Synthesis of Capped Oligoribonucleotides by Use of Protected 7-Methylguanosine 5'-Diphosphate Derivatives

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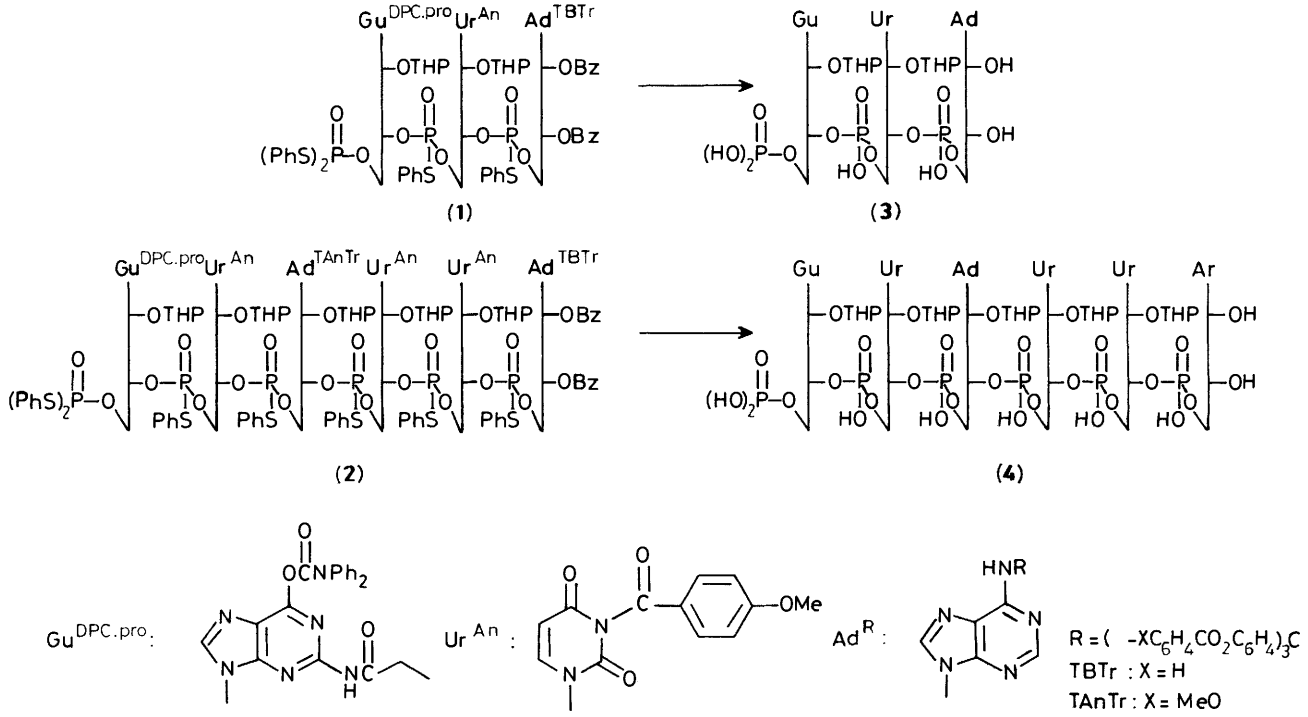
This paper describes the capping reaction of 5'-phosphorylated oligoribonucleotides protected only by a tetrahydropyranyl group at each 2'-position. By use of the conventional capping agent PhSppm^{7}G^{TMTr}mM, m^{7}G^{5}pppGUA and m^{7}G$^{5'}$pppGUAUUA were synthesized from the corresponding oligoribonucleotide 5'-monophosphate derivatives. Since the latter was obtained in low yield because of poor solubility of the partially protected pUUA in DMF, the oligomer component was converted into a phosphorimidazolidate species which was found to be soluble in DMF and allowed to react with a newly designed capping agent, ppm^{7}G^{TMTr}mM. This triphosphate bond formation proceeded smoothly to give m^{7}G$^{5'}$pppGUAUUA in good yield.

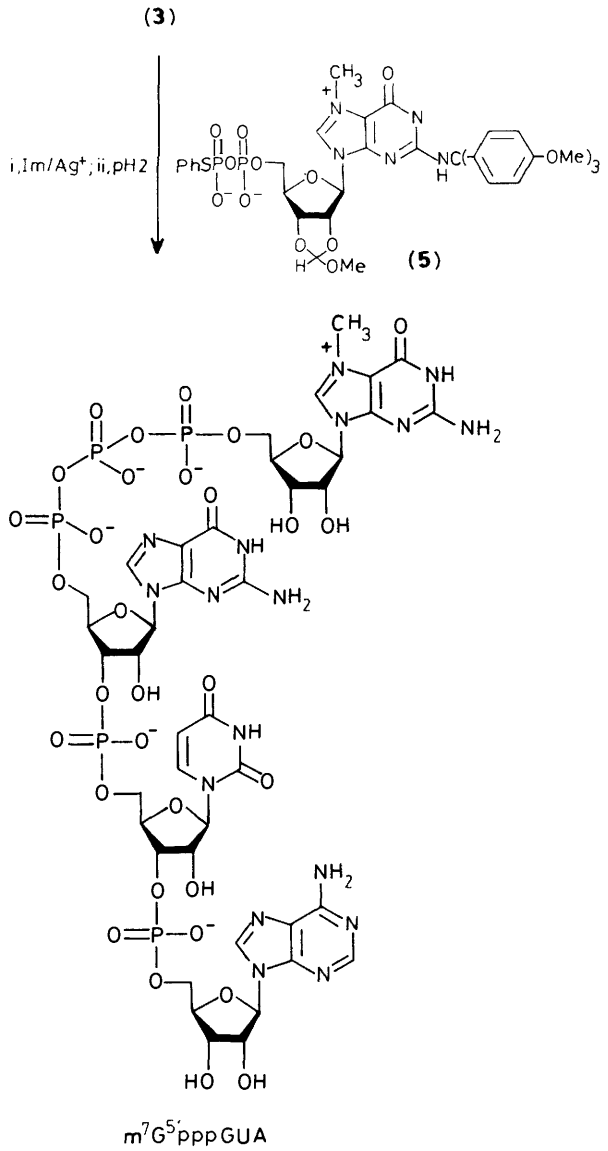
Eukaryotic mRNAs have a unique structure, m^{7}G$^{5'}$ppp-NupNupNu . . ., called cap', at their 5'-termini.[1-7] Since the discovery of this structure, a number of research groups have studied extensively its biological significance.[8-19] Thus the synthesis of capped mRNAs was an interesting target and in previous work we improved the synthesis[20-25] and other groups have described their own approaches.[26,27] In this paper, we report further studies on the chemical synthesis of capped mRNAs.[28]

Results and Discussion

Recently, we reported the synthesis of oligonucleotides using the 4,4',4''-tris(benzoyloxy)trityl (TBTr)[29,30] and 4,4',4''-tris(*p*-methoxybenzoyloxy)trityl (TAnTr)[31] groups as base-labile protecting groups for the 6-amino group of deoxyadenosine[32] or adenosine.[31] These protecting groups enabled us to make partially protected oligoribonucleotides with a 5'-terminal phosphate and a tetrahydropyranyl (THP) group at each 2'-position.[31] We therefore employed two partially protected oligoribonucleotide derivatives (**3**) and (**4**), obtained from the fully protected trimer (**1**) and hexamer (**2**) respectively, in order to ascertain whether the base-unprotected oligomers could be used as substrates for the capping reaction. The synthesis of (**1**) and (**2**) has recently been reported.[31]

Firstly, the synthesis of m^{7}G$^{5'}$pppGUA was performed as outlined below (Scheme 1). Reaction of (**3**) with an appropriately protected diphosphate derivative of 7-methylguanosine, (**5**),[22] was carried out in the presence of silver nitrate and imidazole[25] for 42 h. In this case, protection of the base residues was unnecessary as far as the solubility problem was concerned. After work-up, the mixture was analysed by anion-exchange h.p.l.c. as shown in Figure 1. Anion-exchange h.p.l.c. was simultaneously monitored by u.v. and fluorescence



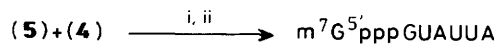


Scheme 1. *Reagents:* i, Im, Ag; ii, pH 2

detectors. Consequently, a peak at 31 min was determined to be the desired product of m^7G^5'pppGUA based on its enzymatic assay (Figures 2A and 2B and see Experimental section).

