where the alkaline treatment was used because of its easy post-treatment. Nevertheless sufficient amounts of the capped oligomers for biological studies were successfully obtained. We are now studing improved procedures.

Experimental

^{1}H NMR spectra were recorded at 100 MHz on a JEOL UNM PS-100 spectrometer using tetramethylsilane as an internal standard. UV spectra were obtained on a Hitachi 124 spectrophotometer. Paper chromatography was performed by use of a descending technique with Whatman 3MM papers or Toyo Roshi No. 50 papers using the following solvent system: 2-propanol–concentrated aqueous ammonia–water (Solvent I, 6:1:3, v/v/v). Column chromatography was performed with silica gel C-200 purchased from Wako Co. Ltd., and a minipump for a goldfish basin was conveniently used to gain a medium pressure for rapid chromatographic separation. Thin-layer chromatography was performed on precoated TLC plates Silica Gel 60F-254 (Merck). The R_f values of the protected nucleoside derivatives were measured after development with CH_2Cl_2–MeOH (20:1, v/v) unless otherwise noted. HPLC was performed on a JASCO TRI ROTAR Type II apparatus equipped with a GP-A30 solvent programer, a UVIDEC

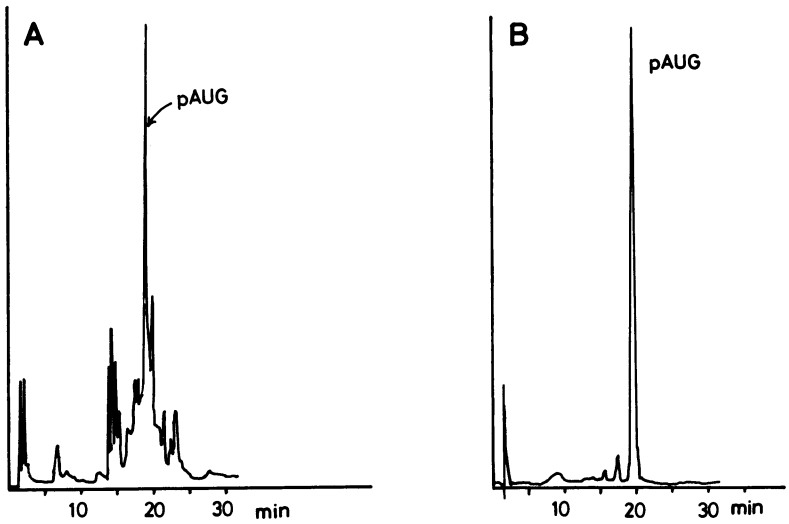


Fig. 1. A: HPLC chromatogram of the mixture after acid treatment of **32**. B: HPLC analysis of pAUG obtained by preparative HPLC shown in A. Conditions of HPLC: System B.

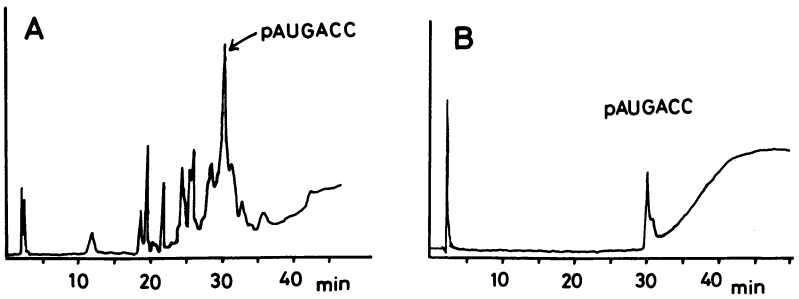


Fig. 2. A: HPLC chromatogram of the mixture after acid treatment of **33**. B: HPLC analysis of pAUGACC separated by preparative HPLC shown in A. Conditions of HPLC: System B.

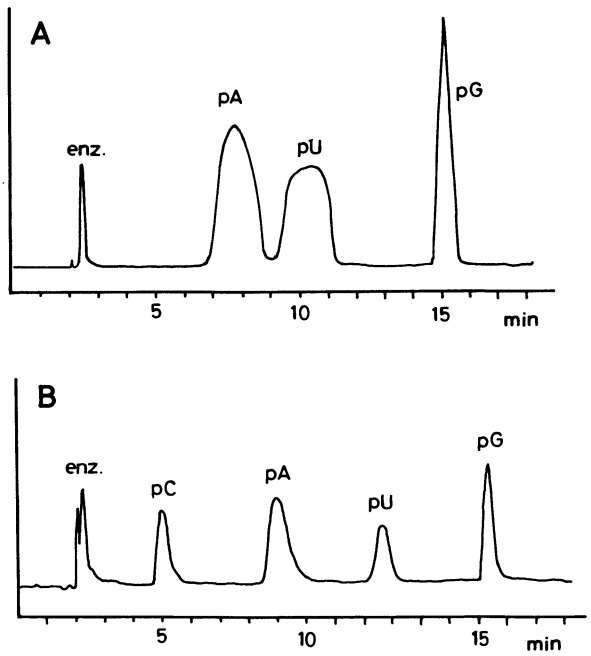


Fig. 3. HPLC analysis of the incubation mixtures of synthetic pAUG (A) and pAUGACC (B) with snake venom phosphodiesterase. Conditions of HPLC: System B.

