Volume 12 Number 6 1984 Nucleic Acids Research

Chemical synthesis of the 5'-terminal part bearing cap structure of messenger RNA of cytoplasmic polyhedrosis virus (CPV): $m^7G^{5'}pppAmpG$ and $m^7G^{5'}pppAmpGpU$

Kazuo Yamaguchi, Iwao Nakagawa, Mitsuo Sekine, Tsujiaki Hata*, Kunitada Shimotohno+, Michiyo Hiruta+ and Kin-ichiro Miura+

Department of Life Chemistry, Tokyo Institute of Technology, Nagatsuta, Midoriku, Yokohama 227, and +National Institute of Genetics, Mishima 411, Japan

Received 23 December 1983; Revised and Accepted 24 February 1984

ABSTRACT

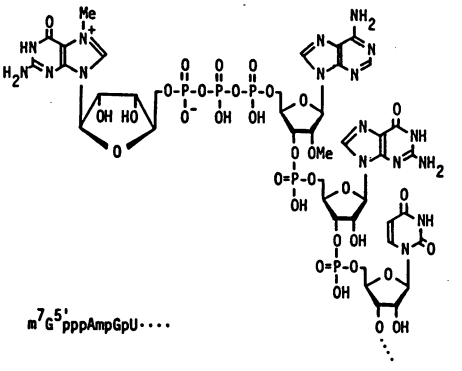
The 5'-terminal structures of mRNA bearing the so-called 'cap' from cytoplasmic polyhedrosis virus (CPV), $m^7G^{5'}pppAmpG$ and $m^7G^{5'}pppAmpGpU$, were first chemically synthesized. S,S-Di(4-methoxyphenyl) N^6-benzoyl-2'-O-methyladenosine 5'-phosphorodithioate ($(ArS)_2pA^{Bz}m$) was prepared by phosphorylation of the 5'-hydroxyl group of N^6-benzoyl-2'-O-methyladenosine with S,S-di(4-methoxyphenyl) phosphorodithioate by TPS. By the triester approach using $(ArS)_2pA^{Bz}m$ as starting material, the protected dinucleotide and trinucleotide bearing 5'-phosphate group were synthesized. The protective groups of the dinucleotide and trinucleotide were removed to obtain pAmpG and pAmpGpU, respectively. By the reaction of a capping agent ($(PhS)ppm^7G$) with pAmpG and pAmpGpU in the presence of silver nitrate or iodine. The 5'-terminal structure of the messenger RNA strand of CPV, which was labelled isotopically, was confirmed completely as $m^7G^{5'}pppAmGpU$ by cochromatography with the materials chemically synthesized here.

INTRODUCTION

Since a blocked structure at the 5'-terminus of messenger RNA (mRNA) of silkworm cytoplasmic polyhedrosis virus was detected by Furuichi and Miura[1] as $m^7G^{5'}pppAm$-G-U---, similar structures have been reported in a variety of mRNAs from eukaryotic cells and viruses, and now it is called as cap structure of eukaryote mRNA.[2-5] It is known that elimination of this structure causes loss of stability of mRNA[5-7], especially against exonuclease degradation, and decrease in ability of the initiation complex formation of mRNA for protein synthesis.[5-8]

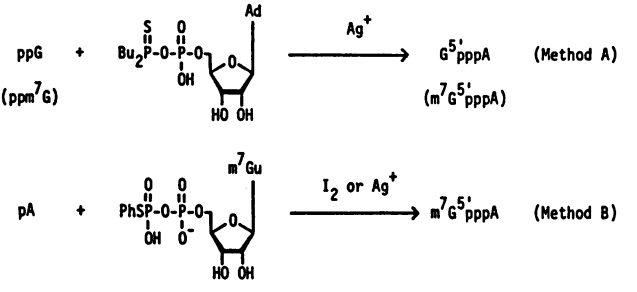
We have already confirmed the cap structure ($m^7G^{5'}pppAm$) by chemical synthesis[9-11] and have clarified CPV methylase specificity by using synthetic non-methylated α,γ-dinucleoside triphosphates as enzyme substrate.[12] In this paper we describe chemical synthesis of cap structure carrying oligoribonucleo-

tides, $m^7G^{5'}pppAmpG$ and $m^7G^{5'}pppAmpGpU$ in order to confirm the 5'-terminal structure of CPV mRNA and to investigate the function of this part.



Here, two synthetic methods for unsymmetrical α,γ-dinucleoside triphosphates have been developed (Scheme 1); 1) 7-methylguanosine 5'-diphosphate is allowed to react with nucleoside 5'-

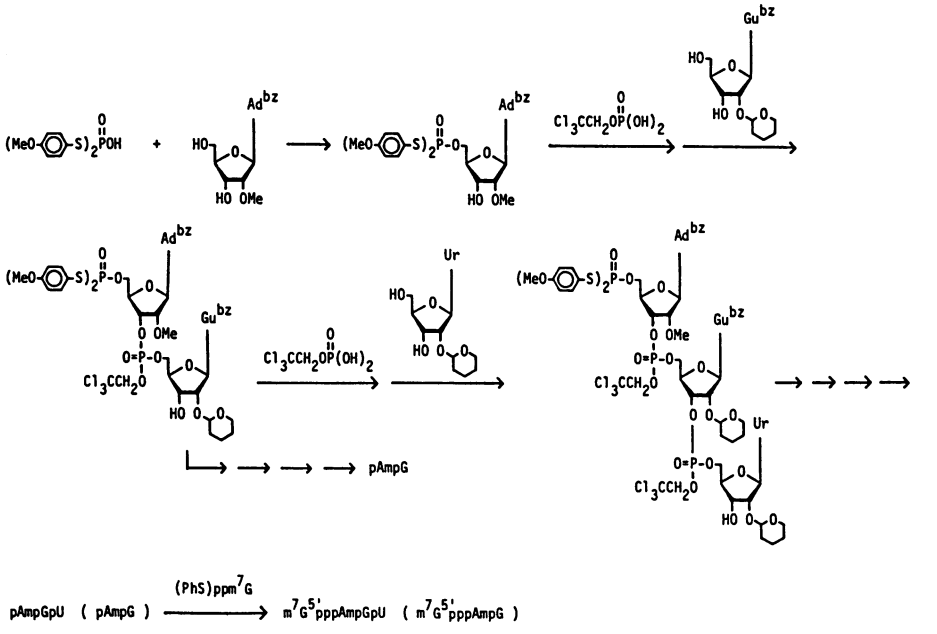
Scheme 1.