The capped trimer m^7G^5'pppGUA, separated by anion-exchange h.p.l.c., was finally desalted on Sephadex A-25; the isolated yield was 11% from (**3**).

In a similar manner, the THP-containing hexaribonucleotide (**4**) was obtained in 69% yield from (**2**). In this case, the ammoniacal treatment required a longer reaction time (24 h at 50 °C) for complete removal of the TAnTr group. The ammonium salt of the partially protected hexamer (**4**) was converted into its trioctylammonium salt for the capping reaction. However, it was difficult to dissolve this salt in dry dimethylformamide (DMF). Nonetheless, the capping reaction was carried out in a heterogeneous solution using the trioctylammonium salt of (**4**) for 66 h. As expected, the reaction was incomplete and the capped hexamer m^7G^5'pppGUAUUA was obtained in only 2.1% yield from (**4**) (Scheme 2).



Scheme 2. *Reagents:* i, Im, Ag^+; ii, H^+

We have previously encountered difficulty in the identification of a desired capped mRNA fragment from the mixture obtained after the capping reaction and the successive deprotection, since h.p.l.c. analysis showed numerous insignificant peaks. In the present work the same problem occurred again, as shown in Figure 3. However, we have found that when the mixture obtained after the deprotection was adsorbed on a Sephadex A-25 column and then roughly eluted with 1M ammonium hydrogen carbonate, h.p.l.c. of the eluant exhibited a markedly simpler pattern, where the capped and unchanged hexamers were seen as the major products (Figure 4). This pretreatment was indeed effective for isolation of m^7G^5'pppGUAUUA by h.p.l.c.

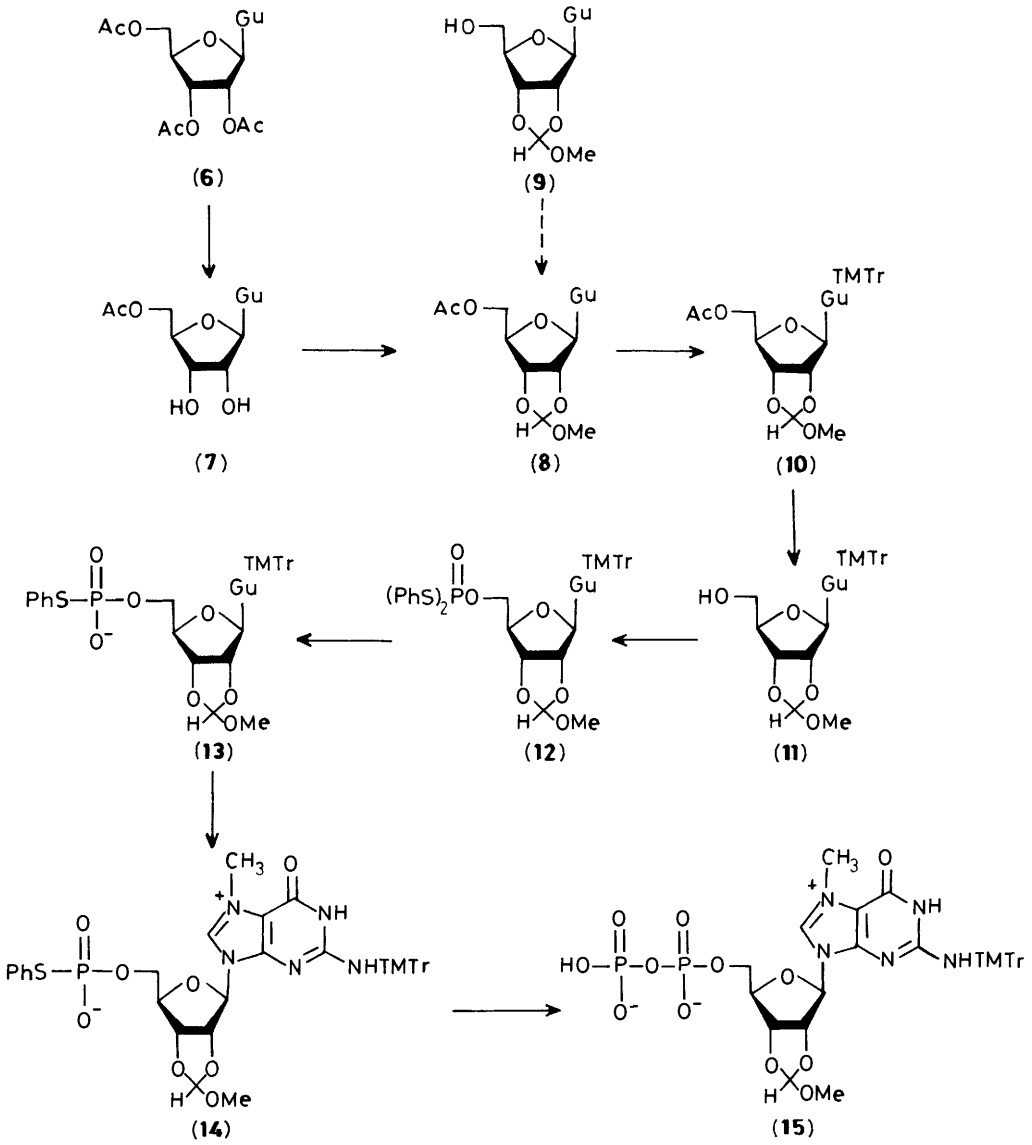
At the trimer level, the h.p.l.c. pattern (Figure 1A) seen after deprotection was similar to that described in the synthesis of m^7G^5'pppAUG.[25] In that case, the capping reaction complications seemed to be due to side reactions between the activated capping reagent and internal phosphates of oligoribonucleotides or once-formed triphosphate linkages. We therefore searched for an alternative synthetic route to capped oligoribonucleotides to overcome these inherent problems.

As another possible reaction mode for the triphosphate bond formation, the condensation between ppm^7G and XpNupNupNu... should also be considered, where the phosphoryl group of the oligomer component is activated. We therefore synthesized an appropriately protected ppm^7G (**15**) as an accepter molecule for the capping reaction in order to examine this possibility. For the synthesis of (**15**), a TMTr-containing pm^7G derivative (**14**) was synthesized as a synthetic intermediate as follows (Scheme 3).

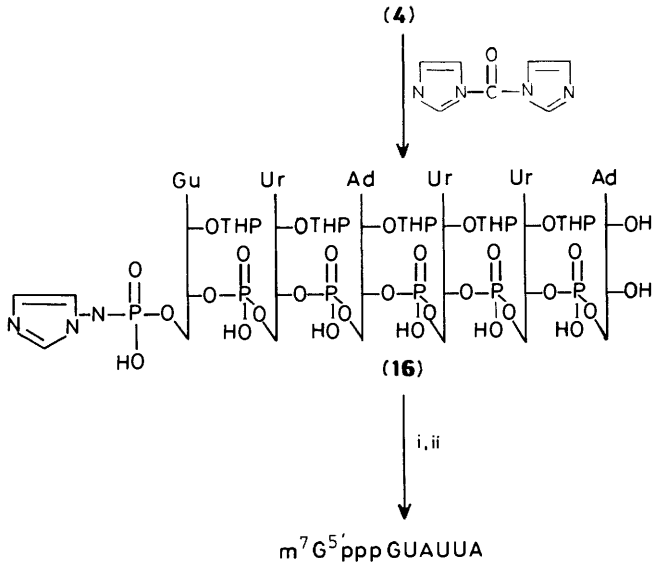
2',3',5'-*O*-Triacetylguanosine (**6**)[33] was partially deacylated by a modification of the method of Ishido[34] using hydroxylamine in methanol to give 5'-*O*-acetylguanosine (**7**), which was easily crystallized from methanol (61%). Compound (**7**) was converted into (**8**) by a trimethylsilyl chloride-catalysed methoxymethylenation recently reported by us.[35] Compound (**8**) was obtained as *ca.* 1:1 diastereoisomers which could not be separated by chromatography. Attempted acetylation of 2',3'-*O*-methoxymethyleneguanosine (**9**)[36] with acetic anhydride in pyridine failed. The reaction did not occur even at 80 °C because of the extremely poor solubility of (**9**) in pyridine. Addition of other polar solvents such as dimethyl sulphoxide or DMF was also ineffective. Compound (**8**) was converted *in situ* into (**10**) by tritylation with TMTrCl in the presence of 4-(*N*,*N*-dimethylamino)pyridine.[37] Treatment of (**10**) with butylamine gave the 5'-free guanosine derivative (**11**) which was obtained in an overall yield of 46% from (**7**). Phosphorylation of (**11**) with cyclohexylammonium *S*,*S*-diphenyl phosphorodithioate[38–40] in the presence of 1,2,3,5-tetramethylbenzenedisulphonyl dichloride (DDS)[41] and 1*H*-tetrazole (Tet)[42] gave the fully protected guanosine derivative (**12**).