100-II UV detecter, a FP-110 fluorescence spectrofluorometer, a RC-225 recorder, and a Ohkura Model R 7005 digital integrator. Analysis and purification of unprotected oligoribonucleotides were performed on a Whatman Partisil 10 SAX column (3.9×250 mm) using the following solvent systems: System A: a linear gradient (0—99%) starting with buffer A (0.005 M KH_2PO_4, pH 4.1) and applying buffer B (0.1 M KH_2PO_4, 1.0 M KCl, pH 4.5) at a flow rate of 2.0 mL/min for 32 min; System B: a linear gradient (0—99%) starting with buffer C (0.005 M KH_2PO_4, 20% CH_3CN, pH 4.1) and applying buffer D (0.5 M KH_2PO_4, 20% CH_3CN, pH 4.5) at a flow rate of 1.5 mL/min for 32 min. Pyridine was distilled two times from *p*-toluenesulfonyl chloride and from calcium hydride and then stored over molecular sieves (3A). DMF was distilled and stored over molecular sieves (3A). Ribonucleosides, 1,1,3,3-tetraisopropyl-1,3-dichlorodisiloxane, and DMTrCl were purchased from Yoshitomi Seiyaku Co. Ltd. Diphenylcarbamoyl chloride and the other reagents were purchased from Tokyo Kasei Co. Ltd. Venom phosphodiesterase was purchased from Boehringer Co. Ltd. and nuclease P_1 from Yamasa Shoyu Co. Ltd. Elemental analyses were performed by the Microanalytical Laboratory, Tokyo Institute of Technology, at Nagatsuta.

N^3-Anisoyl-2′-O-(tetrahydropyran-2-yl)-3′,5′-O-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)uridine (24). Compound **23**[10]

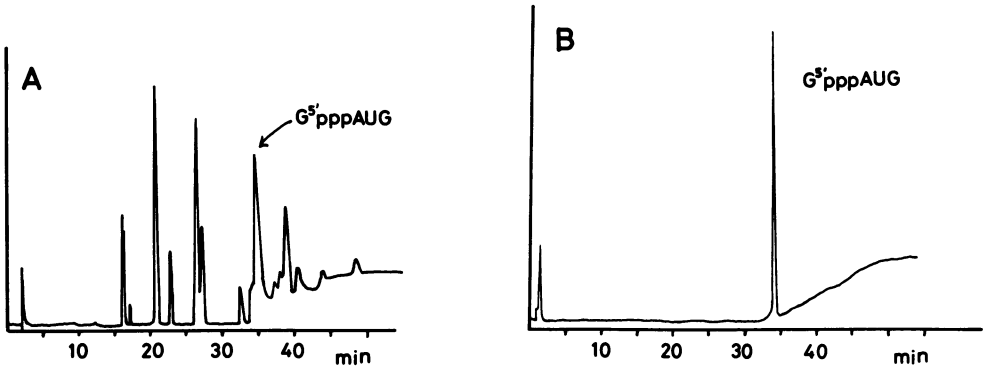


Fig. 4. HPLC chromatogram of the mixture obtained after acid treatment of the reaction mixture derived from condensation of **32** with **1b**. B: HPLC analysis of G⁵'pppAUG separated by preparative HPLC shown in A. Conditions of HPLC: System B.

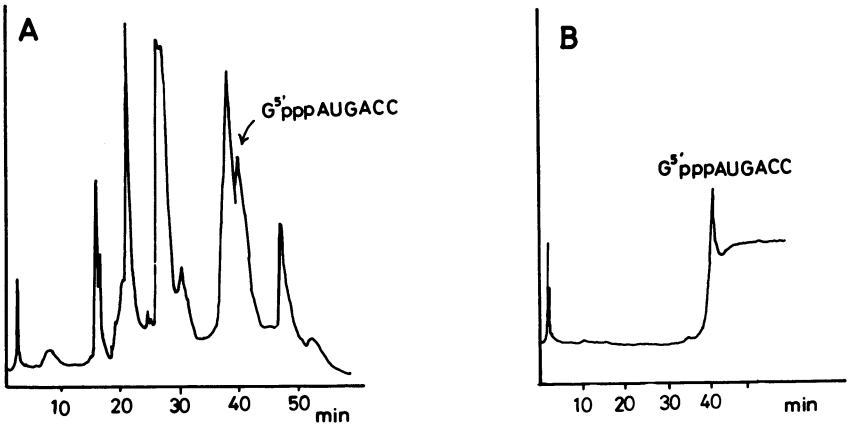


Fig. 5. A: HPLC chromatogram of the mixture obtained after acid treatment of the reaction mixture derived from condensation of **33** with **1b**. B: HPLC analysis of G⁵'pppAUGACC separated by preparative HPLC shown in A. Conditions of HPLC: System B.

