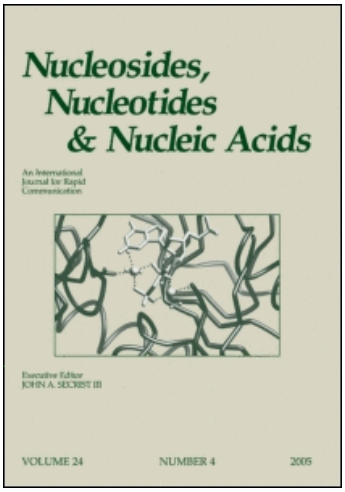

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LARGE SCALE SYNTHESIS OF THE CAP PART IN MESSENGER RNA USING A NEW TYPE OF BIFUNCTIONAL PHOSPHORYLATING REAGENT

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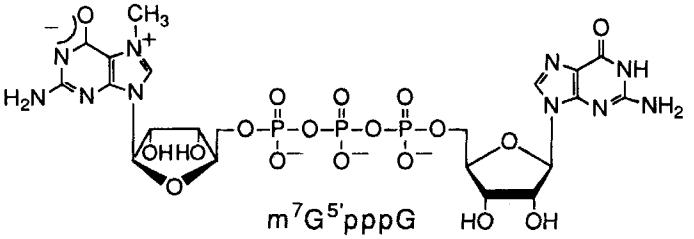
Abstract: Bifunctional phosphorylating reagents **1** and **2** were employed for the synthesis of the cap part, m^7G^5pppG, from guanosine 5'-phosphates on a large scale without any protecting groups.

Eukaryotic mRNAs bear the cap structure at their 5'-termini, which are well known to play an important role in translation. The cap structure consists of P^1-guanosine-5'-yl P^3-7-methylguanosine-5'-yl triphosphate (m^7G^5pppG). m^7G^5pppG has been used as the primer in transcription with T7 or SP6 RNA polymerase *in vitro* to obtain the RNAs having a cap structure in their 5'-termini.[1] This 5'-capped RNA has been currently used for studies on the translation mechanism. When m^7G^5pppG can be obtained on a large



scale, studies on translation will be more rapidly developed. However, m^7G^5pppG is hardly available from the enzymatically degraded products of mRNAs. Therefore, development of a large scale chemical synthesis process of m^7G^5pppG is needed. But adequate chemical methods for the synthesis of P^1, P^3-dinucleoside 5'-triphosphates such

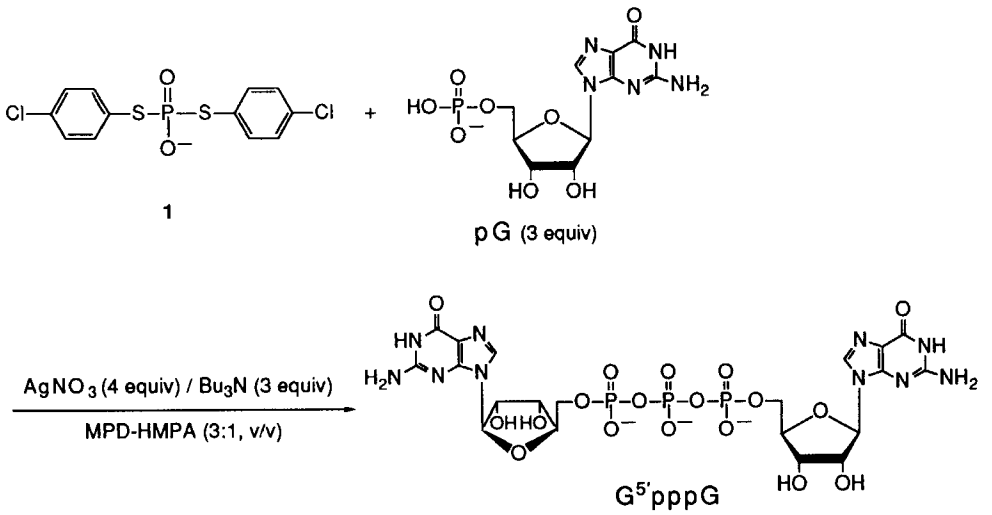
This paper is dedicated to Professor Morio Ikehara on the occasion of his 70th birthday.