phosphoric dibutylphosphinothioic anhydride to obtain α,γ-dinucleoside triphosphate (Method A);[9] 2) reaction of P^1-S-phenyl P^2-7-methylguanosine-5' pyrophosphorothioate ((PhS)ppm^{7}G) with nucleoside 5'-phosphate in the presence of silver salts or iodine afford the same desired product (Method B).[10] The latter is suitable for synthesis of cap structure bearing oligoribonucleotides because the cap structure is synthesized by the reaction of (PhS)ppm^{7}G with oligoribonucleotides bearing 5'-phosphate end group. Synthesis of pAmpG and pAmpGpU was achieved by phosphotriester method using the 4-methoxyphenylthio group as protective

group of 5'-phosphate. The nucleoside 2'-hydroxyl and base amino groups were protected by the tetrahydropyranyl and benzoyl groups, respectively and internucleotidic phosphates were blocked by the 2,2,2-trichloroethyl groups. Subsequently reaction of the deprotected oligoribonucleotides with (PhS)ppm^{7}G gave the desired cap structures as shown in Scheme 2.

Scheme 2.

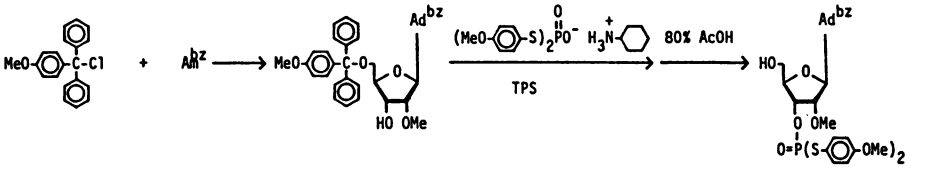


There are only a few examples of 5'-phosphate protecting groups for the phosphotriester approach of oligonucleotide synthesis.[13] Among the protecting groups, the 4-methoxyphenylthio group has enough stability for coupling reaction and is removed under mild conditions.[14] Preparation of 2'-O-methyladenosine 5'-phosphate derivative introducing the 4-methoxyphenylthio group was investigated as starting material for trinucleotide synthesis.

N^6-Benzoylation of 2'-O-methyladenosine (Am) was carried out in a method simila to that described for synthesis of N^6-benzoyldeoxyadenosine:[15] 2'-O-Methyladenosine was treated with 4.4 equiv. of benzoyl chloride in dry pyridine at room temperature to form the tetrabenzoyl derivative, which was partially hydrolyzed

to give Am^{bz}. The reaction mixture was purified by silica gel column chromatography to isolate N^6-benzoyl-2'-O-methyladenosine (Am^{bz}) in 87% yield. The 5'-hydroxyl group of Am^{bz} was phosphorylated with cyclohexylammonium S,S-di(4-methoxyphenyl) phosphorodithioate[16] ($(ArS)_2P(O)O^-C_6H_{11}\overset{+}{N}H_3$, Ar=4-$CH_3OC_6H_4$) in the presence of TPS in dry pyridine at room temperature for 20 h. The desired product was obtained in 51% yield by using silica gel column. The primary hydroxyl group seems to be phosphorylated preferentially, but phosphorylation of the secondary hydroxyl group at the 3'-position is also possible. Actually, diphosphorylated product was detected by TLC of the reaction mixture. Therefore, the 5'-monophosphorylated compound may involve 3'-phosphorylated by-product. The 3'-phosphorylated derivative was obtained separately as shown in Scheme 3. 5'-O-

Scheme 3.



Monomethoxytritylation of Am^{bz} and successive 3'-O-phosphorylation and demonomethoxytritylation gave S,S-di-4-methoxyphenyl N^6-benzoyl-2'-O-methyladenosine 3'-phosphorodithioate ($Am^{bz}p(SAr)_2$). In comparison with the ^1H NMR spectra and chromatographic behavior of both monophosphorylated compounds, the desired 5'-phosphorylated compound ($(ArS)_2pAm^{bz}$) was found to be pure. The spectral data and R_f values of the 2'-O-methyladenosine derivatives are shown in Table 1.

In order to synthesize the dimer, N^2-benzoyl-2'-O-tetrahydropyranylguanosine ($Gthp^{bz}$) was prepared. N^2-Benzoyl-3',5'-di-O-acetylguanosine prepared from guanosine by Ishido's method[17] was treated with dihydropyrane in the presence of p-toluenesulfonic acid in dry dioxane at room temperature for 22 h in the poseve of molecular sieves. The reaction mixture was chromatographed on a silica gel column to provide the diastereoisomers of the 2'-O-tetrahydropyranyl guanosine derivative almost quantitatively. By alkaline hydrolysis of the 3',5'-O-diacetyl

Table 1. Chemical Shift of ^1H NMR Spectrum and R_f Value of TLC for $(ArS)_2pA^{bz}m$ and $A^{bz}mp(ArS)_2$