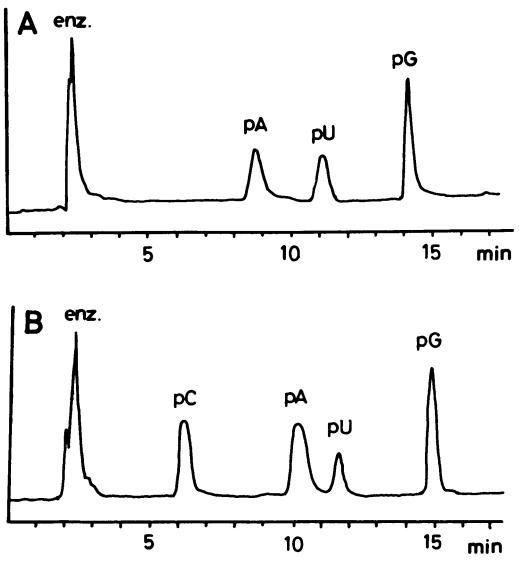


Fig. 6. HPLC analysis of the incubation mixture of synthetic G⁵'pppAUG (A) and G⁵'pppAUGACC (B) with snake venom phosphodiesterase. Conditions of HPLC: System A.

(10.9 g, 19.1 mmol) was rendered anhydrous by coevaporations with dry pyridine (3×20 mL) and finally dissolved in dry pyridine (56 mL). To the solution were added *N,N*-diisopropylethylamine (4.99 mL, 28.7 mmol) and anisoyl chloride (4.12 mL, 38.2 mmol). After being stirred for 10 h, the mixture was treated with ice-water. The agueous solution was extracted with CH_2Cl_2 (3×150 mL), dried over Na_2SO_4, and filtered. The filtrate was concentrated to dryness and coevaporated with toluene (3×20 mL). The residue was chromatographed on a silica-gel column to afford **24** (12.3 g, 91%): R_f 0.63 and 0.70 (benzene–ethyl acetate, 5:1, v/v); ^{1}H NMR ($CDCl_3$) δ=1.02—1.22 (24, m, $(CH_3)_2C$), 1.40—2.00 (10, m, SiCH and CH_2 of THP), 3.52 (2, m, CH_2–O of THP), 3.88 (3, s, CH_3–O), 3.95—4.42 (5, m, 2',3',4',5'-H), 5.15 (1, m, O–CH–O of THP), 5.73 (1, m, 5-H), 5.94 (1, m, 1'-H), 6.92 (2, m, ArH), 7.86 (1, m, ArH), 8.00 (1, m, 6-H). Anal. Calcd for $C_{34}H_{52}N_2O_{10}Si_2$: C, 57.93; H, 7.43; N, 3.94. Found: C, 58.49; H, 7.33; N, 3.36.

N^3-Anisoyl-2'-O-(tetrahydropyran-2-yl)uridine (25). Compound **24** (12.3 g, 17.4 mmol) obtained in the above experiment was dissolved in acetonitrile (350 mL), and tetraethylammonium bromide (21.96 g, 104 mmol), potassium fluoride (5.86 g, 104 mmol), and water (5.22 mL) were

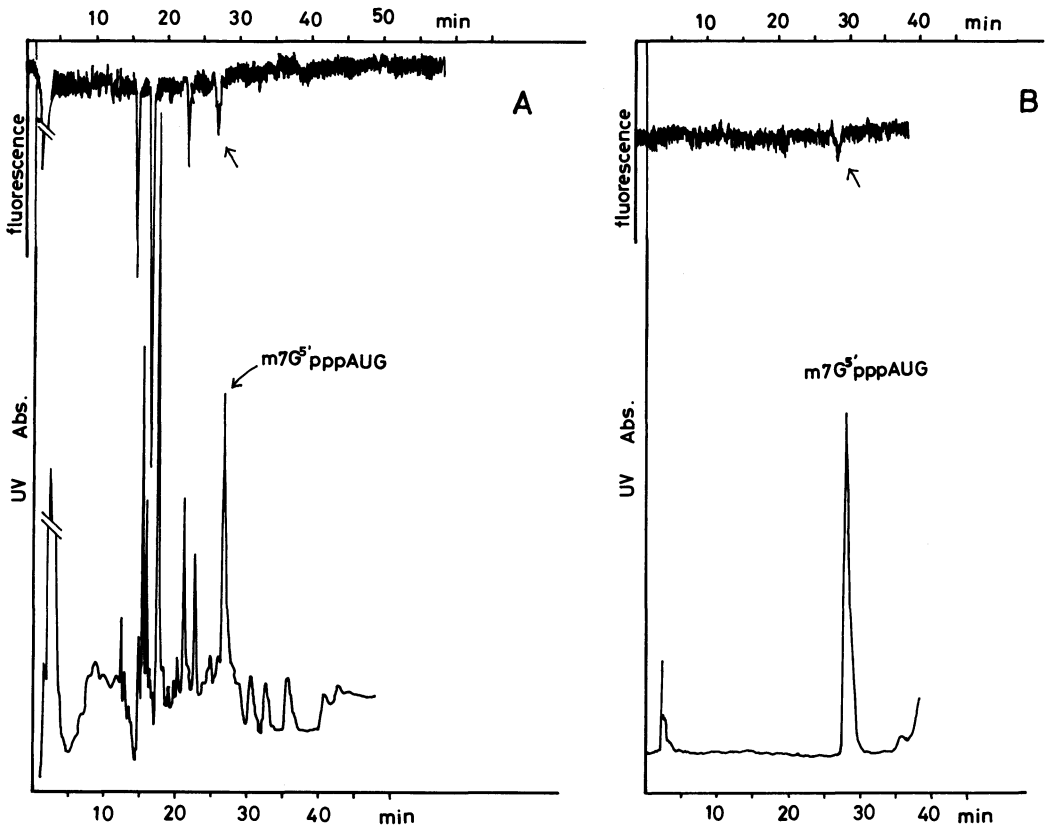


Fig. 7. A: HPLC chromatogram of the mixture obtained after acid treatment of the reaction mixture derived condensation of **32** with **1a**. B: HPLC analysis of m^{7}G^5'pppAUG separated by preparative HPLC shown in A. Conditions of HPLC: System B.