as the cap part have not yet been well established. Because most of the reported methods[2] have involved more than two steps using appropriately protected nucleotides, the deprotected P^1, P^3-dinucleoside 5'-triphosphates have not been obtained on a large scale due to problems in its separation from the side products. In this paper, two simple methods for the large scale synthesis of P^1, P^3-dinucleoside 5'-triphosphates including the cap part without any protecting groups for the starting nucleotides are described.

Unprotected guanosine 5'-phosphate (pG) and 7-methylguanosine 5'-phosphate (pm^7G) are poorly soluble in pyridine and dimethylformamide which have been used for oligonucleotide synthesis. In order to overcome this problem, many kinds of aprotic polar solvents were examined based on the solubility of pG and pm^7G. Finally, a mixture of 1-methylpyrrolidone (MPD)-HMPA (3:1, v/v) was found to be suitable for this purpose.

The synthesis of $m^7G^{5'}pppG$ seemed to be achieved *via* the synthesis of P^1, P^3-diguanosine 5'-triphosphate ($G^{5'}pppG$) followed by the methylation of $G^{5'}pppG$ at the N^7-position. Miura[3] has reported that the CD spectrum of $m^7G^{5'}pppA$ showed a confronting base-stacking conformation. The intramolecular stacking might also be expected in the case of guanosine instead of adenosine since the guanine moiety is known as a typical electron-donating heterocycle. When one of the guanine moieties of $G^{5'}pppG$ is methylated at the N^7-position to give $m^7G^{5'}pppG$, the methylated electron-deficient m^7G and the electron-donating G might be close enough to form a sandwich-like structure. Consequently, the electron-donating feature of the confronting guanosine would cause a decrease in further methylation of the confronting guanosine and the formation of $m^7G^{5'}pppm^7G$ might be retarded.

First, the synthesis of $G^{5'}pppG$ was tried. In our laboratory *S*, *S'*-diphenyl phosphorodithioate has been frequently used for oligonucleotide synthesis by means of the phosphotriester approach.[4] The P-S bonds of the phosphorodithioate can be cleaved very smoothly by the addition of silver ion at room temperature in the presence of water under neutral conditions. Therefore, the phenylthio group has been employed not only as a protecting group in oligonucleotide synthesis but also as a leaving group in the substitution reaction with phosphate derivatives to form compounds having a P-O-P bond.[5] Since *S*, *S'*-diaryl phosphorodithioates might be regarded as bifunctional phosphorylating reagents having two arylthio groups, which are activated with silver ion, the symmetrical P^1, P^3-dinucleoside 5'-triphosphate, $G^{5'}pppG$ would be prepared by the one-pot reaction of *S*,*S'*-diaryl phosphorodithioate with pG in the presence of silver nitrate. To find a suitable arylthio group, several phosphorodithioates were tested, and finally *S*, *S'*-bis(4-chlorophenyl) phosphorodithioate **1**[6] was found to be the most suitable for the synthesis of $G^{5'}pppG$.