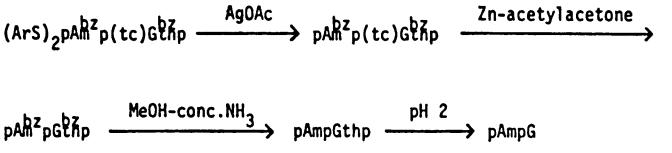
Nucleotide	^1H NMR in $CDCl_3$, δ (ppm)					R_f value of TLC
	H_8	H_2	H_1,(J, Hz)		2'-OCH_3	($CHCl_3$/MeOH=9/1)
$(ArS)_2pA^{bz}m$	8.75	8.20	6.17(4)	3.68	3.47	0.47
$A^{bz}mp(ArS)_2$	8.75	8.15	5.93(8)	3.83	3.35	0.51

groups $G^{bz}mp$ was obtained. The diastereoisomers were separated by silica gel column to isolate the high R_f isomer in 24% and the low R_f isomer in 51% yield respectively. ^1H NMR spectra of two isomers were identical with the reported ones.[14] The procedure of the tetrahydropyranylation was similar to Neilson's method[18] except for the purification process. Since the tetrahydropyranylated product was separated before deacetylation, two isomers were isolated in higher yields than Neilson's method. This process is generally applied to preparation of other tetrahydropyranyl nucleoside diastereoisomers of which are isolated in low yields.

$(ArS)_2pA^{bz}m$ and $G^{bz}mp$ (the high R_f isomer) were used for the dinucleotide synthesis. Phosphorylation of the 3'-hydroxyl group of $(ArS)_2pA^{bz}m$ with trichloroethyl phosphate was carried out by using TPS as condensing agent in dry pyridine at room temperature for 18 h. After excess of trichloroethyl phosphate was removed by extraction, the crude 3'-phosphorylated product without isolation was allowed to react with the guanosine derivative ($G^{bz}mp$) by using 3 equiv. of p-nitrobenzenesulfonyltriazolide (NBST) as coupling agent in dry pyridine at room temperature for 2 days. The condensed dinucleotide ($(ArS)_2pA^{bz}mp(tc)G^{bz}mp$) was obtained in 53% yield by silica gel column chromatography. The product was identified by elemental analysis and ^1H NMR spectrum.

Deprotection of $(ArS)_2pA^{bz}mp(tc)G^{bz}mp$ was investigated to obtain pAmpG as shown in Scheme 4. The protected dinucleotide in pyridine-H_2O (2/1) was treated with 20 equiv. of silver acetate for 20 h at room temperature for deprotection of the two 4-methoxyphenylthio groups.[14] Subsequently, removal of the

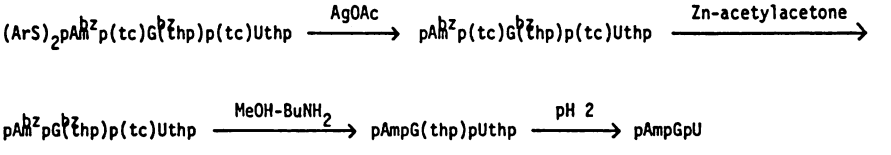
Scheme 4.



trichloroethyl group was achieved by treatment of $pA^{bz}_{m}p(tc)G^{bz}_{thp}$ with zinc powder in DMF-pyridine (2/1) by addition of acetylacetone for 17 h.[19] The solvent was removed under reduced pressure followed by treatment of the residue with methanol-conc. ammonia (1/1) at room temperature for 3 days. The debenzoylated compound was allowed to stand at pH 2 at room temperature for 2 days for removal of the tetrahydropyranyl group. The reaction mixture was applied to paper chromatography to provide pAmpG in 42% yield. The structure of pAmpG was confirmed by enzymatic degradation. Treatment of pAmpG with snake venom phosphodiesterase at 37°C for 9.5 h gave pAm and pG with the ratio of 1.0:1.2.

The protected dinucleotide ($(ArS)_2pA^{bz}_{m}p(tc)G^{bz}_{thp}$) and 2'-O-tetrahydropyranyluridine (Uthp) were employed for trinucleotide synthesis. $(ArS)_2pA^{bz}_{m}p(tc)G^{bz}_{thp}$ was phosphorylated with trichloroethyl phosphate and TPS in a manner similar to that described in the case of $(ArS)_2pA^{bz}_{m}$. The phosphorylated dinucleotide was successively condensed with Uthp by NBST at room temperature for 5 days. The desired trinucleotide $(ArS)_2pA^{bz}_{m}p(tc)G^{bz}_{(thp)}p(tc)Uthp$, which was isolated by silica gel column chromatography in 43% yield, was identified by elemental analysis and ^1H NMR. Deprotection of the fully protected trinucleotide was carried out as in case of dinucleotide (Scheme 5). The protected trimer was treated with silver acetate in pyridine-H_2O (2/1) and with zinc-acetylacetone in DMF-pyridine to lead to trinucleotide without phosphate protective groups. The benzoyl

Scheme 5.



groups of the trinucleotide were removed by methanol-butylamine[20] (1/1) instead of methanol-conc.ammonia at room temperature for 3 days. Finally the tetrahydropyranyl group was removed at pH 2 for 2 days and the reaction mixture was applied to paper chromatography to obtain pAmpGpU in 57% yield. The yield of pAmpGpU was better than that of pAmpG because of improvement of deprotection conditions for the benzoyl groups. The trinucleotide pAmpGpU was digested by snake venom phosphodiesterase to provide mononucleotides, pAm, pG and pU (1.0:0.96:1.0).

