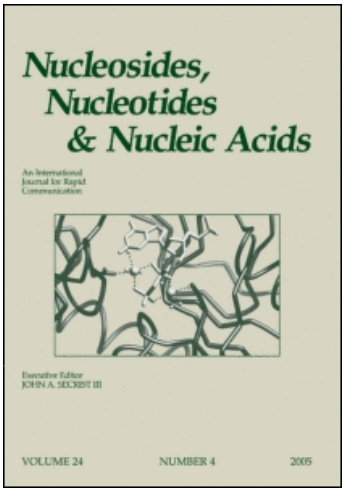

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Chemical Synthesis of RNA Fragments Related to the C4N Hypothesis

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CHEMICAL SYNTHESIS OF RNA FRAGMENTS RELATED TO THE C4N HYPOTHESIS

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This paper is dedicated to Professor Morio Ikehara for the occasion of his retirement from Osaka University on March, 1986.

Summary. Several oligoribonucleotide fragments which are related to the C4N hypothesis, were synthesized by both the liquid and solid phase methods via the phosphotriester approach, in which the combination of the 2'-Thp group with the 5'-DMTr group was used for chain elongation.

INTRODUCTION

It is well known that tRNAs can recognize precisely the corresponding amino acids in aminoacylation of the 3'-terminal CCA in prebiotic conditions. However, there have been reported only a few papers[1-3] dealing with the mutual interaction between tRNAs and amino acids. Among them Shimizu has proposed the so-called C4N hypothesis[4] for 1:1 complex formation of amino acids with tRNAs. According to his papers, a cavity is formed at the initial stage by C4N (Complex of Four Nucleotides), which composes of the anticodon of three letters and the discriminator of one letter for tRNAs. This cavity serves as the acceptor site which can recognize the shape and functional side chain of an amino acid.

On the other hand, we have shown in our previous papers[5-9] that the dimethoxytrityl (DMTr) group introduced to the 5'-oxygen could be rather selectively removed by

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treatment with trifluoroacetic acid (TFA) in $CHCl_3$ in the presence of the 2'-O-tetrahydropyranyl (Thp) group in the liquid phase synthesis of oligoribonucleotides. As the next step, we tried to apply the two protecting groups to the synthesis of RNA fragments on solid supports based on the follwing consideration. If the Thp group is simultaneously eliminated on the removal of the 5'-terminal DMTr group, the 2',3'-cyclization of the phosphate group is expected to occur owing to the neighboring group participation of the free 2'-hydroxyl group which would attack on the phosphorus resulting in elimination of the phenylthio group. Since internucleotidic cyclic triester intermediates tend to be cleaved between the P-$O^{5'}$ bond upon alkali treatment,[10] partial loss of the Thp group during acid treatment will lead mainly to the cleavage of internucleotidic bonds. Consequently, the contamination of the 2'-5' linkage in synthesized RNA oligomers can be minimized.

In this paper, we wish to report the chemical synthesis of RNA fragments, which will be employed as substrates for physicochemical studies to elucidate the C4N hypothesis. These oligoribonucleotides consist of the sequence $G(A)_n$ (n=2,3,4 and 5), which may form a cavity for L-phenylalanine by the anticodon GAA and the discriminator A.

RESULTS AND DISCUSSION

Synthesis of RNA fragments in the liquid phase method

First, we have synthesized three oligoribonucleotides of $G(A)_n$ (n=2,3 and 4) via the solution method. We chose here the tetrahydropyranyl and benzoyl groups for the protection of the 2'-hydroxyl and amino groups. The key intermediate of 2'-O-(tetrahydropyran-2-yl)-N^6-benzoyl-adenosine (2) has been prepared by benzoylation of 2'-O-(tetrahydropyran-2-yl)- adenosine followed by selective debenzoylation of the 3',5'-di-O-benzoate.[11] In this study, to facilitate chromatographic separation of synthetic intermediates, the benzoyl group was introduced into the