On treatment of (**12**) with phosphinic acid–Et_3N in pyridine (PSA reagent),[30,43] the *S*-phenylguanosine 5'-phosphorothioate derivative (**13**) was obtained. This compound was methylated *in situ* with methyl iodide to afford an inner salt of the phosphorylating reagent (**14**) in an overall yield of 85% from (**12**).

As described earlier the reaction of the octylammonium salt of (**4**) with (**5**) in the presence of imidazole was not completed because of the insolubility of (**4**) in DMF. In an effort to improve the solubility of the oligonucleotide residue, the 5'-terminal phosphate of (**4**) was allowed to react with carbonyldiimidazole (Scheme 4).[44–48] Fortunately, it was found that the conversion of (**4**) into the corresponding phosphorimidazolidate (**16**) led to solubility in DMF. This enabled us to examine the capping reaction in a reverse manner where the partially protected oligoribonucleotide (**16**) could be activated as a donor molecule at the 5'-terminal phosphate residue, so that a 7-methylguanosine 5'-diphosphate derivative (**15**) could be



Scheme 3.



Scheme 4. *Reagents:* i, (15); ii, H^+

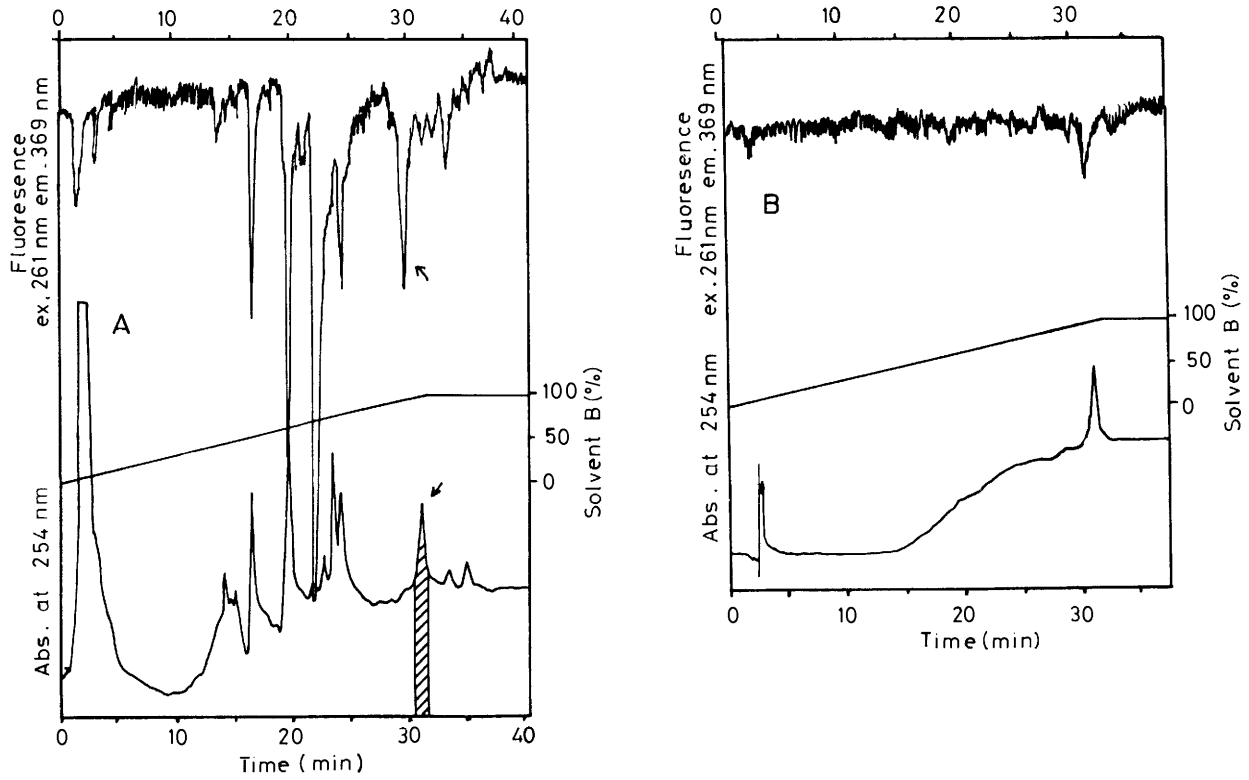


Figure 1. A: The anion-exchange h.p.l.c. profile of the mixture obtained following the reaction of (**3**) with (**5**). B: The h.p.l.c. profile of purified $m^7G^{5'}pppGUA$

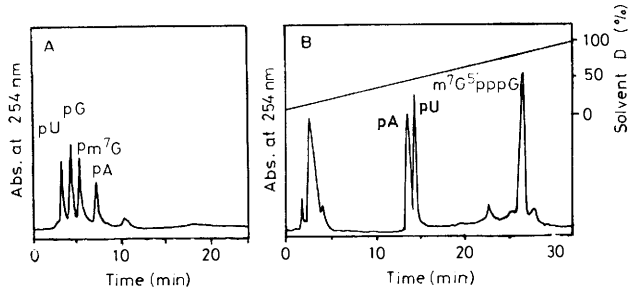


Figure 2. A: The reverse-phase h.p.l.c. profile of the digestion products obtained after the incubation of $m^7G^{5'}pppGUA$ with snake venom phosphodiesterase. In this case, 0.1M NH_4OAc was used as eluant at a flow rate of 2.0 ml min^{-1}. B: The anion-exchange h.p.l.c. profile of the digestion protects obtained after the incubation of $m^7G^{5'}pppGUA$ with nuclease P1

used as the accepter molecule for the triphosphate bond formation.

For the synthesis of such an accepter molecule, (**14**) was allowed to react with tributylammonium phosphate in the presence of silver nitrate. This reaction gave quantitatively (**15**), which was extracted after removal of silver ion by treatment with hydrogen sulphide and used for the capping reaction without further purification (single spot on t.l.c. after extraction). Compound (**15**) was partially soluble in DMF.

Since 7-methylguanosine (m^7G) is known to be unstable under basic conditions, and even under neutral conditions in certain cases, synthesis of the capping reagent should involve only simple operations, otherwise decomposition more or less accompanies the chemical transformation. Thus $ppm^7G_{mM}^{TMTr}$ was readily obtained without damage of the methylated purine ring.