Synthesis of the cap structures containing oligonucleotides was performed by reaction of pAmpG and pAmpGpU with (PhS)ppm^{7}G. Tributylammonium salts of pAmpG and (PhS)ppm^{7}G in pyridine-DMSO (2/1) were treated with iodine at room temperature for 32 h. After removal of excess iodine by extraction with ether, the aqueous layer was applied to SP Sephadex column and eluted with water. The eluate was neutralized with lithium hydroxide and was chromatographed on Sephadex G-10 column eluted with 10% ethanol. The eluted solution was rechromatographed to give the fraction A with the ratio of 0.65 (A_{280}/A_{260}) and the fraction B with the ratio of 0.60. The fraction A was purified on AG-1 column in 0.01 N HCl with gradients of NaCl (0-0.26, 0.26-1 M) as eluent. The elution pattern is shown in Figure 1. The peak of fraction No. 104-108 was consistent with m^{7}G$^{5'}$pppAmpG obtained by digestion of CPV mRNA. The same peak appeared on chromatogram of the fraction B. The total yield of m^{7}G$^{5'}$pppAmpG was about 10%. Instead of iodine silver nitrate in capping reaction was employed to form the cap structure in a similar yield.

In order to synthesize m^{7}G$^{5'}$pppAmpGpU reaction of pAmpGpU with (PhS)ppm^{7}G in the presence of silver nitrate was undertaken analogously to synthesis of m^{7}G$^{5'}$pppAmpG. After a similar workup the fraction eluted from Sephadex G-10 was chromatographed on a AG-1 column to elute with a linear gradient of NaCl (0.2-0.7 M) in 0.01 N HCl as shown in Figure 2. The peak of fraction No. 35-40 was consistent with that of [^{3}H-methyl] labelled m^{7}G$^{5'}$pppAmpGpU, which was obtained by digestion with pancreatic ribonuclease I from CPV mRNA. The chemically obtained m^{7}G$^{5'}$pppAmpGpU in 0.4% yield was treated with Nuclease P_1 and

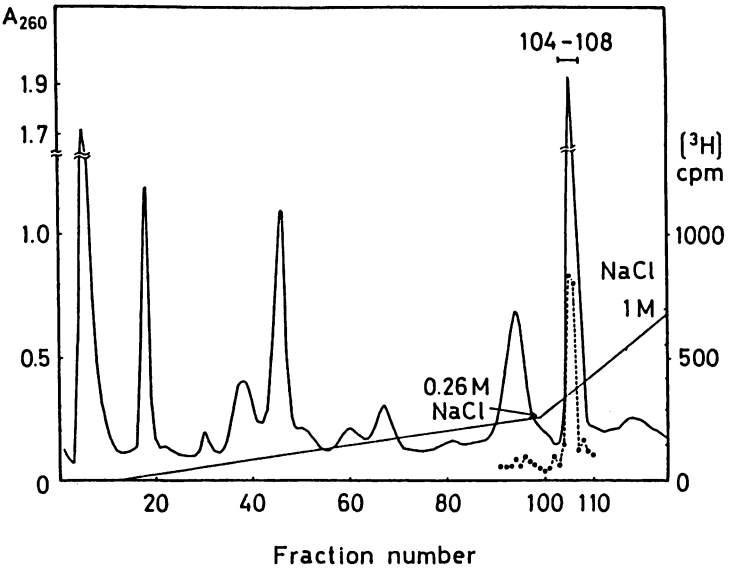


Fig. 1 Identification of $m^7G^{5'}pppAmpG$

[methyl ^3H]labeled CPV mRNA —nuclease T_1→ —phosphomonoesterase→ $[^3H]m^7G^{5'}pppAmpG$

subsequently with phosphomonoesterase followed by chromatographed on a AG-1 column. The result is shown in Figure 3. The mixture of uridine and guanosine was first eluted. The second peak of α,β

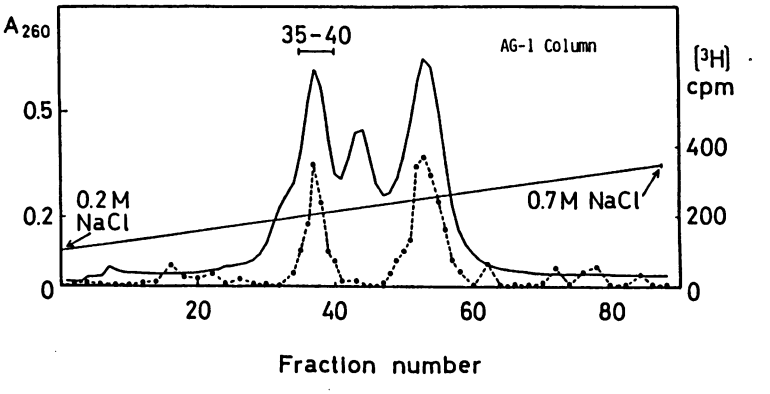


Fig.2 Identification of $m^7G^{5'}pppAmpGpU$

[methyl ^3H] labeled CPV mRNA —panc. RNaseI→ $[^3H]\ m^7G^{5'}]pppAmpGpU$

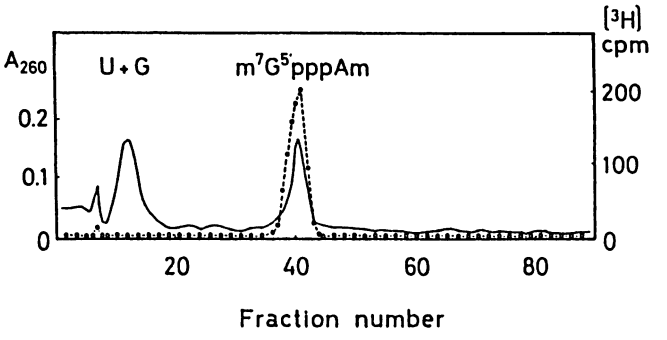


Fig.3 Enzymatic Digestion of $m^7G^{5'}$pppAmpGpU

$m^7G^{5'}$pppAmpGpU
↓ nuclease P_1
$m^7G^{5'}$pppAm + pG + pU
↓ phosphomonoesterase
$m^7G^{5'}$pppAm + G + U + 2Pi

-dinucleoside triphosphate $m^7G^{5'}$pppAm was identical with the one provided by enzymatic degradation of CPV mRNA.