amino group prior to removal of the tetraisopropyl-disiloxane-1,3-diyl group which was used for the simultaneous protection of the 3' and 5'-hydroxyl groups. Takaku et. al[11] has employed the Thp and benzoyl groups for protection of the 2'- hydroxyl and amino groups of an adenosine building unit. 2',3'-O-Methoxymethylidene-N^6-benzoyladenosine[12] was used as 3'-terminal unit, and the protected oligomers were synthesized from the building blocks as shown in FIG 1. Mesitylenedisulfonyl chloride (MDS)[13] and 3-nitro-1,2,4- triazole (NT) were chosen as a combined coupling reagent. Coupling was performed by a procedure similar to that reported previously.[9] The yields and coupling conditions of the fragment condensations are listed in TABLE 1. As shown in FIG 1 the 5'-OH components were prepared by removal of the 5'-DMTr group with 0.5% TFA in $CHCl_3$ at 0°C.[6] The 2',3'-O-methoxymethylidene (mM) and 2'-O-Thp groups were found to be sufficiently stable under these conditions. The yields and conditions are summarized in TABLE 2. Deblocking of the fully protected RNA fragments was performed by the following procedure: 1) 0.5 M 1,1,3,3-tetramethylguanidium 4-nitrobenzaldoximate (TMG-NBAO) in pyridine—water (9:1, v/v) at r.t. for 5 h. 2) conc. ammonia—pyridine (5:1, v/v) at r.t. for 12 h. and at 60°C for 6 h. 3) anion exchange with Dowex 50W X2 (pyridium form) 4) washing with ether—ethyl acetate (3:2, v/v) 5) dilute hydrochloric acid (pH 2.0) at r.t. for 30 h. The deblocked products were separated by paper chromatography with Whatman 3MM papers developed with 2-propanol—conc. ammonia—water (6:1:3, v/v/v) and further purification was carried out by reversed phase HPLC (μBondapak C_{18} column) (FIG 2). The pure trimer (250 OD), tetramer (132 OD) and pentamer (191 OD) were obtained in 67%, 56% and 64% yields, respectively. These yields were estimated by assuming the hypochromicity of 15% and 20% for the trimer and the latter two, respectively. The isolated oligomers were completely hydrolyzed with nuclease P_1 to G and pA in the correct ratios.

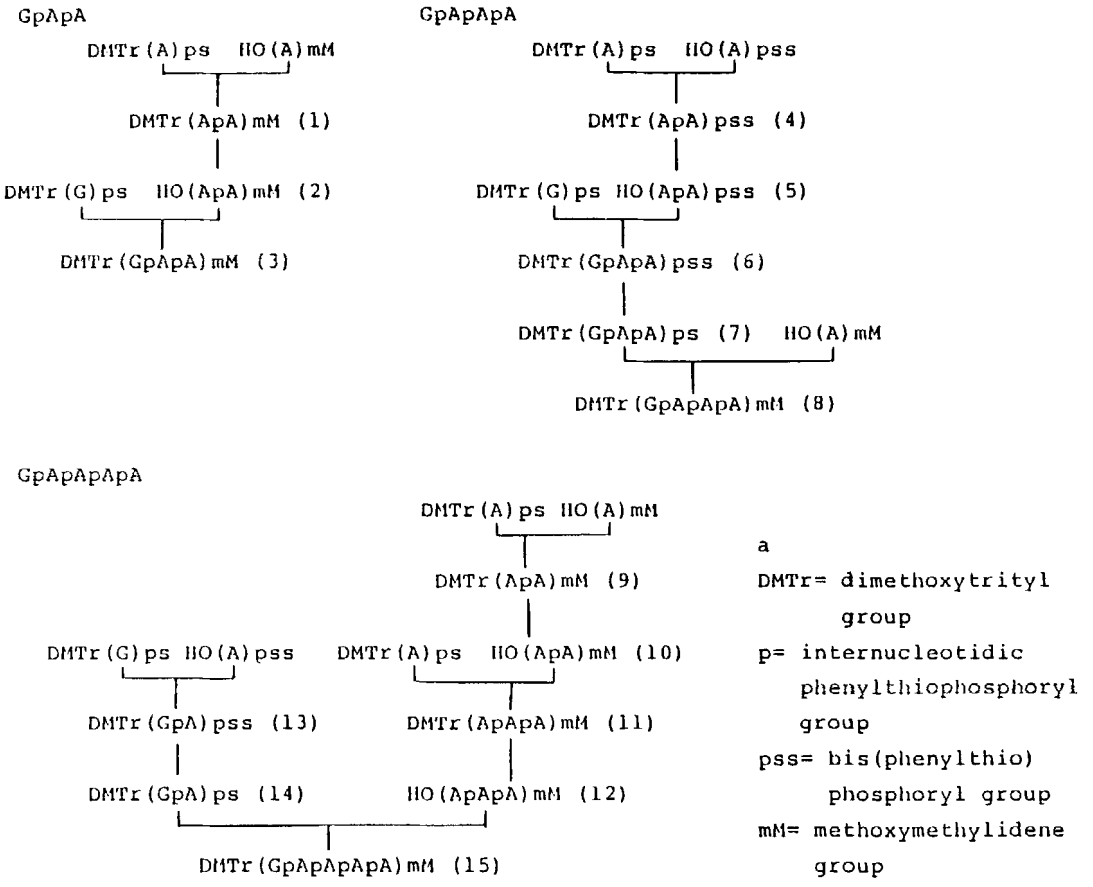


FIG 1. Synthetic scheme for trimer, tetramer and pentamer[a]

Synthesis of RNA fragments in the solid phase method

Only a few papers have been published about the synthesis of RNA fragments by the use of polymer supports.[14-18] This is due to the additional requisite of protection of the 2'-hydroxyl function present in ribonucleotide molecules. An appropriate protecting group at the 2'-position must be chosen in combination with the 5'-hydroxyl protecting group that is selectively removed. There have not been reported any papers dealing with the synthesis of RNA fragments using the combination of the 2'-Thp and 5'-DMTr groups in the solid phase approach.[19]