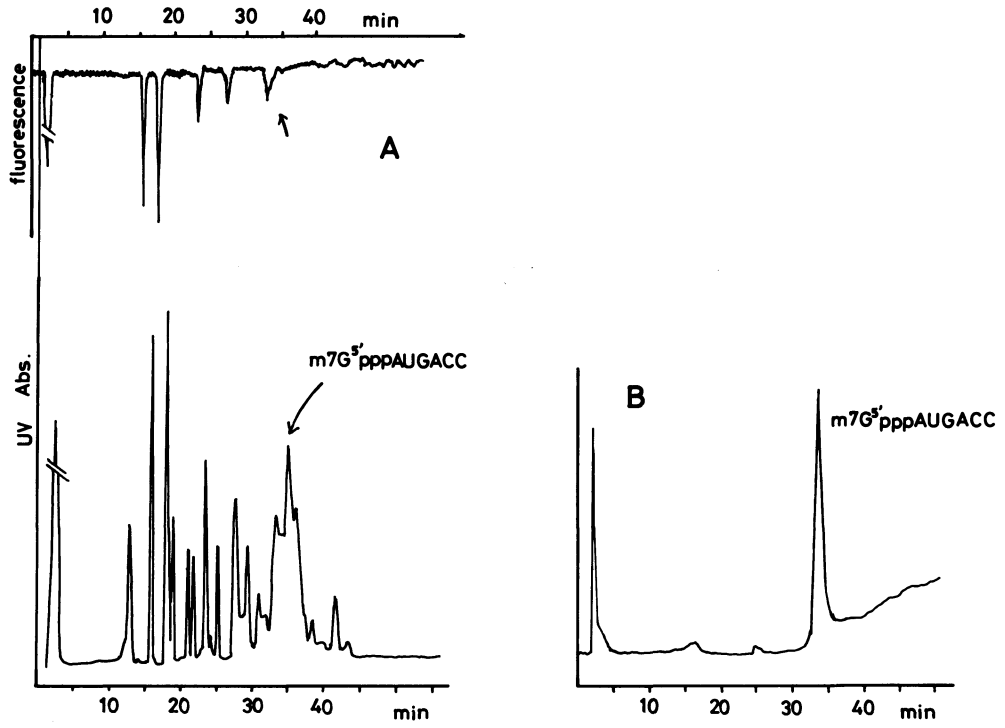


Fig. 8. A: HPLC chromatogram of the mixture obtained after acid treatment of the reaction mixture obtained by condensation of **33** with **1a**. B: HPLC analysis of m^{7}G^5'pppAUGACC preparative HPLC shown in A. Conditions of HPLC: System B.

added. The resulting mixture was stirred vigorously at 60 °C for 5.5 h. Then the precipitate was filtered and the filtrate was concentrated under reduced pressure. The residue was partitioned between CH_2Cl_2 and water. The aqueous layer was extracted further two times with CH_2Cl_2. The combined CH_2Cl_2 extracts were dried over Na_2SO_4, filtered, and evaporated. The residue was chromatographed to give **25** (7.3 g, 90%): R_f 0.11 and 0.16: ^{1}H NMR ($CDCl_3$): δ=1.10—2.05 (6, m, CH_2 of THP), 2.90 and 3.09 (2, br, OH), 3.54 (2, m, CH_2-O of THP), 3.64—4.78 (5, m, 2′,3′,4′,5′-H), 3.87 (3, s, CH_3-O), 5.77 (1, d, J=8 Hz, 5-H), 6.91 (2, d, J=8 Hz, Ar), 7.67 and 7.85 (1, d, J=8 Hz, 6-H), 7.85 (2, d, J=8 Hz, ArH). Anal. Calcd for $C_{22}H_{25}N_2O_9$: C, 57.14; H, 5.67; N, 6.06. Found: C, 56.91; H, 5.78; N, 5.71.