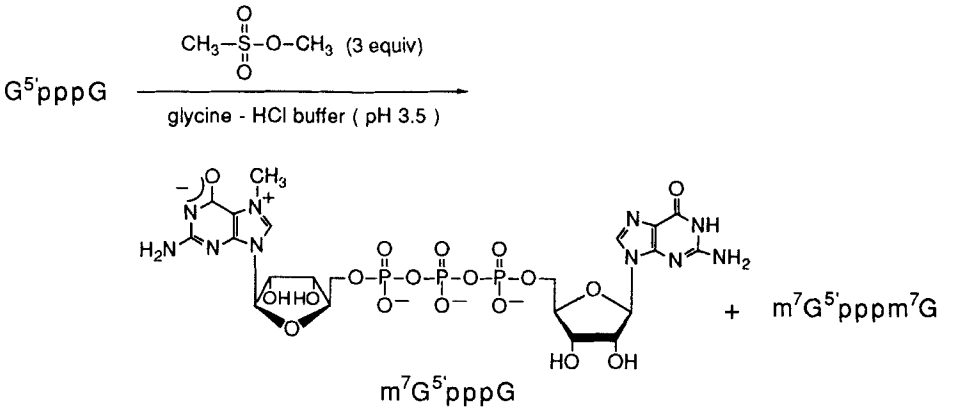
Scheme 1

When a mixture of **1** and 3 equiv of pG was allowed to react with 4 equiv of silver nitrate in MPD-HMPA (3:1, v/v) at 0 °C for 5 h and then allowed to stand at room temperature for 2 h, $G^{5'}$pppG was isolated in 71% yield using DEAE Sephadex A-25 column chromatography (Scheme 1). Although the 4-chlorophenylthio group of **1** could be activated in MPD without the addition of HMPA, small amounts of P^1, P^2-diguanosine 5'-diphosphate ($G^{5'}$ppG) and guanosine 5'-diphosphate (ppG) were formed and the desired $G^{5'}$pppG could not be separated from the side products. Therefore, the addition of HMPA was essential and remarkably effective to avoid the formation of any side products.

In a similar manner, $A^{5'}$pppA was obtained in 80% yield (135 mg) based on **1** after the purification procedure as described above.

Next, the N^7-methylation of $G^{5'}$pppG was performed to obtain $m^7G^{5'}$pppG. Since the formation of the stacking between m^7G and G might be effective in polar solvents, the methylation of $G^{5'}$pppG was carried out in aqueous solution.

When $G^{5'}$pppG was treated with 3 equiv of methyl methanesulfonate in a buffer solution of 1.0 M glycine - HCl (pH 3.5) at room temperature for 4 d, $m^7G^{5'}$pppG was obtained along with a recovery of unreacted $G^{5'}$pppG and per-methylated $m^7G^{5'}$pppm^{7}G (FIG. 1). $m^7G^{5'}$pppG was isolated by DEAE Sephadex A-25 column chromatography in 37% yield.



Scheme 2

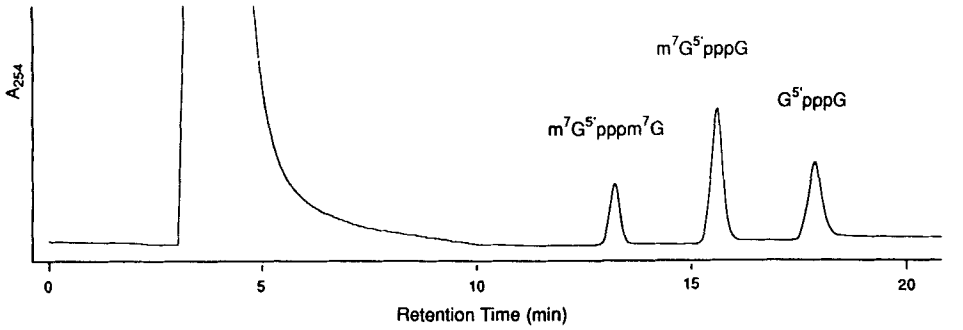


FIG. 1 Anion exchange HPLC profile of the reaction mixture

Such a post-methylation strategy for the synthesis of $m^7G^{5'}pppG$ as described above has resulted in undesirable side products and it was difficult to control the selective methylation of $G^{5'}pppG$. In view of the results so far achieved, pm^7G should be employed in the one-pot reaction using **1** to eliminate the crucial problems from the post-methylation reaction. However, the reactivity between two arylthio groups of **1** could not be easily distinguished even when each of the nucleotides, pG and pm^7G, were carefully added step by step. Therefore, a new type of bifunctional activatable phosphorylating reagent, which has two different leaving groups activated by different methods, was required. We reported that 5-chloroquinolyl-8-oxy group could be activated by copper(II) ion.[7] If a bifunctional phosphorylating reagent which carried an arylthio group and a 8-quinolyloxy group could

be synthesized, it could be selectively activated; the former by silver ion and the latter by copper(II) ion. According to this assumption, *O*-8-(5-chloroquinolyl) *S*-phenyl phosphorothioate **2** was designed as a new type of bifunctional phosphorylating reagent for the synthesis of $m^7G^{5'}pppG$ in order to eliminate the crucial methylation process.