Although the present approach provided for the first time the chemically capped oligonucleotides, we feel that several points must be improved during their synthesis.

We are now investigating the improved procedure, which will be reported in the near future.

EXPERIMENTAL

^1H NMR spectra were recorded at 60 MHz on a Hitachi R-24B spectrometer. UV spectra were obtained on a Hitachi 124 spectrophotometer.

Paper chromatography was performed by descending technique using Toyo Roshi No. 51 and Whatman 3 MM papers. The solvent system used was 1-propanol-conc. NH_3-water (55:10:35, v/v/v). Thin layer chromatography (TLC) was performed on plates of Merck Silica gel 60 F_{254} developing by chloroform-methanol (9:1, v/v). For columns Wakogel C-200 was used.

Pyridine was distilled from p-toluenesulfonyl chloride and stored over calcium hydride.

Snake venom phosphodiesterase (1 mg/1 ml) was purchased from Boehringer Mannheim GmbH.

N^6-Benzoyl-2'-O-methyladenosine (Am^{bz}).

To 2'-O-methyladenosine[21] (1.97 g, 6.99 mmol) in dry pyridine (12 ml) at 0°C was added benzoyl chloride (3.60 ml, 30.8 mmol) dropwise, and the reaction mixture was stirred at room temperature for 2 h. It was poured into ice water (50 g) and the aqueous solution was extracted with chloroform (50 ml x 3). The organic solution was dried over sodium sulfate and evaporated in vacuo. The residue dissolved in a mixture of pyridine (14 ml) and ethanol (21 ml) was treated with 2M NaOH (28 ml) and ethanol (28 ml) at room temperature for 5 min and then neutralized with Dowex 50 W (pyridinium form). The resin was removed by filtration and the filtrate was concentrated in vacuo. The residue was chromatographed on silica gel column (98:2 $CHCl_3$-MeOH) to give Am^{bz} (2.57 g, 87%): UV_{max}^{EtOH} 281 nm (ε=18000), UV_{min}^{EtOH} 250 nm (ε=9900); NMR ($CDCl_3$) δ 3.33(3H, s, 2'-O-CH_3), 3.7-4.7(5H, m, sugar ring), 5.92(1H, d, J=6Hz, H-1'), 7.08(3H, m, aromatic), 7.95(2H, m, aromatic), 8.05(1H, s, H-1), 8.68(1H, s, H-8), 9.40 (1H, s, PhC(O)NH). Anal. Calcd for $C_{18}H_{19}N_5O_5$ $2H_2O$: C, 51.30; H, 5.50; N, 16.62. Found: C, 51.41; H, 5.27; N, 16.83%.

S,S,-Di-4-methoxyphenyl N^6-benzoyl-2'-O-methyladenosine 5'-phosphorodithioate ($(ArS)_2pAm^{bz}$).

Cyclohexylammonium S,S-di(4-methoxyphenyl) phosphorodithioate[22] (1.46 g, 3.31 mmol) and Am^{bz} (1.33 g, 3.15 mmol) were rendered anhydrous by repeated coevaporation with dry pyridine and finally dissolved in dry pyridine (15 ml). TPS (2.01 g, 6.62 mmol) was added and the mixture was stirred at room temperature for 20 h. Methylene chloride (150 ml) and water (150 ml) were added and the organic layer was separated and the aqueous layer was extracted with methylene chloride (150 ml) again. The organic extracts were combined, dried over sodium sulfate, and evaporated to dryness. The residue was chromatographed on silica gel column (99:1 CH_2Cl_2-MeOH) to give $(ArS)_2pAm^{bz}$ (1.14 g, 51%): NMR($CDCl_3$) δ3.47 (3H, s, 2'-O-CH_3), 3.68(6H, s, CH_3O-C_6H_4S), 3.7-5.2(5H, m, sugar ring), 6.17(1H, d, J=4Hz, H-1'), 6.7-7.7(11H, m, aromatic), 8.00 (2H, m, aromatic), 8.20(1H, s, H-2), 8.75(1H, s, H-8). Anal. Calcd for $C_{32}H_{32}N_5O_8S_2P$: C, 54.15; H, 4.55; N, 9.87. Found: C, 54.06; H, 4.57; N, 9.50%.

S,S-Di(4-methoxyphenyl) N^6-benzoyl-2'-O-methyladenosine 3'-phosphorodithioate ($Am^{bz}p(ArS)_2$).