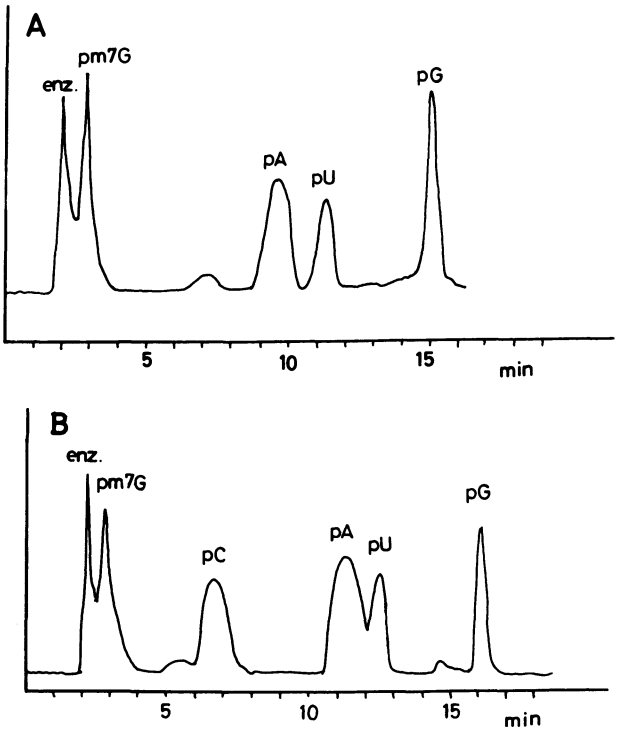


Fig. 9. HPLC analysis of the incubation mixture of synthetic m^{7}G^5′pppAUG (A) and m^{7}G^5′pppAUGACC (B) with snake venom phosphodiesterase.

N^3-Anisoyl-2′-O-(tetrahydropyran-2-yl)-5′-O-dimethoxytrityluridine (26). Compound **25** (7.3 g, 15.7 mmol) was dried by coevaporations with dry pyridine (3×20 mL) and finally dissolved in dry pyridine (47 mL). Dimethoxytrityl chloride (6.42 g, 19 mmol) was added and the mixture was stirred for 2.5 h. Then, the usual workup followed by chromatography gave **26** (74%): R_f 0.20, 0.37 ^{1}H NMR ($CDCl_3$): δ=1.38—2.00 (6, m, CH_2 of THP), 2.72 (1, br, OH), 3.52 (2, m, CH_2-O of THP), 3.78 (3, s, CH_3-O), 3.68—4.85 (5, m, 2′,3′,4′,5′-H), 5.36 (1, m, O-CH-O of THP), 5.86 and 6.11 (1, m, 1′-H), 6.68—6.96 (6, m, ArH), 7.10—7.44 (9, m, ArH), 7.82 (2, d, J=8 Hz, ArH), 8.06 (1, d, J=8 Hz, 6-H). Anal. Calcd for $C_{43}H_{44}N_2O_{11}$: C, 67.53; H, 5.80; N, 3.66. Found: C, 67.69; H, 6.27; N, 3.37.

S,S-Diphenyl N^3-Anisoyl-2′-O-(tetrahydropyran-2-yl)-5′-O-dimethoxytrityluridine 3′-Phosphorodithioate (6). Cyclohexylammonium *S,S*-diphenyl phosphorodithioate (1.15 g, 3.01 mmol) was coevaporated with dry pyridine (3×10 mL) and finally dissolved in dry pyridine (20 mL), and then **MDS** (0.97 g, 3.01) was added. After being kept for 30 min, the solution was added to **26** (1.92 g, 2.51 mmol) dried in

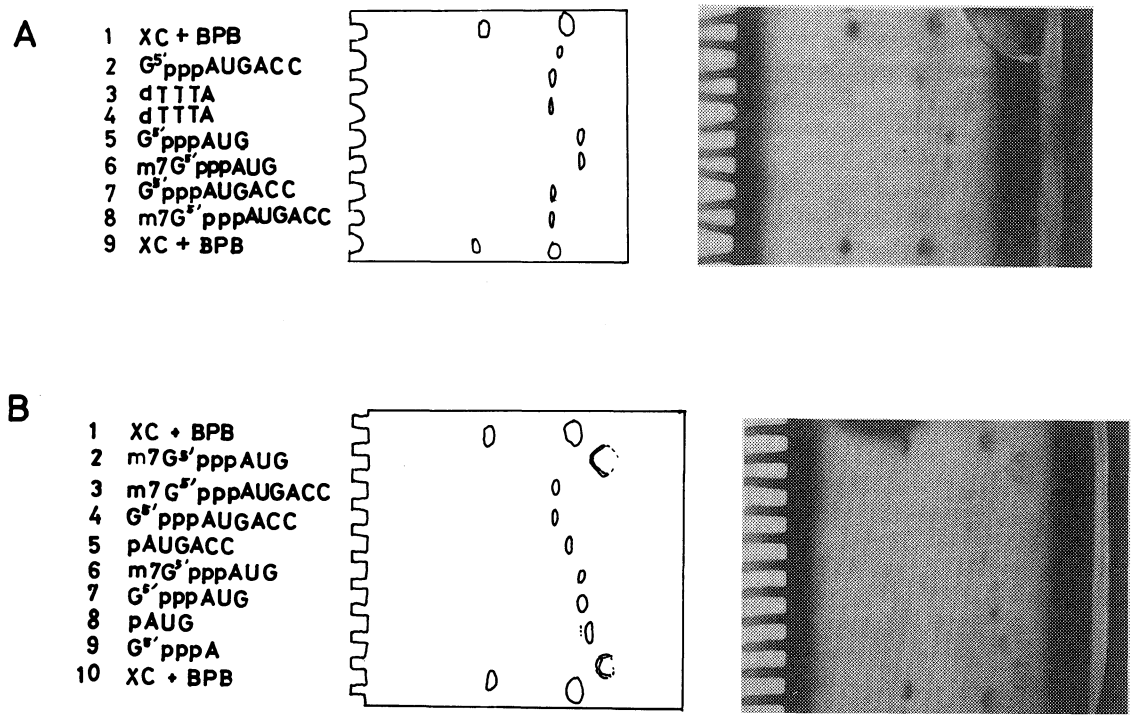


Fig. 10. 20% Polyacrylamide gel electrophoresis of the synthetic capped oligoribonucleotides and related compounds. The electrophoresis was performed at 300 V (10 mA) for 3—4 h. Markers, XC and BPB, refer to Xylene Cyanol and Bromophenyl Blue, respectively.