The homogeneous reaction of (**16**) with (**15**) in DMF was now

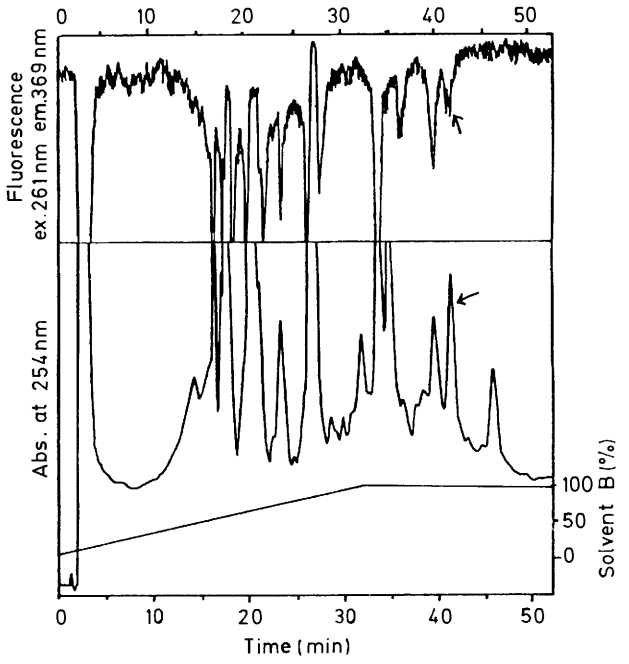


Figure 3. The anion-exchange h.p.l.c. profile of $m^7G^{5'}pppGUAUUA$ obtained following the reaction of (**4**) with (**5**)

accomplished. After 1 day, the mixture was treated with 0.01M HCl to remove the TMTr and THP groups. Anion-exchange chromatography of the mixture using Sephadex A-25 gave a crude coupling product of $m^7G^{5'}pppGUAUUA$ in a considerably improved yield of 61%. The elution profile is shown in

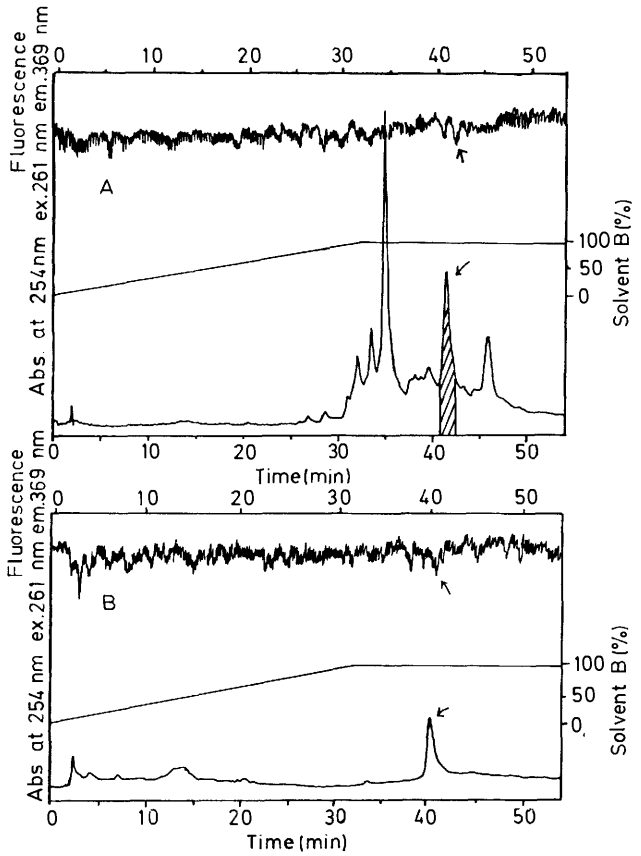


Figure 4. A: The anion-exchange h.p.l.c. profile of the mixture containing $m^7G^{5'}pppGUAUUA$ obtained from Sephadex A-25 column chromatography of the mixture obtained following the reaction of (**4**) with (**5**). B: The anion-exchange h.p.l.c. profile of purified $m^7G^{5'}pppGUAUUA$

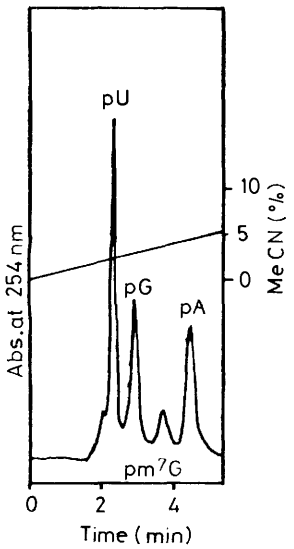


Figure 5. The reverse-phase h.p.l.c. profile of the digestion products obtained after the incubation of $m^7G^{5'}pppGUAUUA$ with snake venom phosphodiesterase

Figure 6. H.p.l.c. of the crude material shared some impurities, as shown in Figure 7A, which were finally separated by preparative h.p.l.c. using a Whatman 10 SAX column as shown

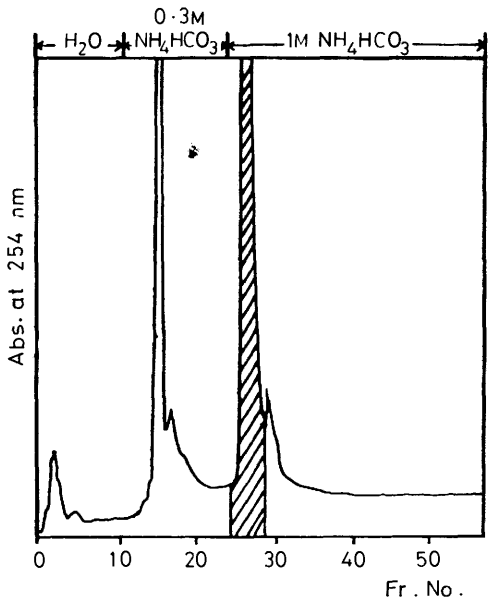


Figure 6. The elution profile for Sephadex A-25 column chromatography of the mixture containing $m^7G^{5'}pppGUAUUA$ obtained by the reaction of (**15**) with (**16**)

in Figure 7B. Subsequent desalting on Sephadex A-25 gave purified $m^7G^{5'}pppGUAUUA$ in 27% yield (Figure 8).

Although considerable loss of material was observed during extensive purification of the capped hexamer, the present reaction was very clean and gave the desired product as the major component. It should be noted that such a clear result was accomplished for the first time in our study on the chemical synthesis of capped mRNAs. This is indeed a considerable improvement because the previous condensation between (**2**) and (**5**) for the capping reaction resulted in complex h.p.l.c. patterns as reported.[25]

Since the sensitivity of the fluorescence detector used for h.p.l.c. is rather low compared with that of the u.v. detector, samples containing less than 1 OD per $m^7G^{5'}pppGUAUUA$ could not be detected (Figure 7A). Therefore, in the case of $m^7G^{5'}pppGUAUUA$, more than 5 OD of the capped hexamer was required. $m^7G^{5'}pppGUAUUA$ could be monitored by fluorescence detector when purified on a preparative scale by h.p.l.c. (Figure 7B). The capped hexaribonucleotide was analysed by enzymatic assay with snake venom phosphodiestease and nuclease P_1. The former gave a mixture of pU, pG, pA, and pm^7G in the correct ratio (Figure 9A). The latter digested the hexamer to a mixture of pA, pU, and $m^7G^{5'}pppG$ in the expected ratio (Figure 9B).

The TMTr group of (**14**) and (**15**) was sufficiently stable in several reactions. This is an additional advantage of using the diphosphate-type capping reagent over the previous reagent (**5**). The latter often lost the TMTr group during methylation and subsequent separation using reversed-phase chromatography.[24] The loss of the TMTr group made it more difficult to analyse the capping reaction by t.l.c. Indeed, the capping reaction of (**3**) or (**4**) with (**5**) gave a complex t.l.c. pattern so that we could not judge which spot corresponded to the desired coupling product. On the other hand, the capping reaction of (**16**) with (**15**) could be readily monitored by t.l.c. which exhibited only the starting materials and the coupling product. The intermediate $m^7G^{TMTr}mM^{5'}pppG(thp)U(thp)A(thp)U(thp)U(thp)A$ was clearly detected as shown in the Table which gave the R_F values of the partially protected oligoribonucleotide derivatives studied here.

Table. The R_F values of partially protected oligoribonucleotide derivatives, capping reagents, and related compounds[a]

Compound	R_F Value on Avicel t.l.c plates		R_F value on Whatman 3MM papers
	Solvent E	Solvent F	Solvent E
pG(thp)pU(thp)A(thp)U(thp)U(thp)A	0.46	—	0.23
ImpG(thp)pU(thp)A(thp)U(thp)U(thp)A	0.66	0.85	
m^7G^{TMTr} $mM^{5'}$pppG(thp)pU(thp)A(thp)U(thp)U(thp)pA	0.60	0.77	
pG(thp)pU(thp)pA	0.31	—	0.25
$PhSpm^{7TMTr}mM$	0.69	0.85	
$ppm^{7TMTr}mM$	0.51	0.36	
$PhSppm^{7TMTr}mM$	0.71	0.81	

[a]Solvent E: Pr^iOH–conc. aqueous ammonia–H_2O(6:1:3, v/v/v). Solvent F: EtOH–1M NH_4OAc (7:3, v/v).