Am^{bz} (1.08 g, 2.79 mmol) was rendered anhydrous as described above and dissolved in dry pyridine (6 ml). Monomethoxytrityl chloride (1.04 g, 3.35 mmol) was added and the mixture was stirred at room temperature for 40 h. The reaction was stopped by addition of ethanol and the solution was extracted with chloroform (30 ml) and water (30 ml). The organic layer was separated and the aqueous solution was washed with chloroform (30 ml). The organic extracts were combined, dried over sodium sulfate, and concentrated *in vacuo*. The residue was chromatographed on silica gel column (ethyl acetate) to afford $MMTrAm^{bz}$ (1.10 g, 60%): NMR($CDCl_3$)δ 3.52(3H, s, 2'-O-CH_3), 3.77(3H, s, CH_3O-Ar), 6.21(1H, d, J=3Hz, H-1'), 6.7-8.1(14H, m, aromatic), 8.25(1H, s, H-2), 8.75(1H, s, H-8), 9.35(1H, s, PhC(O)NH). Anal. Calcd for $C_{38}H_{35}N_5O_6$: C, 69.39; H, 5.36; N, 10.65. Found: C, 68.66; H, 5.77; N, 9.96%. To a mixture of $MMTrAm^{bz}$ (146 mg, 0.22 mmol) and cyclohexylammonium S,S-di-4-methoxyphenyl phosphorodithioate (1.19 g, 0.27 mmol) in dry pyridine (1 ml) was added TPS (160 mg, 0.53 mmol) and the solution was stirred at room temperature for 3 days. The mixture was poured into ice water (10 ml) and the aqueous solution was extracted with chloroform (10 ml). The organic layer was dried over sodium sulfate, concentrated to dryness, and ./chromatographed on silica gel column (99:1 $CHCl_3$-MeOH) to give $MMTrAm^{bz}p(ArS)_2$ (193 mg, 91%). $MMTrAm^{bz}p(ArS)_2$ was subsequently treated with 80% acetic acid (10 ml) at room temperature for 16 h and the reaction mixture was concentrated *in vacuo* and chromatographed on silica gel column (95:5 $CHCl_3$-MeOH) to obtain $Am^{bz}p(ArS)_2$ (67 mg, 84%): NMR($CDCl_3$)δ 3.35(3H, s, 2'-O-CH_3), 3.83 (6H, s, CH_3O-C_6H_4S), 4.2-5.6(5H, m, sugar ring), 5.93(1H, d, J= 8Hz, H-1'), 6.7-7.7(11H, m, aromatic), 8.02(2H, m, aromatic), 8.15(1H, s, H-2), 8.75(1H, s, H-8), 9.4(1H, bs, PhC(O)NH).

N^2-Benzoyl-2'-O-tetrahydropyranylguanosine ($Gthp^{bz}$).

To N^2-benzoyl-3',5'-di-O-acetylguanosine[17] (4.02 g, 8.54 mmol) suspended in dry dioxane (40 ml) was added p-toluenesulfonic acid monohydrate (0.40 g, 2.2 mmol) in dry dioxane (40 ml)

previously dried over molecular sieve 4A (0.8 g) and dihydropyrane (17 ml, 0.19 mol), and the reaction mixture was stirred at room temperature. After 22 h, the reaction was quenched by addition of sodium ethoxide and the precipitated sodium p-toluenesulfonate was filtered. The filtrate was concentrated in vacuo and chromatographed on silica gel column (98:2 $CHCl_3$-MeOH) to afford the tetrahydropyranylated product almost quantitatively. To the product dissolved in a mixture of ethanol (40 ml) and pyridine (20 ml) was added a mixture of 2M NaOH (20 ml) and ethanol (20 ml) and the solution was stirred at room temperature for 10 min. The solution was neutralized with Dowex 50W (pyridinium form) and the resin was filtered. The filtrate was concentrated and chromatographed on silica gel column (96:4 $CHCl_3$-MeOH) to give a mixture of diastereoisomers of $G^{bz}tnp$ and the low R_f isomer of $G^{bz}tnp$ (1.04 g). The mixture was rechromatographed on silica gel column (97:3-96:4 $CHCl_3$-MeOH) to give high R_f isomer (987 mg) and low R_f isomer (1.07 g).

Synthesis of $(ArS)_2pA^{bz}p(tc)G^{bz}tnp$.

2,2,2-Trichloroethyl phosphate[23] (305 mg, 1.33 mmol) and $(ArS)_2pA^{bz}$ (789 mg, 1.11 mmol) were rendered anhydrous as described above and dissolved in dry pyridine (10 ml). TPS (806 mg, 2.66 mmol) was added and the mixture was stirred at room temperature for 19 h. Chloroform (20 ml) and water (20 ml) were added and the organic layer was separated. The aqueous solution was extracted with chloroform (20 ml x 2) and the organic extracts were combined, dried over sodium sulfate, and evaporated to dryness. Without isolation of the resulting 3'-phosphorylated derivative of $(ArS)_2pA^{bz}$, a mixture of the compound and $G^{bz}tnp$ (815 mg, 1.67 mmol) were rendered anhydrous as described above and dissolved in dry pyridine (10 ml). NBST[24] (846 mg, 3.33 mmol) was added and the mixture was stirred at room temperature for 2 days. To the reaction mixture was added monomethoxytrityl chloride (514 mg, 1.67 mmol) and the solution was stirred subsequently for 22 h. Chloroform (30 ml) and water (30 ml) were added and the organic layer was separated. The aqueous solution was washed with chloroform (20 ml x 2) and the organic solutions were combined, dried over sodium sulfate, and concentrated. The residue was chromatographed on silica gel column (97:3 $CHCl_3$-

MeOH) to afford $(ArS)_2pA^{bz}m^{bz}p(tc)G^{bz}thp$ (803 mg, 53%): NMR($CDCl_3$) δ 1.1-1.9(6H, m, thp ring), 3.2-3.4(2H, m, thp ring OCH_2), 3.3(3H, s, 2'-OCH_3 of Ado), 3.68(6H, s, CH_3O-ArS), 3.5-5.5(13H, m, sugar ring, thp acetal, and Cl_3CCH_2O), 5.88(1H, d, J=4Hz, H-1' of Ado), 5.98(1H, d, J=6Hz, H-1' of Guo), 6.5-8.1(18H, m, aromatic). Anal. Calcd for $C_{56}H_{57}N_{10}O_{17}Cl_3S_2P_2$ $2H_2O$: C, 47.68; H, 4.36; N, 9.93. Found: C, 47.41; H, 4.18; N, 9.62%.

Deprotection of $(ArS)_2pA^{bz}m^{bz}p(tc)G^{bz}thp$.