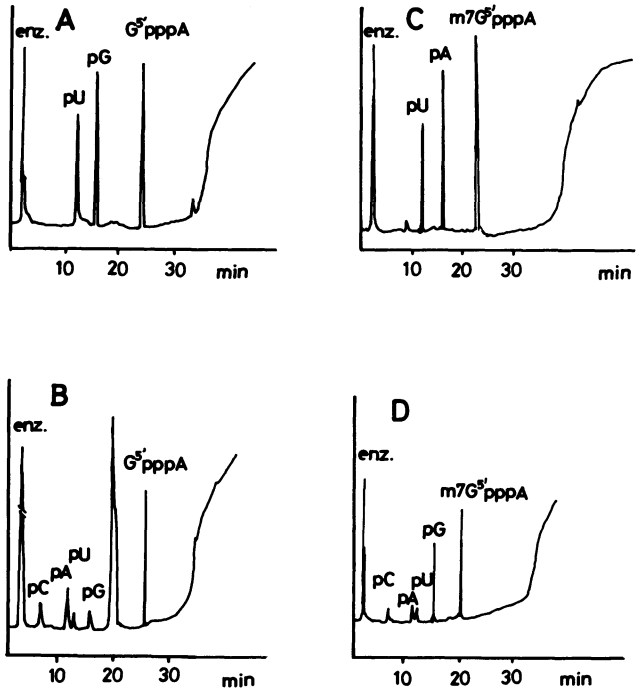


Fig. 11. HPLC analysis of the incubation mixtures of synthetic G5'pppAUG (A), G5'pppAUGACC (B), m7G5'pppAUG (C), and m7G5'pppAUGACC (D) with nuclease P_1. Conditions of HPLC: System A.

advance by coevaporations with dry pyridine. The mixture was stirred for 75 min, and then ice-water was added and extracted with CH_2Cl_2 (3×50 mL). The usual workup followed by chromatography gave **7** (1.95 g, 76%). The ^1H NMR spectrum of this compound was consistent with that of an authentic sample synthesized by the previous route.[11]

N^4-Anisoyl-2'-O-(tetrahydropyran-2-yl)-3'-O-benzoyl-5'-O-dimethoxytritylcytidine (31). Compound **30**[11] (2.29 g, 3 mmol) was coevaporated with dry pyridine (3×10 mL) and finally dissolved in dry pyridine (15 mL). Benzoyl chloride (0.42 mL, 3.6 mmol) was added and the mixture was stirred for 3.5 h. Then the usual workup involving extraction, dryness, filtration, evaporation, and chromatography gave **31** (2.19 g, 86%): R_f 0.34 and 0.37; ^1H NMR (d^6-DMSO): δ=1.20—1.88 (6, m, CH_2 of THP), 3.60 (2, br, CH_2-O of THP), 3.76 (6, s, CH_3-O of DMTr), 3.83 (3, s, CH_3-O of An), 4.53 (2, br, 5'-H), 4.72—4.92 (2, br, 3',4'-H), 5.21, (1, br, 2'-H), 5.67 (1, br, O-CH-O of THP), 6.18 (1, br, 1'-H), 6.45 (1, d, J=4 Hz, 5-H), 6.74—7.03 (6, m, ArH), 7.36—7.60 (13, m, 6-H and ArH), 7.90 (2, d, J=5 Hz, ArH), 8.05 (2, d, J=4 Hz, ArH). Anal. Calcd for $C_{50}H_{49}O_{11}N_3 \cdot 1/2H_2O$: C, 68.48; H, 5.75; N, 4.79. Found: C, 68.63; H, 5.65; N, 4.67.

N^4-Anisoyl-2'-O-(tetrahydropyran-2-yl)-3'-O-benzoylcytidine (10). Compound **31** (2.19 g, 2.58 mmol) was dissolved in CH_2Cl_2 (15 mL) and 2% trifluoroacetic acid in CH_2Cl_2 (15 ml) was added at −20 °C. After the mixture was stirred at −20 °C for 55 min, an additional amount of trifluoroacetic acid (150 μL) was added. After 2 min, pyridine was added, and then extraction was performed with CH_2Cl_2-water. The organic extract was dried over Na_2SO_4, filtered, and evaporated to dryness. The residue was chromatographed on a silica gel column to give **10** (1.02 g, 74%): R_f 0.29; ^1H NMR (d^6-DMSO): δ=1.20—1.88 (6, br, CH_2 of THP), 3.51 (2, m, CH_2-O of THP), 3.85 (3, s, CH_3-O of An), 4.02 (2, br, 5'-H), 4.50 (1, br, 4'-H), 4.72—5.12 (2, br, 2',3'-H), 5.65 (1, m, O-CH-O of THP), 5.80 (1, m, 1'-H), 6.18 (1, m, 5-H), 6.94 (2, d, J=5 Hz, ArH), 7.28—7.64 (4, m, 6-H and ArH), 7.91 (2, d, J=5 Hz, ArH), 8.07 (2, d, J=5 Hz, ArH). Anal. Calcd for $C_{29}H_{31}N_3O_9 \cdot C_6H_{14}$: C, 64.50; H, 6.96; N, 6.45. Found: C, 64.69; H, 7.23; N, 6.13.