TABLE 1. Conditions and results for the synthesis of the RNA fragments[a,b]

	3'-phospho-diester component	(μmol)	5'-OH component	(μmol)	MDS (μmol)	NT (μmol)	Time (min.)	Product	Yield (%)
(1)	DMTr(A)	185	(A)mM	154	308	462	45	DMTr(AA)mM	95
(3)	DMTr(G)	96	(AA)mM	80	160	240	60	DMTr(GAA)mM	70
(4)	DMTr(A)	217	(A)pss	181	362	543	45	DMTr(AA)pss	98
(6)	DMTr(G)	199	(AA)pss	166	332	498	60	DMTr(GAA)pss	75
(8)	DMTr(GAA)	120	(A)mM	100	200	300	80	DMTr(GAAA)mM	66
(9)	DMTr(A)	450	(A)mM	300	600	900	60	DMTr(AA)mM	81
(11)	DMTr(A)	315	(AA)mM	210	420	630	60	DMTr(AAA)mM	88
(13)	DMTr(G)	240	(A)pss	200	400	600	60	DMTr(GA)pss	81
(15)	DMTr(GA)	136	(AAA)mM	110	220	330	70	DMTr(GAAAA)mM	89

a DMTr= dimethoxytrityl group, mM= methoxymethylidene group, pss= bis(phenylthio)phosphoryl group,

b Coupling was performed in pyridine.

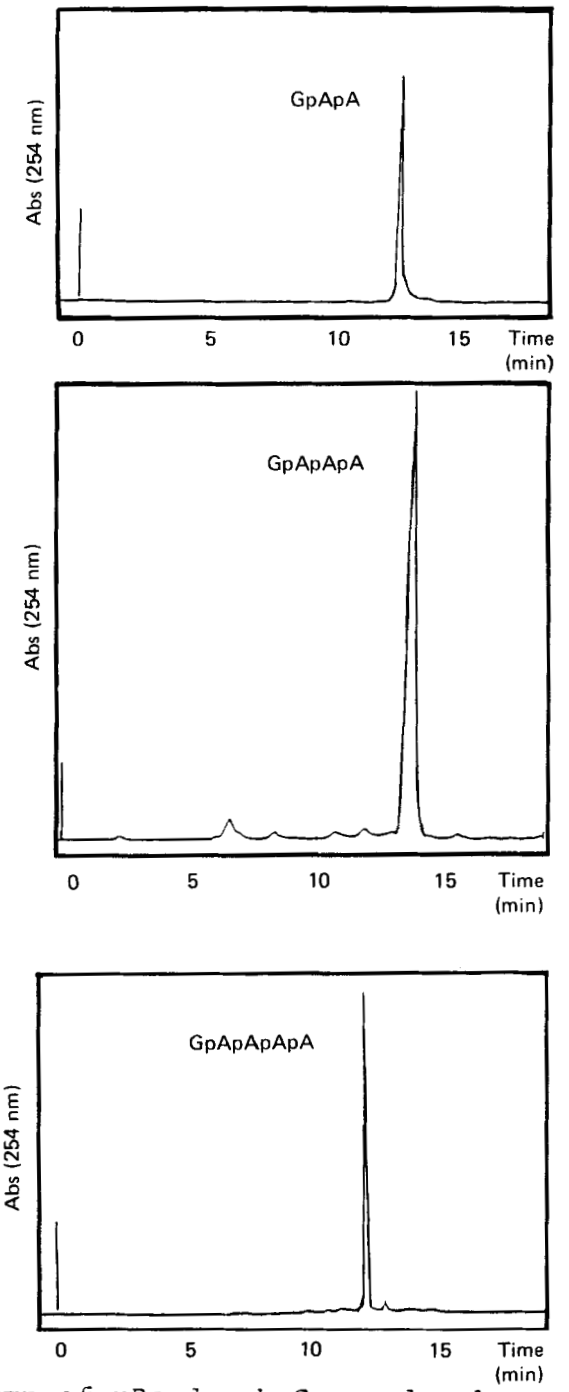
TABLE 2. Removal of the 5'-dimethoxytrityl group

	Substrate	(μmol)	Temperature (°C)	Time (min)	product	Yield (%)
(2)	DMTr(AA)mM	90	0	15	HO(AA)mM	90
(5)	DMTr(AA)mM	181	0	15	HO(AA)mM	92
(10)	DMTr(AA)mM	243	0	17	HO(AA)mM	87
(12)	DMTr(AAA)mM	185	0 (5min) r.t (10min)		HO(AAA)mM	85

Detritylation was performed with 0.5% trifluoroacetic acid in $CHCl_3$ (50ml/mmol)

Therefore, we considered that, if the Thp group could be used with the DMTr group even on polymer supports, this combination would allow a very practical access to the solid phase RNA synthesis since 2,3-dihydropyran was commercially available as the introducing agent. We decided to use the combination of the 2'-Thp and 5'-DMTr groups in our polymer supported synthesis.