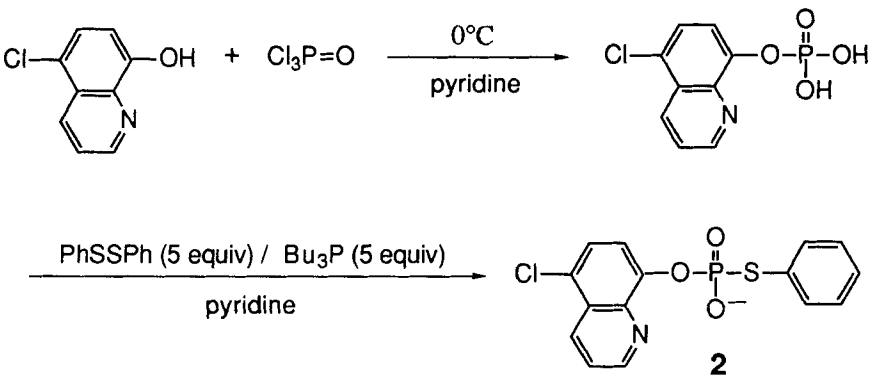
A new type of bifunctional phosphorylating reagent **2** was synthesized as shown in Scheme 3: When a mixture of one equiv of *O*-8-(5-chloroquinolyl) phosphate[8] and 5 equiv of diphenyl disulfide in dry pyridine was treated with 5 equiv of tributylphosphine[9] at room temperature for 2 h, **2** was obtained in 85% yield as a white powder. It was stable enough to store in a refrigerator.

Compound **2** was used for the synthesis of $m^7G^{5'}pppG$. To a solution of one equiv of **2** in the presence of a stoichiometric amount of pG in MPD-HMPA (3:1, v/v), a solution of 1.2 equiv of silver nitrate in dry pyridine was added at room temperature for 30 min. Compound **2** was activated and reacted with pG to form **3** and AgSPh. By monitoring the reaction mixture by ^{31}P NMR and anion exchange HPLC, the intermediate **3** was found to be quantitatively formed and be stable in solution (Scheme 4).

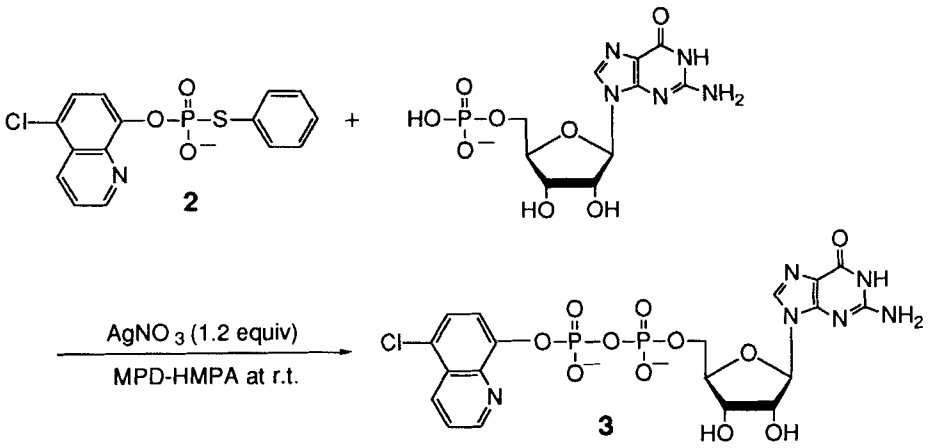
Without isolating **3**, the reaction mixture was further treated with 2.3 equiv of pm^7G and 5.1 equiv of anhydrous copper(II) chloride in MPD-HMPA at room temperature for 24 h. $m^7G^{5'}pppG$ was isolated in 57% yield(108 mg) by means of DEAE Sephadex A-25 column chromatography (Scheme 5). This method gave $m^7G^{5'}pppG$ in a larger amount than the known methods. When the 5-chloroquinolyl-8-oxy group of **3** was activated at a high temperature (60 ℃) for 1 h, the yield of the desired $m^7G^{5'}pppG$ was decreased. When **2** was first treated with copper(II) chloride then with silver nitrate, P^1-*S*-phenyl P^2-*O*-guanosine 5'-diphosphorothioate and **3** were formed due to the activation of both the 5-chloroquinolyl-8-oxy group and phenylthio group. Therefore, the order of addition of the metal salts is important for the synthesis of $m^7G^{5'}pppG$. In a similar manner, when adenosine 5'-phosphate(pA) was employed in place of pG, $m^7G^{5'}pppA$ was obtained in 53% yield based on **2** after purification.