To a solution of $(ArS)_2pA^{bz}m^{bz}p(tc)G^{bz}thp$ (137 mg, 0.097 mmol) in pyridine (2 ml) and water (1 ml) was added silver acetate (325 mg, 1.94 mmol) and the mixture was stirred in the dark at room temperature for 20 h. Hydrogen sulfide was bubbled to the mixture at 0°C and the precipitated silver sulfide was removed by centrifugal filtration. The filtrate was concentrated in vacuo and the residue was dissolved in a mixture of DMF (12 ml), pyridine (6 ml), and acetylacetone (1.8 ml) followed by addition of Zn powder (900 mg). After the mixture was stirred at room temperature for 17 h, Zn was removed by filtration and the filtrate was evaporated under reduced pressure. The residue was treated with methanol (5 ml) and conc.NH_3 (5 ml) at room temperature for 3 days. The mixture was concentrated followed by addition of water to give white precipitate. Dowex 50W (H^+) was added and the mixture was stirred for 30 min at room temperature with a clear supernatant had been obtained. The resin was filtered and the filtrate was allowed to stand at pH 2 at room temperature for 2 days. The aqueous solution was evaporated and chromatographed on Whatman 3 MM paper (55:10:35 1-propanol-conc. NH_3-H_2O) to give pAmpG (0.041 mmol, 42%): $UV_{max}^{pH\ 7.0}$ 256 nm (ε= 28000), $UV_{min}^{pH\ 7.0}$ 237 nm. R_f 0.25 (Toyo Roshi No.51).

Synthesis of $(ArS)_2pA^{bz}mp(tc)G^{bz}(thp)p(tc)Uthp$.

Similarly to synthesis of $(ArS)_2pA^{bz}m^{bz}p(tc)G^{bz}thp$, 2,2,2-trichloroethyl phosphate (101 mg, 0.44 mmol) and $(ArS)_2pA^{bz}m^{bz}p(tc)G^{bz}thp$ in dry pyridine (6 ml) were allowed to react with TPS (212 mg, 0.70 mmol) at room temperature for 17 h. After work up as mentioned above, the phosphorylated compound was condensed with Uthp (high R_f diastereisomer) (144 mg, 0.44 mmol) by NBST (221 mg, 0.87 mmol) in dry pyridine (6 ml) for 5 days at room temperature. After a similar work up, the mixture was chromato-

graphed on silica gel column (95:5 $CHCl_3$-MeOH) to obtain $(ArS)_2pA^{bz}m(tc)G^{bz}(thp)p(tc)$-Uthp (238 mg, 43%):NMR($CDCl_3$)$\delta$1.1-1.9 (12H, m, thp ring), 3.0-3.5(4H, m, thp ring OCH_2), 3,33(3H, s, 2'-OCH_3 of Ado), 3.5-5.0(19H, m, sugar ring and Cl_3CCH_2O), 3.75 (6H, s, CH_3O-C_6H_4S), 5.0-6.5(6H, m, H-1' of Ado, Guo, and Ura and H-5 of Ura and thp acetal), 6.7-8.2(19H, aromatic and H-6 of Ura). Anal. Calcd for $C_{72}H_{77}N_{12}O_{26}Cl_6S_2P_3$ $2H_2O$: C, 44.76; H, 4.23; N, 8.70. Found: C, 44.58; H, 4.22; N, 8.58%.

Deprotection of $(ArS)_2pA^{bz}m(tc)G^{bz}(thp)p(tc)$Uthp.

Deprotection of $(ArS)_2pA^{bz}m(tc)G^{bz}(thp)p(tc)$Uthp (133 mg, 0.069 mmol) was carried out under conditions similar to those of the dimer except for debenzoylation by methanol (5 ml) and butylamine (5 ml) for 3 days. The trimer pAmpGpU was obtained in 57% yield by paper chromatography: $UV_{man}^{pH\ 7.0}$ 257 nm (ε=32800), $UV_{min}^{pH\ 7.0}$ 228 nm. R_f 0.19 (Toyo Roshi No.51).

Enzymatic degradation of pAmpG and pAmpGpU.

The dimer pAmpG (18 OD) was incubated with snake venom phosphodiesterase (10 l) in 0.1M Tris buffer (pH 8.0, 0.2 ml) at 37°C for 9.5 h. A small amount of toluene was added to the mixture and the solution was evaporated *in vacuo*. The residue was applied to Toyo Roshi No.51A paper and developed to analyze digested 5'-mononucleotides. The trimer pAmpGpU was digested and analyzed as in the case of pAmpG.

Synthesis of $m^7G^{5'}pppAmpG$.

To a solution of pAmpG (420 OD, 0.015 mmol) in aqueous pyridine was added methanol and tributylamine (0.10 ml) and the solution was concentrated to dryness. The aqueous solution of $(PhS)pm^7G$ (0.075 mmol) was added to the residue followed by evaporation. Tributylamine (0.10 ml) and methanol were added to the residue dissolved in DMSO (0.30 ml). The mixture was rendered anhydrous as described above and dissolved in dry pyridine (0.60 ml). Iodine (40 mg, 0.15 mmol) was added to the solution. The mixture was stirred vigorously at room temperature for 32 h. Water was added and the aqueous solution was extracted with ether to remove excess ammount of iodine employed. The aqueous layer concentrated was applied to a SP Sephadex (H^+) column and the 8 ml fractions eluted with water were collected. Fraction No.5-30 were neutralized with 0.1M lithium hydroxide

followed by evaporation. The residue was then applied to a Sephadex G-10 column and eluted with 10% ethanol to collect 3 ml fractions. Fraction No.17-20 were concentrated and rechromatographed on the same column. Fraction No.15-17 and No.18-22 contained 330 OD (A_{280}/A_{260}=0.65) and 200 OD (A_{280}/A_{260}=0.60) respectively. The former fractions were applied to a AG-1 column and eluted with a linear concentration gradient of sodium chloride from 0 M through 0.26M to 1M in 0.01N HCl. Concentration of No.104-108 of 1 ml fractions gave $m^7G^{5'}$pppAmpG (4.7 OD). The latter fractions were chromatographed similarly to afford $m^7G^{5'}$pppAmpG. The total yield of the product was 60 OD (10 %): $UV_{max}^{pH\ 5.5}$ 257 nm, $UV_{min}^{pH\ 5.5}$ 230 nm, ε_{260} 38000.