We chose controlled pore glass (CPG)[20-22] as a solid support to realize rapid synthesis of RNA fragments with an automatic synthesizer.[23] The 3'-terminal adenosine unit was introduced to CPG (CPG Biogel, Mean Pore Dia (Å) 569, Particle size 120/200) having an aminopropyl functionality of 97 μmol/g via the succinate linker in the usual manner.[24] The loading amount of the adenosine unit introduced to the resin was 34 μmol/g. The conditions for the manipulation of automatic synthesizer (Solid Phase Synthesizer Model 25A, Genetic Design Co.) are summarized in TABLE 3 and the coupling yields are listed in TABLE 4. The



A column of μBondapak C_{18} and a linear gradient of acetonitrile (0—30%) in 0.1 M ammonium acetate.

FIG 2. Chromatography of GAA, GAAA, and GAAAA.

TABLE 3. Program of reaction cycle

step	solvent or reagent	Time (min)
1	pyridine	5
2	pyridine-Ac_2O-DMAP	10
3	pyridine	5
4	CH_2Cl_2	10
5	1% TFA-CH_2Cl_2	1X2
6	CH_2Cl_2	5
7	pyridine	5
8	N_2	3
9	coupling reaction	40

Solid Phase Synthesizer Model 25A,
Genetic Desighn Co.

TABLE 4. Conditions for the solid phase synthesis[a,b]

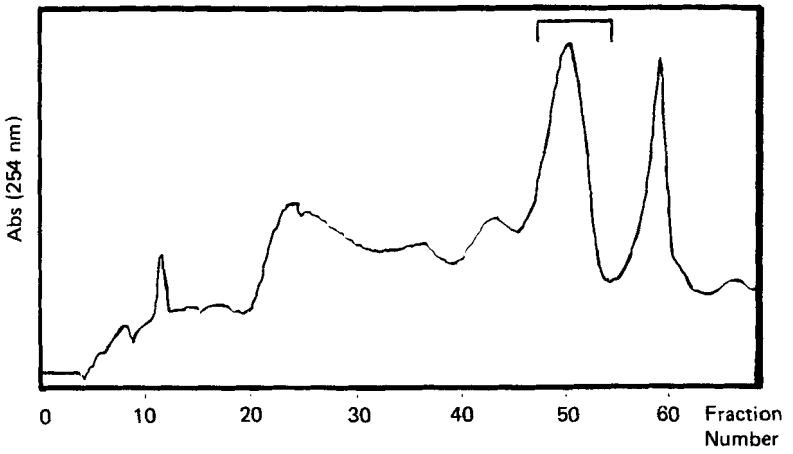
support (mg, umol)	diester (equiv.,M)	MSNT (equiv., M)	Time (min.)
100, 3.4	15, 0.2	75, 1.0	40

DMTr(G)ps(A)	ps(A)	ps(A)	ps(A)	ps(A)	CPG
(%)	82	92	96	100	

DMTr(G)ps(A)	ps(A)	ps(A)	ps(A)	ps(A)	CPG
(%)	75	88	82	91	100

a (G) = 2'-O-(tetrahydropyran)-2-yl-N^2-propionyl-O^6-diphenylcarbamoylguanosine,
(A) = 2'-O-(tetrahydropyran)-2-yl-N^6-benzoyl-adenosine,
ps= phenylthio group

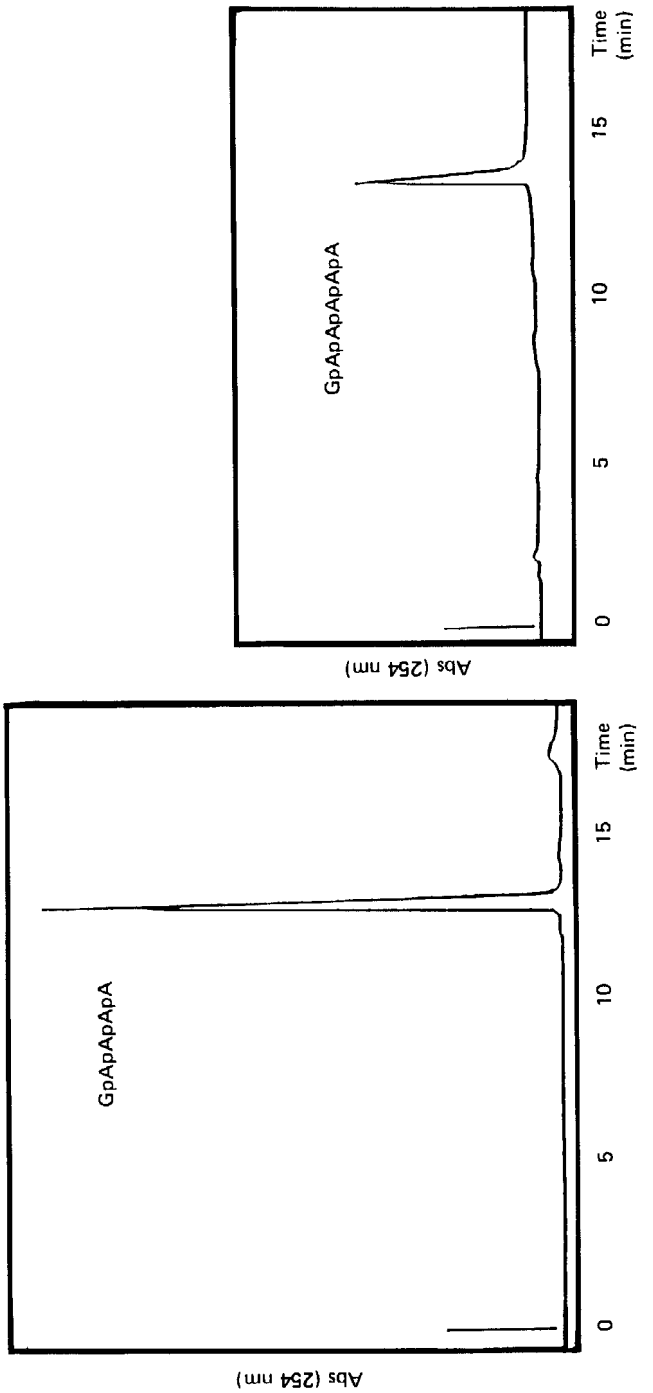
b Coupling was performed in CH_3CN.