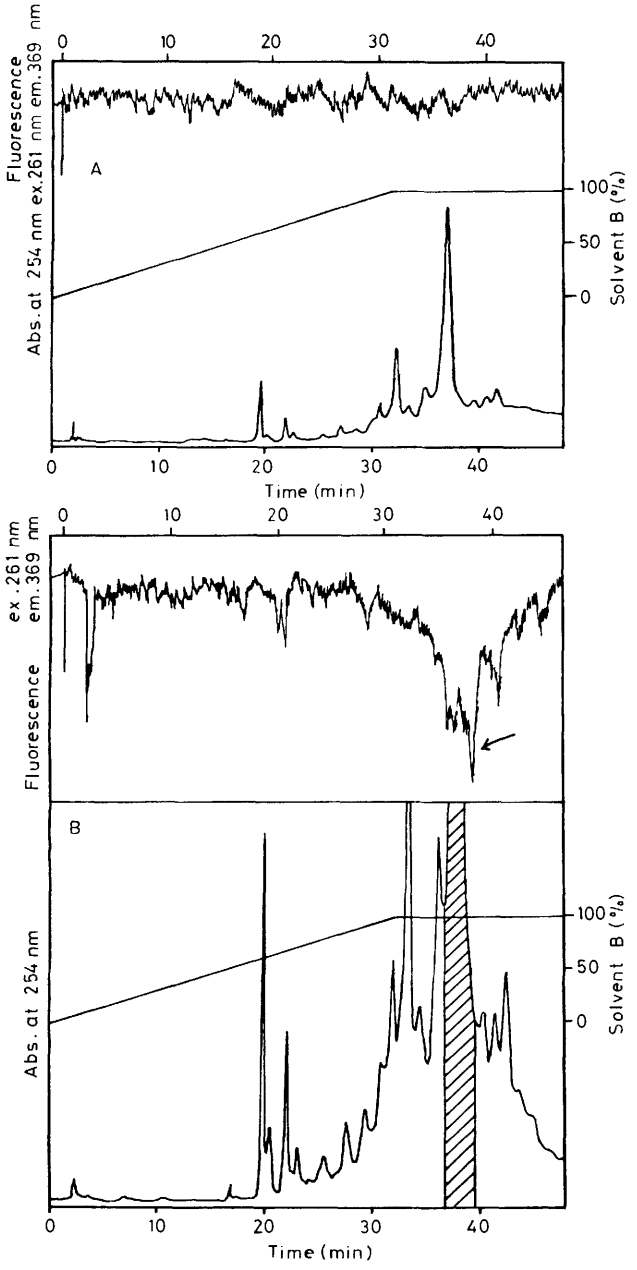


Figure 7. A: The anion-exchange h.p.l.c. profile of crude $m^7G^{5'}pppGUAUUA$ obtained after chromatography on a Sephadex A-25 column. B: The anion-exchange h.p.l.c. profile of the same sample as shown in Figure 7A on a preparation scale

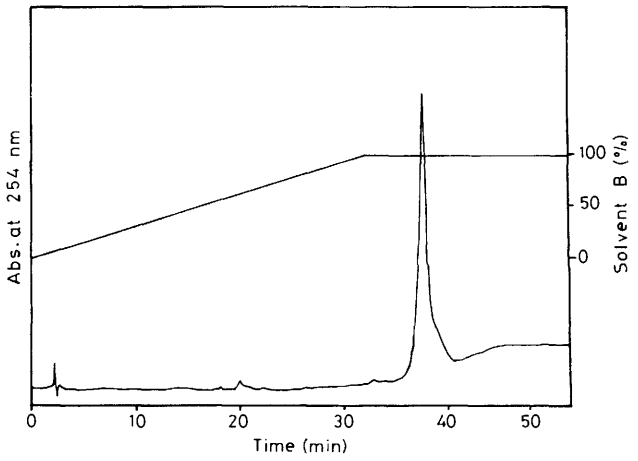


Figure 8. The anion-exchange h.p.l.c. profile of $m^7G^{5'}pppGUAUUA$ purified by h.p.l.c.

As one of the possibilities for the triphosphate bond formation, one can consider the capping reaction of ppG(thp)U(thp)A(thp)U(thp)U(thp)A with (**13**) in the presence of an activating agent. However, our preliminary study on this possibility suggested that the diphosphorylated hexamer was extremely insoluble in DMF so that the reaction did not proceed effectively.

In conclusion, at the hexamer level, it is necessary to use a combination of the 5′-terminal phosphoroimidazolidate derivative (**16**) and the capping accepter molecule (**15**) for smooth reaction. The synthetic mRNA fragment of $m^7G^{5'}$ pppGUA has recently been used as a substrate for ligation with oligoribonucleotide 5′-phosphates in order to obtain enzymatically longer capped mRNA fragments which were used for biological studies on the binding with 18*S* ribosome. These results will be soon reported elsewhere. In view of the results obtained in this study, we are studying an improved approach to a high-yield chemical synthesis of capped mRNAs.

Experimental

^1H N.m.r. spectra were recorded at 100 MHz on a JEOL UNM PS-100 spectrometer using tetramethylsilane as an internal standard. U.v. 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. Column chromatography was performed with silica gel C-200 purchased from Wako Co. Ltd., and a minipump (intended for goldfish bowls) was conveniently used to gain a medium pressure for rapid chromatographic

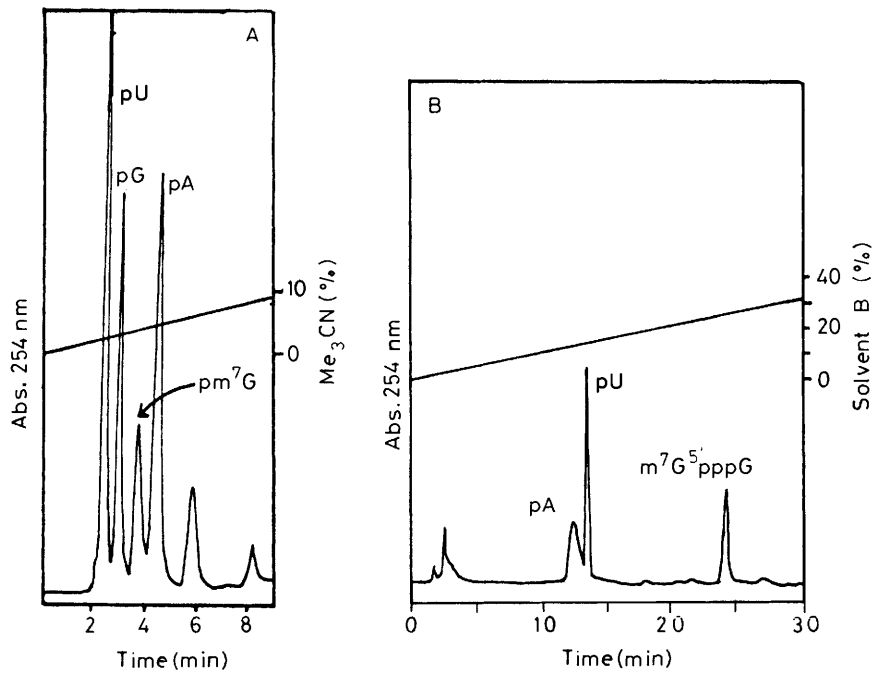


Figure 9. A: The reverse-phase h.p.l.c. profile of the digestion product obtained after the incubation of $m^7G^{5'}$pppGUAUUA with snake venom phosphodiesterase. The peaks at 6 min and 8.2 min in A were impurities derived from the enzyme solution. B: The anion-exchange h.p.l.c. profile of the digestion product obtained after the incubation of $m^7G^{5'}$pppGUAUUA with nuclease P

separation. Thin-layer chromatography (t.l.c.) was performed on plates precoated with Silica gel $60F_{254}$ (Merck). Anion-exchange h.p.l.c. was performed on a JASCO TRI ROTAR Type II apparatus equipped with a GP-A30 solvent programmer, a UVIDEC 100-II detector, a FP-110 fluorescence spectrofluorometer, a RC-225 recorder, and an Ohkura Model R 7005 digital integrator. Analysis and purification of unprotected oligoribonucleotides was 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 (0.005M KH_2PO_4, 20% MeCN, pH 4.1) and applying buffer B (0.5M KH_2PO_4, 20% MeCN, pH 4.5) at a flow rate of 1.5 ml min^{-1} for 32 min; System B, a linear gradient (0—99%) starting with buffer C (0.005M KH_2PO_4, pH 4.1) and applying buffer D (0.1M KH_2PO_4, 1.0M KCl, pH 4.5) at a flow rate of 1.5 ml min^{-1} for 32 min. Reversed-phase h.p.l.c. was performed on a Waters Model-440 apparatus equipped with a Model-660 solvent programmer, a M-45 pump, a column oven, and a Pantos U-228 recorder. Analysis of degradation products obtained by enzymatic treatment of capped oligoribonucleotides was performed on a μ-Bondapak C_{18} column (Waters) using a linear gradient of 0.1M NH_4OAc to 30% MeCN in the same buffer for 30 min at a flow rate of 1.5 ml min^{-1} at 50 °C or Whatman Partisil 10 SAX column using system B.