The 5-chloro substituent of the quinolyl group of **2** was found to be effective for the substitution reaction with pm^7G in the presence of copper(II) chloride. For example, non-substituted *S*-phenyl *O*-8-quinolyl phosphorothioate was prepared and applied to the synthesis of $m^7G^{5'}pppG$ in the same manner. However, the activation of the 8-quinolyloxy group by means of anhydrous copper(II) chloride was sluggish and $m^7G^{5'}pppG$ was obtained in ca. 50% yield. In addition, an attempt to prepare the 5, 7-dichloroquinolyl-8-oxy derivative of the corresponding phosphorothioate was unsuccessful because of its instability.

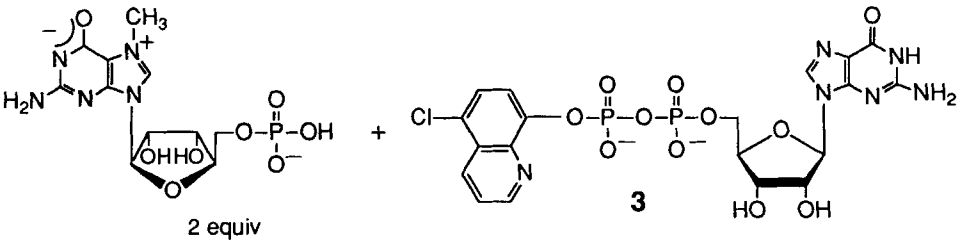
In conclusion, it is emphasized that pG and pm^7G could be dissolved in a mixture of MPD-HMPA. Bifunctional and activatable phosphorylating reagents **1** and **2** were



Scheme 3



Scheme 4



Scheme 5

prepared and they were stable enough during storage. The cap part and the related triphosphates such as $m^7G^{5'}pppG$, $m^7G^{5'}pppA$, $G^{5'}pppG$ and $A^{5'}pppA$ were prepared as white powders in good yields on a large scale using **1** or **2** in a one-pot reaction without any protecting groups for the starting nucleotide and all the reactions proceeded at ordinary temperature under neutral conditions.