A DEAE Sephadex A-25 column was employed and the elution was performed a linear gradient of triethylammonium bicarbonate buffer (0.05-1.0 M).

FIG. 3. Chromatography of the GAAAAA.

deblocking of the 5'-DMTr group was done by the use of 1% TFA in CH_2Cl_2. The capping reaction was performed by employing acetic anhydride in the presence of a catalytic amount of N,N-dimethylaminopyridine in pyridine. The mesitylenesulfonyl-3-nitrotriazole (MSNT)[25] was used as condensing reagent in CH_3CN—pyridine. Deblocking of the protected RNA fragments on the solid support was performed in the same way as described in the liquid phase method. The hexamer GAAAAA was separated by ion exchange chromatography on Sephadex A25. FIG 3 shows the elution profile of the hexamer from the column. The product was further purified by reversed phase HPLC (μBondapak C_{18} column) (FIG 4). The pure pentamer GAAAA (9.75 OD) and the hexamer GAAAAA (4.37 OD) were obtained in 15% and 3.5% yields, respectively, based on the assumption of 20% hypochromicity. Both pentamer and hexamer were completely hydrolyzed with nuclease P_1 to G and pA in the correct ratios.



A column of μBondapak C_{18} and a linear gradient of acetonitrile (0—30%) in 0.1 M ammonium acetate.

FIG 4. Chromatography of the GAAAA and GAAAAA.

CONCLUSION

Most studies in the field of modern molecular biology require only small quantities of synthetic oligoribonucleotides. Our experiments described here were directed toward a practical approach foward the preparation of RNA fragments, which would be useful for elucidation of the mechanism of biological pathways. Our initial purpose in this direction was achieved, although the isolated yields of G(A)n were not satisfactory especially in the solid support synthesis. The most serious problem in our strategy, which would be expected in the case of the combination of the 2'-Thp and 5'-DMTr groups, was the contamination of 2'-5' linkages in synthetic RNA fragments. However, the oligoribonucleotides obtained finally by the usual deblocking procedure have proved to be free of 2'-5' linked regioisomers, as suggested by nuclease P_1 digestion. This result implies that the splitting between the P-$O^{5'}$ bond occurs predominantly over that of the P-$O^{3'}$ or P-$O^{2'}$ bond from the cyclic triester intermediate as discussed previously. Therefore, we concluded that the combination of the 2'-Thp group with the 5'- DMTr group would provide a practical and rapid system for the synthesis of RNA fragments.

We are now studying the synthesis of various oligoribonucleotides containing the common four bases in this direction. Physicochemical studies on the C4N hypothesis using these synthetic RNA fragments will be reported in the near future.

EXPERIMENTAL

^{1}H-NMR spectra were recorded at 100MHz on a JNM-PS-100 spectrometer. UV spectra were obtained on a Hitachi 220A spectrophotometer. 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 a medium pressure for rapid chromatographic separation.

HPLC was performed on a μBondapak C_{18} column using 0.1M ammonium acetate (pH 7.0) at the flow rate of 2.0 ml/min. Pyridine was distilled twice from p-toluenesulfonyl chloride and from CaH_2 and then stored over molecular sieves 4A. Elemental analysis was performed by the Microanalytical Laboratory, Tokyo Institute of Technology, at Nagatsuta.