The yield of capped oligoribonucleotides was calculated on the assumption of 15% hypochromicity.

Pyridine was distilled twice from toluene-*p*-sulphonyl chloride and from calcium hydride and then stored over molecular sieves (4 Å). DMF Was distilled and stored over molecular sieves (4 Å). Snake venom phosphodiesterase was purchased from Boehringer Co. Ltd. Nuclease P_1 and ribonucleosides were purchased from Yamasa Shoyu Co. Ltd. Elemental analyses were performed by the Microanalytical Laboratory, Tokyo Institute of Technology, at Nagatsuta.

Synthesis of $m^7G^{5'}$pppGUA.—The ammonium salt of pG(thp)U(thp)A (10 μmol) was converted into the pyridinium salt by cation-exchange resin (Dowex 50 W × 2, pyridinium form, 1 ml). Elution was performed with pyridine–water (2:1, v/v). The eluate was concentrated and co-evaporated five times with dry pyridine. The residue was dissolved in dry pyridine (93 μl) containing tributylamine (7.2 μl, 30 μmol) and co-evaporated several times with dry pyridine. Compound (**5**) (58.1 mg, 51 μmol) was co-evaporated twice with dry DMF and finally dissolved in dry DMF (2.5 ml). Methyl iodide (93 μl, 1.5 mmol) was added and stirring was continued for 15 h. After removal of the solvent under reduced pressure, imidazole (69 mg, 1 mmol) was added and the mixture was dissolved in dry DMF (3 ml). The solution was added to the tributylammonium salt of pG(thp)U(thp)A, previously dried as described above. The mixture was evaporated and co-evaporated several times with dry pyridine and finally dissolved in dry DMF (660 μl). The solution was evaporated under reduced pressure for several minutes to remove almost all the remaining pyridine. The resulting mixture was treated with silver nitrate (257 mg, 1.5 mmol) and stirred at room temperature for 42 h. The reaction was quenched by the addition of pyridine–water (2:1, v/v, 15 ml). Hydrogen sulphide was bubbled into the solution at 0 °C for 10 min. The excess gas was removed on stirring with an aspirator. The precipitate was removed by centrifugation, and the supernatant was concentrated to a gum. The gummy material was co-evaporated three times with toluene–methanol (1:1, v/v) to remove the last traces of pyridine. To the residue was added 0.01M HCl in 50% aqueous dioxan (20 ml). The mixture was stirred at room temperature for 22 h. Dilute aqueous ammonia was added to neutralise the acid solution. The resulting neutral solution was evaporated and water was added. The aqueous solution was washed with ether (× 3). The aqueous solution was concentrated under reduced pressure. Part of the concentrate (37.5%) was subjected to an anion-exchange h.p.l.c. column to isolate $m^7G^{5'}$pppGUA. The peak at 31 min was pooled and the solvent removed under reduced pressure. The capped trimer was dissolved in water and applied to a column of Sephadex A-25 (1 ml) for desalting. Elution was performed with 1M TEAB buffer. The eluate was evaporated and co-evaporated several times with water to give

$m^7G^{5'}$pppGUA (16.6 A_{257}, 11%); $\lambda_{max.}(H_2O)$ 257 nm (ε 3.96 × 10^4), $\lambda_{min.}$ 233 nm.

Enzymatic Analysis of $m^7G^{5'}$pppGUA.—The sample obtained by h.p.l.c. in the above experiment was further purified by paper chromatography with 95% ethanol as eluant to remove completely the last traces of TEAB which sometimes seriously inhibited the enzyme reaction. The purified material (0.5 O.D. at 254 nm), which did not move on the paper and was eluted with water, was incubated with snake venom phosphodiesterase (1 mg ml^{-1}, 4 μl) in Tris-HCl buffer (pH 8.0, 40 μl) 1M $MgCl_2$ (2 μl) at 37 °C for 3 h. H.p.l.c. of the incubation mixture showed a mixture of pm^7G, pG, pU, and pA in a ratio of 1.2:1.0:1.1:0.8. This ratio was calculated by using the following extension coefficients (dm^3 mol^{-1} ml^{-1}) at 254 nm: pm^7G, 10.3 × 10^3; pG, 13.2 × 10^3; pU, 8.2 × 10^3; pA, 13.6 × 10^3.

The TEAB-free sample (0.5 A_{254}) was incubated with nuclease p_1 (2 mg ml^{-1}, 10 μl) in acetate buffer (pH 5.4, 90 μl) at 37 °C for 6 h. The h.p.l.c. analysis of the mixture showed a mixture of $m^7G^{5'}$pppG, pU, and pA in a ratio of 0.94:1.13:1.00. For calculation of the degradation products, the following extension coefficients (dm^3 mol^{-1} ml^{-1}) at 257 nm were used: $m^7G^{5'}$pppG, 17.1 × 10^3; pU, 9.1 × 10^3; pA, 14.6 × 10^3.

5′-O-*Acetylguanosine* (**7**).—To a solution of 2′,3′,5′-tri-*O*-acetylguanosine (**6**) (140 mg, 0.34 mmol) in methanol (5 ml) were added hydroxylammonium sulphate dihydrate[24] (62.5 mg, 0.38 mmol) and triethylamine (106 μl, 0.76 mmol). The resulting mixture was stirred at room temperature for 13 h, at which time hydroxylammonium sulphate dihydrate (50.5 mg, 0.31 mmol) and triethylamine (85 μl, 0.61 mmol) were added. After being stirred for an additional 20 h, the mixture was treated with acetone (1.7 ml). The solution was stirred for 75 min and then evaporated under reduced pressure. Addition of methanol to the residue gave the crystalline product (**7**) (67 mg, 61%); R_F 0.00 (CH_2Cl_2–MeOH, 9:1, v/v); m.p. 188.5—189 °C (Found: C, 43.3; H, 5.0; N, 21.5. $C_{12}H_{15}NO_6 \cdot 0.5H_2O$ requires C, 43.1; H, 4.5; N, 20.95); $\delta_H(CDCl_3)$ 2.08 [3 H, s, $C(O)CH_3$]. 4.00—4.36 (4 H, m, 3′-, 4′-, and 5′-H), 4.44—4.60 (1 H, m, 2′-H), 5.77 (1 H, m, 1′-H), and 7.90 (1 H, s, 8-H).