EXPERIMENTAL

^1H NMR spectra were recorded at 270.05 MHz on a JEOL JNM-EX 270 spectrometer. ^1H chemical shifts were given in ppm (δ) relative to tetramethylsilane(TMS) as internal standard in $CDCl_3$ or relative to sodium 3-(trimethylsilyl) propanesulfonate as external standard in D_2O. ^{13}C NMR and ^{31}P NMR spectra were measured using a JEOL JNM-EX 270 spectrometer at 67.80 MHz and 109.25 MHz, respectively. ^{13}C chemical shifts were given in ppm (δ) relative to TMS as internal standard. ^{31}P chemical shifts were given in ppm (δ) relative to 85% H_3PO_4 as external standard. Thin layer chromatography was performed on precoated TLC plates Silica Gel 60F-254(Merck). Anion exchange HPLC was performed using a Waters LC Module I apparatus equipped with a Waters 470 scanning fluorescence detector and a Waters 741 data module. Analysis of P^1, P^3-dinucleoside 5'- triphosphates such as $G^{5'}pppG$, $A^{5'}pppA$, $m^7G^{5'}pppG$ and $m^7G^{5'}pppA$ was performed on a Whatman Particil 10 SAX column(25 cm) using the following solvent system; system A, a linear gradient of 5 mM KH_2PO_4 (20% CH_3CN, pH 4.1) to 0.5 M KH_2PO_4 (20% CH_3CN, pH 4.5) for 30 min; flow rate, 1 ml/min; system B, a linear gradient of 5 mM KH_2PO_4 (20% CH_3CN, pH 4.1) to 0.5 M KH_2PO_4 (20% CH_3CN, pH 4.5) for 20 min; flow rate, 1 ml/min. Chromatography on DEAE Sephadex A-25(3 x 43 cm, HCO_3^- form) was carried out at 4 °C at a flow rate of 2.5 ml/min. Pyridine was distilled from *p*-toluenesulfonyl chloride and redistilled from calcium hydride and then stored over molecular sieves(4A). Toluene was distilled from calcium hydride and stored over molecular sieves(4A). 1-Methylpyrrolidone(MPD) was distilled and stored over molecular sieves(4A). HMPA was distilled from calcium hydride and stored over molecular sieves(4A). *O*-8-(5-chloroquinolyl) phosphate was prepared according to the literature procedure.[8]

Preparation of *S*, *S'*-bis(4-chlorophenyl) phosphorodithioate (1). Compound **1** was prepared according to a modification of the procedure of Yamaguchi.[6] m.p. 197.5-210.0 °C (decomp.) ^1H NMR (DMSO-d_6) 7.70-7.87 (br s, 3 H), 7.50 (d, J = 8.1 Hz, 4 H), 7.30 (d, J = 8.1 Hz, 4 H), 2.80-2.96 (br s, 1 H), 1.50-1.94 (m, 5 H), 0.97-1.35 (m, 5 H). ^{13}C NMR (DMSO-d_6) 134.3, 133.2, 131.5, 128.2, 49.3, 30.3, 24.5, 23.7. ^{31}P NMR (DMSO-d_6) 21.75(s).

Preparation of *O*-8-(5-chloroquinolyl) *S*-phenyl phosphorothioate (2). A mixture of *O*-8-(5-chloroquinolyl) phosphate (1.0 g, 3.9 mmol) and diphenyl disulfide (3.4 g, 15.4 mmol) was coevaporated three times with dry pyridine and finally dissolved in dry pyridine (50 ml). To the solution was added tributylphosphine (3.8 ml, 15.4 mmol). After being stirred for 2 h, the mixture was treated with water (500 µl) and then concentrated under reduced pressure. The oily residue was dissolved in CH_2Cl_2 (10 ml) and then cyclohexylamine (2.2 ml, 19.3 mmol) and CH_3CN (10 ml) were added. The solution was concentrated and a precipitate appeared. It was collected by filtration and dissolved in $CHCl_3$ (80 ml). The solution was washed with sat. $NaHCO_3$aq (50 ml). The organic phase was dried over $MgSO_4$, filtered, and concentrated. The residue was dissolved in CH_2Cl_2 (10 ml) and CH_3CN (10 ml) was added. The solution was concentrated and a precipitate appeared. It was collected by filtration. The monocyclohexylammonium salt of **2** was obtained in 85% yield(1.47 g). m.p. 150-151 °C (decomp.) ^{1}H NMR ($CDCl_3$) 8.61 (br s, 3 H), 8.40 (dd, J = 1.3, 4.3 Hz, 1 H), 8.23 (dd, J = 1.3, 8.6 Hz, 1 H), 7.72 (d, J = 8.6 Hz, 1 H), 7.41 (dd, J = 1.3, 6.8 Hz, 2 H), 7.36 (d, J = 8.6 Hz, 1 H), 7.05-7.22 (m, 4 H), 2.63 (br s, 1 H), 0.92-1.85 (m, 10 H). ^{13}C NMR ($CDCl_3$) 148.9, 147.4, 140.0, 134.9, 133.2, 130.4, 128.5, 127.7, 126.7, 126.4, 124.2, 121.7, 117.5, 50.1, 31.6, 25.1, 24.7. ^{31}P NMR ($CDCl_3$) 11.98(s). MS(FAB) calcd. for $(M+H)^+$ 451.1012, found 451.0995.