General Procedure for the Condensation of Oligoribonucleotide Building Blocks. A fully-protected oligomer used as a diester component was treated with a 4 M pyridine solution of pyridinium phosphinate (20—30 equiv to the oligomer) and triethylamine (20—30 equiv to the oligomer), until the oligomer had been converted to a base-line material on TLC. Then, the mixture was quenched with pyridine-water (1:1, v/v) and extracted three times mith hexane-ether (1:1, v/v) to remove thiophenol. The aqueous layer was extracted three times with CH_2Cl_2. The combined CH_2Cl_2 extracts were washed with 0.2 M triethylammonium hydrogencarbonate (TEAB, pH 7.5) and water and then dried over Na_2SO_4. After the solution was filtered and evaporated, the residue was used as the diester component for the coupling reaction.

Removal of the DMTr group from oligomers was performed with 1% trifluoroacetic acid in CH_2Cl_2 at −10 °C for the times listed in Table 1. The workup was done in the same manner as described in the synthesis of **10**.

Condensation was carried out by mixing the two components, a diester and a hydroxyl components, followed by repeated coevaporations with dry pyridine, dissolving in dry pyridine (20 mL/1 mmol of the latter), and addition of **MDS** and 3-nitro-1,2,4-triazole or *N*-methylimidazole. After the condensation was complete, the mixture was quenched with pyridine-water (1:1, v/v) and extracted several times with CH_2Cl_2. The combined CH_2Cl_2 extracts were washed with 0.1 M TEAB (pH 7.5) and water. Then the usual workup followed by chromatography gave the coupling product as listed in Table 1.

5'-Phosphorylation of 14 and 20. Cyclohexylammonium *S,S*-diphenyl phosphorodithioate was coevaporated three times with dry pyridine and finally dissolved in dry pyridine (3 mL/0.1 mmol of **PSS**). **MDS** was added and the mixture was stirred for 30 min. Then the solution was added to **14** or **20** predried by repeated coevaporations with dry pyridine. The mixture was stirred for the time listed in Table 2. After the reactions was complete, the usual workup gave **15** or **21**.

Full Deprotection of 15. The trimer **15** (12.3 mg, 5 mol) was treated with 0.4 M NaOH-pyridine (1:3, v/v, 2 mL) at room temperature for 40 min. Then, the solution was passed through Dowex 50 W×2 (pyridinium form, 5 mL) and the resin was washed with pyridine-water (1:1, v/v, 50 mL). The eluent was evaporated under reduced pressure and the residue was treated with concentrated aqueous ammonia-pyridine (5:1, v/v, 18 mL) at room temperature for 12 h. Then, the solvent and ammonia were removed by evaporation and the residue was dissolved in pyridine-water (2:1, v/v, 0.5 mL). To the solution was added silver acetate (41.7 mg, 0.15 mmol). The mixture was stirred in the dark for 12 h. Then, the solution was diluted with pyridine-water (1:1, v/v, 20 mL) and hydrogen sulfide gas was bubbled into the solution at 0 °C for several minutes. The precipitate of silver sulfide was removed by centrifugation and the supernatant was concentrated to a gum. The gum was coevapo-

rated three times with methanol–toluene to remove the last traces of pyridine. The residue was dissolved in 0.02 M HCl–dioxane (1:1, v/v, 10 mL). The solution was adjusted exactly to pH 2.0 by addition of 0.1 M HCl. The resulting solution was stirred vigorously at room temperature for 48 h.

Then, concentrated aqueous ammonia (100 μL) was added and the mixture was concentrated to a gum. The gummy residue was partitioned between water (10 mL) and ether (10 mL). The ether was removed and the aqueous layer was further washed with ether (2×10 mL). The aqueous solution was concentrated to a small volume. HPLC analysis of this mixture exhibited a main peak at 19 min as seen in Fig. 1A. This peak was collected and the solvent was removed. The residue was diluted with water so as to decrease the salt concentration to less than 0.1 M, and applied to a column on DEAE-Sephadex A-25 (1×5 cm). Elution with water was performed and then elution with 1 M TEAB buffer (pH 7.5) gave fractions containing the product. Most of TEAB was removed under reduced pressure by coevaporations with water and finally the product was desalted by paper chromatography on Toyo Roshi No 51A developed with 95% ethanol. A band at the origin was eluted with water and the solution was filtered and concentrated to give pApUpG (38.0 OD, 20%): HPLC 20 min (A): UV (H_2O) λ_{max} 255 nm, λ_{min} 277 nm.