5′-O-*Acetyl*-2′,3′-O-*methoxymethylene*-N^2-(4,4′,4″-*trimethoxytrityl*)*guanosine* (**10**).—To a suspension of (**7**) (1.45 g, 4.46 mmol) in dry DMF (10 ml) were added methyl orthoformate (4.92 ml, 44.6 mmol) and trimethylsilyl chloride (1.13 ml, 8.92 mmol). After the mixture had been stirred for 4.5 h, pyridine (20 ml) was added. The solution was evaporated under reduced pressure. At this stage, t.l.c. showed a single spot of (**8**), R_F 0.21 [twice developed with CH_2Cl_2–MeOH (7:1, v/v)]. The residue was dissolved in dry DMF (10 ml). To the solution were added successively triethylamine (1.86 ml, 13.4 mmol), *N*,*N*-dimethylaminopyridine (22 mg, 0.18 mmol), and 4,4′,4″-trimethoxytrityl chloride (4.93 g, 13.4 mmol). After being stirred for 30 min, the mixture was filtered to remove insoluble material and the filtrate was partitioned between CH_2Cl_2 and water. The organic phase was collected and the aqueous layer was back extracted with CH_2Cl_2 (× 4). The extracts were combined, dried (Mg_2SO_4), filtered, and evaporated under reduced pressure. The residue was chromatographed on a column of silica gel with CH_2Cl_2–MeOH to give (**10**) (1.87 g, 60%) (Found: C, 60.25; H, 4.95; N, 10.15. $C_{36}H_{25}O_{19}N_5 \cdot 2H_2O$ requires C, 59.75; H, 4.04; N, 9.68%); $\delta_H(CDCl_3)$ 1.94 and 1.96 [3 H, s, diastereoisomeric $C(O)CH_3$], 3.18 and 3.21 (3 H, s, CH_3CH), 3.75 (9 H, s, $ArOCH_3$), 3.92—4.24 (3 H, m, 4′- and 5′-H), 4.56 (1 H, m, 3′-H), 4.88 (1 H, m, 2′-H), 5.68 (1 H, m, 1′-H), 5.98 (1 H, s, CH_3CH), 6.64—7.32 (12 H, m, ArH), and 7.60 (1 H, br, 8-H).

2′,3′-O-*Methoxymethylene*-N^2-(4,4′,4″-*trimethoxytrityl*)-*guanosine* (**11**).—Compound (**11**) (1.87 g, 2.67 mmol) was treated with butylamine–MeOH (1:1, v/v, 56 ml) at room temperature for 6 h. After the solvent and reagent had been removed under reduced pressure, the residue was chromatographed on a column of silica gel with CH_2Cl_2–MeOH to give (**11**) (1.30 g, 71%); R_F 0.25 (CH_2Cl_2–MeOH, 9:1, v/v) (Found: C, 61.8; H, 5.1; N, 10.35. $C_{34}H_{35}O_9N_5$ requires C, 62.1; H, 5.4; N, 10.6%); $\delta_H(CDCl_3)$ 3.25 (3 H, s, OCH_3), 3.62 (9 H, s, OCH_3), 3.98—4.30 (3 H, m, 4′- and 5′-H), 4.45—4.73 (1 H, m, 3′-H), 4.86—5.06 (1 H, m, 2′-H), 5.73 (1 H, s, 1′-H), 5.80 (1 H, s, $OCHOCH_3$), and 6.33—7.27 (13 H, m, ArH and 8-H).

S,S-*Diphenyl* N^2-(4,4′,4″-*Trimethoxytrityl*)-2′,3′-O-*methoxymethyleneguanosine* 5′-*Phosphorodithioate* (**12**).—A mixture of (**11**) (1.20 g, 1.82 mmol), cyclohexylammonium *S*,*S*-diphenyl phosphorodithioate (1.04 g, 2.73 mmol), and 1*H*-tetrazole (255 mg, 3.64 mmol) was rendered anhydrous by repeated co-evaporation with dry pyridine and finally dissolved in dry pyridine (20 ml). To the solution was added DDS (1.21 g, 3.64 mmol). After being stirred for 30 min, the mixture was partitioned between CH_2Cl_2 (150 ml) and water (100 ml). The CH_2Cl_2 layer was washed successively with water (100 ml), 5% aqueous sodium hydrogen carbonate (100 ml, × 2), and water (100 ml). Each aqueous phase was back-extracted using 50 ml aliquots of CH_2Cl_2. The combined CH_2Cl_2 extracts were dried (Na_2SO_4), filtered, and evaporated to a gum. The gummy material was chromatographed on a column of silica gel with CH_2Cl_2–MeOH to give (**12**) (1.29 g, 77%) R_F 0.39 (CH_2Cl_2–MeOH, 9:1, v/v) (Found: C, 58.4; H, 4.8; N, 7.35; S, 7.5. $C_{46}H_{44}N_5O_{10}S_2P$ requires C, 59.9; H, 4.81; N, 7.6; S, 6.95%); $\delta_H(CDCl_3)$ 3.23 (3 H, s, OCH_3), 3.63 (9 H, s, $ArOCH_3$), 4.00—4.50 (4 H, m, 3′-, 4′-, and 5′-H), 5.45 (1 H, s, $CHOCH_3$), 5.73 (1 H, m, 1′-H), 6.40—7.50 (22 H, m, ArH), and 8.50 (1 H, br, 8-H).

S-*Phenyl* N^2-(4,4′,4″-*Trimethoxytrityl*)-2′,3′-O-*methoxymethylene*-7-*methylguanosine* 5′-*Phosphorothioate* (**14**).—Compound (**12**) (49 mg, 53 μmol) was rendered anhydrous by repeated co-evaporation with dry pyridine and finally dissolved in pyridine (1.35 ml). To the solution were added 5M phosphinic acid in pyridine (0.62 ml) and triethylamine (0.18 ml, 1.28 mmol). The mixture was kept at 40 °C for 1 h and then partitioned between CH_2Cl_2–water. The CH_2Cl_2 layer was washed successively with water (10 ml), a 0.2M TEAB buffer (10 ml × 3), and water (10 ml). Each aqueous layer was back-extracted with the same CH_2Cl_2 (20 ml) in another separating funnel. The combined CH_2Cl_2 extracts were dried (Na_2SO_4), filtered, and evaporated under reduced pressure to give (**13**); $\delta_H(CDCl_3)$ 1.27 (9 H, t, *J* 7 Hz, NCH_2CH_3), 3.07 (6 H, q, *J* 7 Hz, NCH_2CH_3), 3.43 (3 H, s, $CHOCH_3$), 3.83 (3 H, s, $ArOCH_3$), 4.28—4.52 (2 H, m, 4′- and 5′-H), 4.60—4.76 (1 H, m, 3′-H), 5.26—5.36 (1 H, m, 2′-H), 5.88—6.04 (2 H, m, 1′-H and CH_3OCH), 6.80—6.96 (6 H, m, ArH), 7.12—7.44 (11 H, m, ArH), and 8.10 (1 H, s, 8-H). The residue containing (**13**) was dissolved in DMF (2.5 ml) and methyl iodide (48 μl, 0.77 mmol) was added. After being stirred at room temperature for 2 h, sodium dihydrogen phosphate dihydrate (18 mg, 0.11 mmol) was added since t.l.c. suggested formation of the ring opening by-product. The mixture was vigorously stirred for an additional 20 h and then extracted with CH_2Cl_2–H_2O. The aqueous layer was extracted with CH_2Cl_2 (20 ml × 4). The CH_2Cl_2 extracts were combined, dried (Na_2SO_4), filtered, and evaporated under reduced pressure. The residue was dissolved in CH_2Cl_2–MeOH (9:1, v/v) and applied to a preparative t.l.c. plate (Merck 5717) developed with CH_2Cl_2–MeOH (4:1, v/v). Elution of the strongest u.v. band with CH_2Cl_2–MeOH followed by evaporation gave (**14**) (38 mg, 85%), R_F 0.50 (CH_2Cl_2–MeOH, 4:1, v/v) (Found: C, 57.05; H, 4.8; N, 8.1; S, 4.05.

$C_{41}H_{42}O_{11}N_5PS{\cdot}H_2O$ requires C, 57.15; H, 5.15; N 8.15; S, 3.7%); $\delta_H(CDCl_3)$ 3.23 and 3.27 (3 H, s, $CHOCH_3$), 3.66 (3 H, s, N^+CH_3), 3.76 (3 H, s, $ArOCH_3$), 4.00—4.38 (3 H, m, 4′- and 5′-H), 4.61—4.75 (1 H, m, 3′-H), 4.93—5.10 (1 H, m, 2′-H), 5.62 (1 H, s, 1′-H), 5.74 and 5.87 (1 H, s, $CHOCH_3$), 6.76—6.88 (6 H, m, ArH), 7.08—7.32 (11 H, m, ArH), 8.52 (1 H, br, 8-H), and 9.12 (1 H, br, NH).