2'-O-(Tetrahydropyranyl)-3',5'-O-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-N^6-benzoyladenosine (1)

To a solution of 2'-O-(tetrahydropyranyl)-3',5'-O-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)adenosine[9] (2.47 g, 4.16 mmol) in dry pyridine (12 ml) was added benzoyl chloride (0.73 ml, 6.24 mmol). After it was stirred for 3 h, 5% $NaHCO_3$ solution was added. The aqueous solution was extracted with $CHCl_3$ (3 X 50 ml). The combined $CHCl_3$ extracts were evaporated to dryness under reduced pressure. The residue was coevaporated several times with toluene and chromatographed on a column of silica gel to give (1) (2.44 g, 84%)

Elemental analysis for $C_{34}H_{51}N_5O_7Si_2-H_2O$

Calcd C 57.04, H 7.46, N 9.78;

found C 56.80, H 7.58, N 9.13.

^{1}H-NMR ($CDCl_3$)

8.35(s,1H,H-8) 8.22(s,1H,H-2) 8.01(m,2H, o-H of benzoyl) 7.55(m,3H,m and p-H of benzoyl) 6.14(d,1H,H-1') 5.05(m,1H, H-3') 4.67(m,1H,H-2') 4.50(m,1H,H-4') 4.12(m,2H,H-5') 3.50(m,2H,O-methylene of Thp) 1.70(m,6H,C-methylene of Thp) 1.08(d,28H,iPr_2Si)

2'-O-(Tetrahydropyranyl)-N^6-benzoyladenosine (2)

To a solution of (1) (1.86 g, 2.67 mmol) in acetonitrile (53 ml) was added tetraethylammonium bromide (3.37 g, 16.0 mmol) potassium fluoride (0.90 g, 16.0 mmol) and water (0.80 ml, 16.0 mmol) and the solution was kept at 50°C for 5 h. The precipitate was filtered and the filtrate was evaporated under reduced pressure. The residue was dissolved in $CHCl_3$ and washed three times with 5% $NaHCO_3$ solution. The organic

phase was evaporated and chromatographed to give (2) (1.19 g 98%).

Elemental analysis for $C_{22}H_{25}N_5O_6$

Calcd C 58.02, H 5.53, N 15.38;

found C 57.65, H 5.49, N 15.52.

^{1}H-NMR ($CDCl_3$)

9.36(bs,1H,-NH) 8.05(m,2H,o-H of benzoyl) 8.00(s,s,2H,H-8 and H-2) 7.52(m,3H,m and p-H of benzoyl) 6.00(m,1H,H-1') 5.10(m,1H,H-3') 4.90(m,1H,acetal H of Thp) 4.60(m,1H,H-2') 4.36(m,1H, H-4') 3.89(m,2H,H-5') 3.25(m,2H,O-methylene of Thp) 1.48(m,6H,C-methylene of Thp)

2'-O-(Tetrahydropyranyl)-5'-O-(4,4'-dimethoxytrityl)-N^6-benzoyladenosine (3)

Compound (2) (1.19 g, 2.61 mmol), precoevaporated with dry pyridine, was dissolved in dry pyridine (5 ml) and mixed with DMTrCl (0.97 g, 2.87 mmol). After it was stirred for 3 h, the mixture was extracted with $CHCl_3$. The usual workup gave (3) (1.93 g, 97%).

Elemental analysis for $C_{43}H_{43}N_5O_8$

Calcd C 68.15, H 5.72, N 9.24;

found C 68.05, H 5.84, N 8.93.

^{1}H-NMR ($CDCl_3$)

9.04(bs,1H,-NH) 8.21(s,1H,H-8) 8.18(s,1H,H-2) 8.03(m,2H,o-H of benzoyl) 7.50(m,3H,m and p-H of benzoyl) 7.26(m,9H,Ar-H) 6.80(m,4H,2,2',6,6'-H of trityl) 6.20(d,1H,H-1') 5.00(m,1H, H-3') 4.60(m,1H,acetal of Thp) 4.46(m,1H,H-2') 4.32(m,1H, H-4') 3.80(s,6H,O-CH_3) 3.48(m,4H,O-methylene of Thp and H-5') 1.60(m,6H,C-methylene of Thp)

S,S-Diphenyl-2'-O-(tetrahydropyranyl)-5'-O-(4,4'-dimethoxytrityl)-N^6-benzoyladenosine-3'-phosphorodithioate (4)

Cyclohexylammonium S,S-diphenyl phosphorodithioate (PSS) (1.17 g, 3.06 mmol), pre-coevaporated with dry pyridine, was dissolved in dry pyridine (2.0 ml) and MDS (1.21 g, 3.82 mmol) was added. After the solution was kept for 30 min, (3) (1.93 g, 2.55 mmol) was added. The mixture was stirred

for 4 h. The extractive workup followed by chromatography gave (4) (1.86 g, 72%).

Elemental analysis for $C_{55}H_{52}N_5O_9PS_2$

Calcd C 64.63, H 5.13, N 6.85, S 6.27;

found C 64.90, H 5.36, N 6.73, S 5.87.