Synthesis of G⁵′pppApUpG. The fully protected trimer **15** (5 μmol) was partially deprotected as described in full deprotection of **15**. A mixture of compound **1b** (26.2 mg, 25 μmol) and imidazole (35 mg, 0.5 mmol) was rendered anhydrous by repeated coevaporations with dry pyridine and finally dissolved in dry pyridine (0.5 mL). Silver nitrate (132 mg, 0.75 mmol) was added and the solution was stirred in the dark for 3 h. Then, it was coevaporated with dry DMF (3×5 mL), dissolved in dry DMF (0.3 mL), and finally added to crude **32**, obtained from **15** after treatment with silver acetate, which had been coevaporated with dry pyridine (3×5 mL) and then DMF (3×5 mL). The resulting mixture was stirred in the dark for 48 h. Then pyridine–water (2:1, v/v, 10 mL) was added. Hydrogen sulfide gas was bubbled with stirring into the solution at 0 °C until a clear supernatant had been obtained. The supernatant was concentrated to dryness and coevaporated with methanol–toluene (1:1, v/v 3×5 mL). The residue was dissolved in a solution of 0.02 M HCL–dioxane (1:1, v/v, 10 mL) adjusted exactly to pH 2.0 by 0.1 M HCl. After being stirred for 45 h, the solution was quenched with concentrated aqueous ammonia (0.1 mL) and concentrated to a small volume. The concentrate was chromatographed on Whatman 3 MM paper with Solvent I to give crude G⁵′pppApUpG with R_f 0.09. The crude product was further purified by HPLC as shown in Fig. 4. A peak at 35 min was collected and pooled. The combined fractions were desalted by DEAE-Sephadex A-25 as described in the synthesis of pApUpG to give G⁵′-pppApUpG (25 OD, 10%): HPLC 34 min (System B); UV (H_2O): λ_{max} 255 nm, λ_{min} 232 nm.

Full Deprotection of 21. The same procedure as described in the case of **15** was employed.

The residue, obtained after the acid treatment for removal of the acid-labile protecting groups, was dissoved in water (10 ml) and washed with ether (3×10 mL). The aqueous solution was concentrated, and the residue was applied to Watman 3 MM papers and developed with Solvent I. A band at the origin was cut and eluted with water. The eluent was filtered and concentrated to a small volume. The solution was separated by HPLC. A peak at 30 min (System B) was collected and pooled. The combined fractions were evaporated under reduced pressure and diluted with water. The solution was applied to a column of DEAE-Sephadex A-25 (1×5 cm) and the column was washed with water and then eluted with 1 M TEAB. The eluent was coevaporated repeatedly with water and finally desalted by paper chromatography using Toyo Roshi developed with 95% ethanol. A UV absorbing material at the origin was eluted with water. The eluent was evaporated to give pApUpGpApCpC (22.5 OD, 10%): R_f 0.1 (Solvent I); HPLC 30 min (System B); UV (H_2O) λ_{max} 257 nm, λ_{min} 238 nm.

Synthesis of G⁵′pppApUpGpApCpC. This compound was synthesized from **33** (5 μmol) and **1b** (25 μmol) in the same manner as described in the above experiment: Purification after the acid treatment for removal of the THP, MMTr, and TMTr groups was performed as follows: The residue, obtained by evaporation after quenching of the acid solution used for removed of the acid-labile protecting groups, was dissolved in water (10 mL) and washed with ether (10 mL). The aqueous solution was filtered and separated by preparative HPLC as shown in Fig. 5. A peak at 40 min pooled and desalted by DEAE-Sephadex A-25 as described before to give G⁵′pppApUpGpApCpC (16.7 OD, 5%): R_f 0.14 (Solvent I); HPLC 40 min (System B); UV (H_2O) λ_{max} 258 nm, λ_{min} 230 nm.

Preparation of 1a from 1b. Compound **1b** (30 mg, 25 μmol) was coevaporated with dry DMF (2×5 mL) and finally dissolved in dry DMF (1.25 mL). To the solution was added methyl iodide (19 μL, 0.3 mmol). The mixture was stirred for 16 h and then evaporated under reduced pressure. The residue was coevaporated with dry pyridine (2×5 mL) and then used without further purification in the capping reactions.

Synthesis of m⁷G⁵′pppApUpG. This compound was synthesized from **1a** (5 μmol) and **32** (25 μmol) in the same manner as described in the synthesis of G⁵′pppApUpG. Purification was performed in the same manner as described in the synthesis of pApUpG: R_f 0.14 (Solvent I); HPLC 27 min (System B); UV (H_2O) λ_{max} 230 nm, λ_{min} 230 nm.

Synthesis of m⁷G⁵′pppApUpGpApCpC. This compound was synthesized from **1a** and **33** in the same manner as described in the synthesis of G⁵′pppApUpGpApCpC. Purification was performed in the same manner as described in the synthesis of pApUpG (13 OD, 5%): HPLC 33 min (System B); UV (H_2O) λ_{max} 257 nm, λ_{min} 233 nm.

Enzyme Assay. Treatment with venom phosphodiesterase. A substrate (10 OD) was incubated with venom phosphodiesterase in a mixture of 0.05 M Tris-HCl buffer (pH 8.0, 200 μL) and 1 M $MgCl_2$ (10 μL) at 37 °C for 3 h. When a less amount of substrate than 10 OD was used, the amounts of the enzyme and reagents were decreased to the same degree.

Treatment with Nuclease P_1. A substrate (0.05 OD) was incubated with nuclease P_1 (1 μL) in 0.05 M acetate buffer (pH 5.4, 9 μL) at 37 °C for 30—60 min. The mixture was analyzed by HPLC as described in the text.

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