2′,3′-O-*Methoxymethylene*-N^2-(4,4′,4″-*trimethoxytrityl*)-7-*methylguanosine*-5′-*Diphosphate* (**15**).—Phosphoric acid (17.1 μl, 0.25 mmol) was dissolved in pyridine (1 ml) containing a small amount of water and tributylamine (60 μl, 0.25 mmol) was added. The tributylammonium phosphate was co-evaporated several times with dry pyridine and finally dissolved in dry pyridine (1.5 ml). This solution was added to (**14**) (21.1 mg, 0.025 mmol), which had been dried by repeated co-evaporation with dry pyridine, and silver nitrate (126 mg, 0.75 mmol) was added. The solution was stirred at room temperature for 1 h in the dark and then diluted with pyridine–water (2:1, v/v; 10 ml). Through the solution was bubbled hydrogen sulphide for 10 min. The excess gas was removed by evaporation under reduced pressure. The precipitate was removed by centrifugation and the supernatant was concentrated. The concentrate was dissolved with saturated brine and extraction was performed with CH_2Cl_2–BuOH (5:2, v/v, ×4). The extracts were combined, dried (Na_2SO_4), filtered, and evaporated to dryness under reduced pressure. The residue was used for the subsequent triphosphate bond formation without further purification.

Synthesis of Partially Protected Hexaribonucleotide Derivative (**4**).—To a solution of the fully protected hexaribonucleotide derivative (**2**) (76.4 mg, 14.8 μmol) in pyridine–water (4:1, v/v; 3 ml) was added silver acetate (741 mg, 4.44 mmol). The mixture was stirred at 50 °C for 6 h, cooled, and diluted with pyridine–water (2:1, v/v; 10 ml). Hydrogen sulphide was bubbled through the solution at 0 °C for 10 min. Excess gas was removed by evaporation, the supernatant was passed through Dowex 50W ×2 (pyridinium form, 5 ml), and the resin was washed with pyridine–water (3:1, v/v, 100 ml). The eluate and washing were combined, evaporated under reduced pressure, and co-evaporated several times with pyridine. The residue was dissolved in pyridine–concentrated aqueous ammonia (1:1; 114 ml) and the solution was kept in a sealed flask at 50 °C for 24 h. The solution was diluted with pyridine (60 ml) and concentrated under reduced pressure. The concentrate was partitioned between water and ether. The aqueous layer was collected, concentrated in the presence of a two-fold volume of pyridine to avoid loss of the THP group, and was chromatographed on Whatman 3MM papers with propan-2-ol–concentrated aqueous ammonia–water (6:1:3, v/v). The slowest moving band was eluted with water to give the ammonium salt of (**4**) (481 A_{259}, 54%), $\lambda_{max.}(H_2O)$ 259 nm (ε 60.6 × 10^3), and $\lambda_{min.}$ 232 nm.

The Capping Reaction of (**4**) *with* (**5**) *in the Presence of Imidazole*.—A mixture of the capping reagent (**5**) (29.5 μmol), (**4**) (trioctylammonium salt, 2.95 μmol), and imidazole (40.2 mg, 590 μmol) was rendered anhydrous by repeated co-evaporation with dry pyridine and was finally dissolved in dry DMF (0.7 ml). Silver nitrate (150 mg, 885 μmol) was added to the solution and the solution was stirred at room temperature for 66 h. Work-up as described in the above experiment followed by treatment with 0.01M HCl in dioxane–water (1:1, v/v, 15 ml) at room temperature for 24 h gave the deprotected material. After neutralisation of the acidic solution with 1M aqueous ammonia, the solution was evaporated under reduced pressure and partitioned between water and ether. The aqueous solution was collected, washed with ether (×2), and evaporated under reduced pressure. A quarter of the mixture was separated by anion-exchange h.p.l.c. A peak at 40.5 min was pooled and desalted with DEAE Sephadex A-25 in the usual manner to give $m^7G^{5'}$pppGUAUUA (1.04 A_{257}, 2.1%), $\lambda_{max.}(H_2O)$ 257 nm (ε 67.2 × 10^3), and $\lambda_{min.}$ 238 nm.

Enzymatic Assay of $m^7G^{5'}$pppGUAUUA *Obtained by the Reaction of* (**4**) *with* (**5**).—The capped hexaribonucleotide (0.5 A_{257}) (freeze-dried) was incubated with snake venom phosphodiesterase (1 mg ml^{-1}, 4 μl) in the presence of 1M $MgCl_2$ (2 μl) in Tris-HCl buffer (pH 8.0, 40 μl) at 37 °C for 14 h. Reverse-phase h.p.l.c. showed a mixture of pm^7G, pG, pU, and pA in a ratio of 0.85:1.3:3.00:1.41.

Synthesis of the 5′-Terminal Phosphorimidazolidate Derivative of the Partially Protected Hexaribonucleotide (**16**).—Compound (**4**) (303 A_{259}, ammonium salt, 5 μmol) was converted into the pyridinium salt on Dowex 50W ×2 (pyridinium form, 5 ml) eluted with pyridine–water (4:1, v/v, 50 ml). The eluate was evaporated under reduced pressure and the residue was dissolved in pyridine–water (2:1, v/v, 1 ml) containing trioctylamine (13.1 μl, 30 μmol). The solution was evaporated and co-evaporated with dry pyridine (×5). The residue was dissolved in dry DMF (500 μl) and carbonyldi-imidazole (8.1 mg, 50 μmol) was added. The mixture was stirred for 30 min. Additional carbonyldi-imidazole (8.1 mg, 50 μmol) was added and stirring was continued for 30 min. Methanol (6.5 μl, 160 μmol) was added and the mixture was stirred for 30 min. The solution was evaporated under reduced pressure. The resulting residue was used for the next capping reaction without further purification.

The Capping Reaction of (**16**) *with* (**15**).—Compound (**15**) (25 μmol) was rendered anhydrous by repeated co-evaporation with dry pyridine and dissolved in dry DMF (0.6 ml). This solution was added to compound (**16**) obtained in the above experiment. After being stirred at room temperature for 21 h, the mixture was quenched by addition of water and then evaporated under reduced pressure. The residue was co-evaporated several times with toluene–methanol (1:1, v/v) and 0.01M HCl in dioxan–water (1:1.5, v/v, 12.5 ml) was added. The solution was brought to pH 2.0 by addition of 0.1M HCl. After being stirred at room temperature for 25 h, the mixture was neutralised with 1M aqueous ammonia. The solution was evaporated under reduced pressure and the residue was dissolved in sterilised water (10 ml). The solution was applied to a column of DEAE Sephadex A-25 (ammonium form, 20 ml). Elution was performed successively with sterilised water (100 ml), 0.3M NH_4HCO_3 (200 ml), and 1M NH_4HCO_3 (100 ml). The u.v.-active fractions, obtained when 1M NH_4CO_3 was used for elution, were combined, evaporated under reduced pressure, and co-evaporated several times with sterilised water to give crude $m^7G^{5'}$pppGUAUUA (211 $A_{258.5}$, 61%), $\lambda_{max.}(H_2O)$ 258.5 nm (ε 69.4 × 10^3, and $\lambda_{min.}$ 230 nm.

Part of the crude capped hexaribonucleotide (70 O.D. at 258.5 nm) was purified by anion-exchange h.p.l.c. using a Whatman Partisil 10-SAX column. A peak at 37 min was collected and further desalted with DEAE Sephadex A-25 in the usual manner. This purification gave the pure material [18.6 $A_{258.5}$, recovery: 27%; overall yield from (**4**), 16%].

Enzymatic Assay of $m^7G^{5'}$pppGUAUUA.—The capped hexaribonucleotide (2 $A_{258.5}$), which was freeze-dried, was incubated with snake venom phosphodiesterase (1 mg, ml^{-1}, 8 μl) in the presence of 1M $MgCl_2$ (2 μl) in Tris-Hcl buffer (pH 8.0, 80 μl) at 37 °C for 5 h. Reverse-phase h.p.l.c. of the mixture showed a mixture of pm^7G pG, pU, and pA in a ratio of 0.90:1.06:3.00:1.63.

The same sample (2 $A_{258.5}$) was incubated with nuclease P_1

(2 mg ml^{-1}, 20 μl) in 0.05M sodium acetate buffer (pH 5.4, 180 μl) at 37 °C for 5 h. Anion-exchange h.p.l.c. of the mixture showed a mixture of $m^7G^5'pppG$, pU, and pA in the ratio of 1.00:3.19:1.79.

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