^{1}H-NMR ($CDCl_3$)

9.03(bs,1H,-NH) 8.21(s,1H,H-8) 8.18(s,1H,H-2) 8.01(m,2H,o-H of benzoyl) 7.48(m,3H,m and p-H of benzoyl) 7.27(m,19H,Ar-H) 6.79(m,4H,2,2'6,6'-H of trityl) 6.28(m,1H,H-1') 5.38(m,2H, acetal H of Thp and H-3') 4.45(m,1H,H-2') 4.20(m,1H,H-4') 3.78(s,6H,O-CH_3) 3.30(m,4H,O-methylene of Thp and H-5') 1.54(m,6H,C-methylene of Thp)

General procedure for synthesis of oligomers in the liquid phase method

To a solution of phosphinic acid in pyridine was added a fully protected monomer or oligomer and triethylamine. The mixture was kept at 35—40°C. After the substrate was converted completely to the diester, a mixture of pyridine—water (1:1, v/v) was added. The resulting solution was first extracted twice with hexane—ether (1:1, v/v) for removal of benzenethiol and then several times with $CHCl_3$. The $CHCl_3$ extracts were combined, washed twice with 0.5M triethylammonium bicarbonate (TEAB), and dried over $MgSO_4$. The solvent was removed under reduced pressure and the residual syrup was mixed with an appropriate hydroxyl component and 3-nitro-1,2,4-triazole. The mixture was coevaporated several times with dry pyridine and finally dissolved in dry pyridine. MDS was added and the solution was stirred vigorously. After the reaction was completed, the mixture was treated with 5% $NaHCO_3$ and extracted several times with $CHCl_3$. The $CHCl_3$ layer was concentrated under reduced pressure and the residue was coevaporated three times with toluene. The crude product was purified by chromatography on a column of silica gel (10—20 fold). Elution was performed using CH_2Cl_2—MeOH (97—98:3—2, v/v).

Deprotection of the fully protected pentamer

The pentamer (17 mg, 5 μmol) was dissolved in a 0.5 M solution of N^1,N^1,N^3,N^3-tetramethylguanidium 4-nitrobenzaldoximate in pyridine--water (9:1, v/v, 1.0 ml), and the solution was kept at room teperature for 5 h. Then the solution was dissolved in pyridine (2 ml) and concentrated ammonia (10 ml). The solution was sealed and kept at room temperature for 12 h and at 60°C for 6 h. The solution was evaporated under reduced pressure in the presence of pyridine. Then the solution was passed slowly through a column of Dowex 50W X 2(pyridinium salt, 2 ml), and the column was washed with pyridine—water (1:1, v/v, 50 ml). The eluent and washings were combined and evaporated under reduced pressure. During this evaporation pyridine was added three times to avoid partial loss of the Thp and DMTr groups. The residue was dissolved in water (50 ml) and washed with ether—ethyl acetate (3:2, v/v, 4 X50 ml). Then the aqueous layer was evaporated and coevaporated with toluene under reduced pressure. The oligoribonucleotide containing the DMTr and Thp groups was further dissolved in a 0.01 M hydrochloric acid (20 ml), and the solution was kept at room temperature for 30 h. Then a small amount of diluted ammonia was added to neutralize the solution. The neutralized solution was evaporated under reduced pressure. This residue was applied to Whatman 3MM papers and developed with 2-propanol—conc. ammonia—water(6:1:3, v/v/v).Then further purification was performed with reversed phase HPLC (μBondapak C_{18} column). The pure pentamer GpApApApA (191 OD) was obtained in 64% yield.

General procedure for the attachment of nucleosides to CPG-Biogel

The CPG (Bio 500 Amino Propyl-CPG Mean Pore Dia (Å) 569, Particle size 120/200, 500 mg) was shaken with 2'-O-(tetrahydropyran-2-yl)-5'-O-dimethoxytrityl-N^6-benzoyladenosine-3'-O-succinate (172 mg, 200 μmol), dicyclohexylcarbodiimide

(206 mg, 1 mmol),and triethylamine (28 µl, 200 µmol) in the presence of a catalytic amount of N,N-dimethylaminopyridine in DMF(1 ml) for 72 h. The support was filtered off and washed successively with DMF (10 ml), pyridine (10 ml), methanol (10 ml), and ether (10 ml). The unreacted amino groups were capped by treatment with acetic anhydride—pyridine (1:9, v/v) in the presence of a catalytic amount of N,N-dimethylaminopyridine for 30 min followed by extensive washing of the support with pyridine, methanol, and ether. The CPG gel was dried over P_4O_{10} by a vacum pump. The extent of loading of the first nucleoside on the support was meassured according to the published procedure.[23)]