Synthesis of $m^7G^{5'}$pppAmpGpU.

To a mixture of tributylammonium salt of pAmpGpU (310 OD, 9.5 mol) and (PhS)pm^7G (0.081 mmol) in a mixture of DMSO (0.5 ml) and pyridine (1 ml) was added silver nitrate (276 mg, 1.63 mmol) and the mixture was stirred in the dark at room temperature for 3 days. After addition of water (1 ml) to quench the reaction, hydrogen sulfideto was bubbled to the aqueous solution diluted with water (10 ml) at 0°C. The precipitated silver sulfide was removed by centrifugal filtration and the solution was concentrated in vacuo. The residue was applied to a SP a Sephadex column and the 8 ml fractions eluted with water were collected. Fraction No.3-29 were neutralized with 0.1M lithium hydroxide and evaporated. The residue was applied to Sephadex G-10 column and eluted with 10% ethanol. No.11 and 12 of 3 ml fractions were collected and concentrated to obtain 200 OD products. The residue was applied to a AG-1 column and eluted with a linear concentration gradient of sodium chloride from 0.2M to 0.7M in 0.01 N HCl. No.35-40 of 1 ml fractions were collected and evaporated to give $m^7G^{5'}$pppAmpGpU (1.6 OD, 0.4%): $UV_{max}^{pH\ 5.5}$ 258 nm, $UV_{min}^{pH\ 5.5}$ 236 nm, ε_{260} 42000.

Enzymatic degradation of $m^7G^{5'}$pppAmpGpU. Incubation of $m^7G^{5'}$pppAmpGpU (1.6 OD) with Nuclease P_1 in 0.1M acetate buffer (pH 5.8) was carried out at 37°C for 30 min. Subsequently the solution was treated with phosphomonoesterase at 37°C for 30 min. The digests were applied to a AG-1 column and eluted with a linear concentration gradient of NaCl from 0 M to 0.26M in 0.01N HCl.

Dedicated to Professor Friedrich Cramer on the occasion of his 60th birthday.

*To whom correspondence should be addressed

REFERENCES

1) Furuichi, Y. and Miura, K. (1975) Nature 253, 374-375
2) Cohn, W. E., Volkin, E. Eds., (1976) in Progress in Nucleic Acid Research and Molecular Biology, Academic Press, New York, and references cited therein
3) Griffin, B. (1975) Nature 255, 9
4) Shatkin, A. J. (1976) Cell 9, 645-653
5) Miura, K. (1981) Adv. Biophysics 14, 205-238
6) Shimotohno, K., Kodama, Y., Hashimoto, J. and Miura, K. (1977) Proc. Natl. Acad. Sci. USA 74, 2734-2738
7) Furuichi, Y., LaFiandra, A. and Shatkin, A. J. (1977) Nature 266, 235-239
8) Zan-Kowalczewska, M., Bretner, M., Sierakowska, H., Szczesna, E., Fillipowicz, W. and Shatkin, A. J. (1977) Nucleic Acids Res. 4, 3065-3081
9) Hata, T., Nakagawa, I., Shimitohno, K. and K. Miura, (1976) Chem. Lett., 987-990
10) Nakagawa, I., Konya, S., Ohtani, S. and Hata, T. (1980) Synthesis, 556-557
11) Miura, K., Hiruta, M., Nakagawa, I., Yamaguchi, K. and Hata, T. in preparation.
12) Shimotohno K. and Miura, K. (1976) FEBS Lett., 64, 204-208
13) van Boom, J. H., Burgers, P. M. J. and van Deursen, P. H. (1976) Tetrahedron Lett., 869-872
14) Sekine, M., Hamaoki, K. and Hata, (1979) J. Org. Chem., 44, 2325-2326
15) Schaller, H., Weimann, G., Lerch, B. and Khorana, H. G. J. Am. Chem. Soc., (1963) 85, 3821-3827
16) Hata. T., Yamaguchi, K., Honda, S. and Nakagawa, I. (1978) Chem. Lett., 507-508
17) Ishido, Y., Nakazaki, N. and Sakairi, N. (1976) J. Chem. Soc., Chem., Commun., 832-833
Ishido, Y., Nakazaki, N. and Sakairi, N. (1979) J. Chem. Soc., Perkin I, 2088-2098
18) Neilson, T., Wastrodowski, E. V. and Werstiuk, E. S. (1973) Can. J. Chem., 56, 1068-1074; Gregoire R. J. and Neilson, T. (1978) Can. J. Chem., 56, 487-490
19) Adamiak, R. W., Biaxa, E., Grzeskowiak, K., Kierzek, R., Kraszewski, A., Markiewicz, W. T., Stawinski, J. and Wiewiorowski, M. (1977) Nucleic Acids Res., 4, 2321-2329
20) Weber, H. and Khorana, H. G. (1972) J. Mol. Biol., 72, 219-249
21) Robins, M. J., Naik, S. R. and Lee, A. S. K. (1974) J. Org. Chem., 39, 1891-1899
22) Owen G. R. and Reese, C. B. (1974) Synthesis, 704-705
23) Katagiri, N., Itakaura, K. and Narang, S. A. (1975) J. Am. Chem. Soc., 97, 7332-7337