General procedure for synthesis of oligomers in the solid phase method

The CPG support loaded with the first nucleoside (capacity of 34 µmol/g, 100 mg)was packed in a column which is part of the automatic DNA synthesizer. All manipulations necessary for one complete elongation cycle are summarized in Table 3. TFA (1%) in CH_2Cl_2 solution was flowed twice for 1 min, and the CPG gel was washed with CH_2Cl_2 for 5 min. These solution were collected for determination of coupling yield. N_2 gas was flowed into the column for 3 min to dry the CPG gel. Then a 3'-phosphodiester unit (15 equiv., 51 µmol), pre-dried by repeated coevaporation with pyridine, was treated with MSNT (255 µmol) in CH_3CN—pyridine (4:1, v/v, 250 µl). The resulting mixture was injected into the column. After 40 min, the gel washed with pyridine for 5 min and then was allowed to react with pyridine—acetic anhydride (9:1, v/v,) in the presence of a catalytic amount of N,N-dimethylaminopyridine for 10 min. The CPG gel was washed successively with pyridine for 5 min and CH_2Cl_2 for 10 min. The repetition of the above cycle with appropriate active nucleotides gave the desired sequence of oligoribonucleotides.

Deprotection of the fully protected hexamer attached to the CPG

Pyridine (5 ml) was added to the hexamer linked to the CPG (1.74 μmol). The mixture was kept at 0°C and treated with 0.2 M sodium hydroxide solution (15 ml) at 0°C for 30 min. Then the solution was passed slowly through a column of Dowex 50W X2 (pyridinium salt, 2 ml), and the column was washed with pyridine—water (3:2, v/v, 60 ml). The eluant and washings were combined and evaporated under reduced pressure. The residue was dissolved in pyridine (6 ml) and concentrated ammonia (10 ml). The solution was sealed and kept at 60°C for 3 h and at room temperature for 12 h. The solution was evaporated under reduced pressure, and the residue was dissolved in water (50 ml). The aqueous pyridine solution was washed with ether—ethyl acetate (3:2, v/v, 50 ml X4). The aqueous layer was evaporated under reduced pressure, and the residue was coevaporated with toluene for several times. Then the residue was further dissolved in a 0.01 M hydrochloric acid (20 ml), and the solution was kept at room temperature for 26 h. A small amount of dilute ammonia solution was added to neutralize the solution. The neutralized solution was evaporated and freeze-dried. Then the residue was passed through a column of DEAE Sephadex A25 (HCO_3^- form, 100 ml), and the column was eluted with triethylammonium bicarbonate buffer (500 ml). The eluent containing the hexamer was freeze-dried. Final purification of the hexamer was performed with reversed phase HPLC (μBondapak C_{18} column). The pure hexamer (4.37 OD) was obtained in 3.5% yield.

P_1 nuclease treatment with synthetic RNA oligomers

To a solution of synthetic RNA fragment (0.1 OD) in 0.5 M sodium acetate buffer (18 μl, pH 5.4) was added 0.1 M magnesium chloride (5 μl) and nuclease P_1 (2 μl, 10 mg / 5 ml). The mixture was kept at 37 °C for 1 h, and then the solution was heated at 100 °C for 1 min. The resulting solution was analyzed with reversed phase HPLC (μBondapak

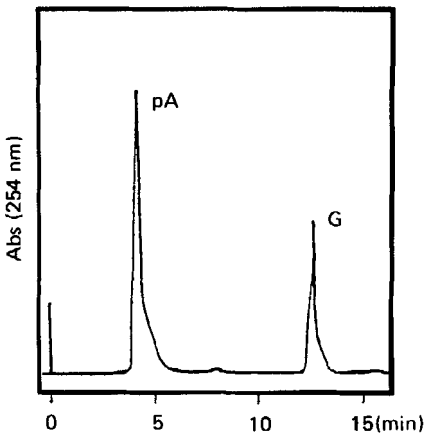


FIG 5. Reversed phase HPLC analysis of the products obtained after digestion of isolated tetramer GAAA with nuclease P_1.

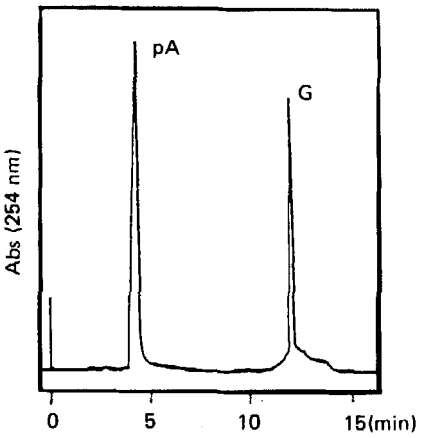


FIG 6. Reversed phase HPLC analysis of the products obtained after digestion of isolated pentamer GAAAA, whiich was synthesized by the solid phase approach, with nuclease P_1.

C_{18} column). The peaks on the HPLC profile were identified with standard sample 5'-AMP and guanosine. FIG 5 and FIG 6 showed HPLC profile of the products after digestion of completely deblocked RNA fragments. The ratios of G and pA were 1:1.88, 1:2.74 and 1:3.86 for digestion of GAA, GAAA and GAAAA respectively, obtained by the liquid phase method, and were 1:3.73 and 1:4.88 for GAAAA and GAAAAA, respectively, obtained by the solid phase synthesis.

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