Synthesis of G$^{5'}$pppG. A mixture of **1** (130 mg, 0.2 mmol) and the pyridinium salt of pG (240 mg, 0.6 mmol) was coevaporated three times with dry pyridine and finally dissolved in dry MPD-HMPA (3:1, v/v, 6 ml). To the solution was added tributylamine (142 µl, 0.6 mmol). A solution of silver nitrate (135 mg, 0.8 mmol) in dry pyridine (3.5 ml) was then added dropwise at 0 °C. The solution was stirred at 0 °C for 5 h and then allowed to stand at room temperature for 2 h. After the addition of water (50 ml), the precipitate was filtered off and the filtrate was washed five times with chloroform. Hydrogen sulfide was bubbled into the aqueous solution with continuous stirring to form Ag_2S. After removal of Ag_2S by filtration, the filtrate was concentrated. The residue was applied to a DEAE Sephadex A-25 column. The elution was performed with 0.30 M NH_4HCO_3 for 7 h and then with a linear gradient of 0.30 - 0.85 M NH_4HCO_3 for 8 h. Fractions containing G$^{5'}$pppG were collected and concentrated to dryness. Ammonium salt of G$^{5'}$pppG was obtained in 71% yield (117 mg) as a white powder. ^{1}H NMR (D_2O) 8.02 (s, 2 H), 5.79 (d, J = 4.9 Hz, 2 H), 4.61 (t, J = 5.0 Hz, 2 H), 4.45 (t, J = 4.3 Hz, 2 H), 4.17-4.35 (m, 6 H). ^{31}P NMR (D_2O) -10.84 (d, J = 19.4 Hz, P^1 and P^3), -22.42 (t, J = 19.4 Hz, P^2).

Synthesis of A$^{5'}$pppA. A mixture of **1** (130 mg, 0.2 mmol) and the pyridinium salt of pA (230 mg, 0.6 mmol) was coevaporated three times with dry pyridine and finally dissolved

in dry MPD - HMPA (3:1, v/v, 6 ml). To the solution was added tributylamine (142 μl, 0.6 mmol) and then a solution of silver nitrate (135 mg, 0.8 mmol) in dry pyridine (3.5 ml) was added dropwise at 0 °C. The mixture was stirred at 0 °C for 3 h and then allowed to stand at room temperature for 2 h. After the addition of water (50 ml), the precipitate was filtered off and the filtrate was washed five times with chloroform. Hydrogen sulfide was bubbled into the aqueous solution with continuous stirring to form Ag_2S. After the removal of Ag_2S by filtration, the filtrate was concentrated. The residue was applied to a DEAE Sephadex A-25 column. The elution was performed with 0.20 M NH_4HCO_3 for 7 h and then with a linear gradient of 0.20 - 0.65 M NH_4HCO_3 for 6 h. Fractions containing $A^{5'}$pppA were collected and concentrated to dryness. Ammonium salt of $A^{5'}$pppA was obtained in 80% yield(135 mg) as a white powder. ^{1}H NMR (D_2O) 8.27 (s, 2 H), 8.04 (s, 2 H), 5.93 (d, J = 4.6 Hz, 2 H), 4.55(t, J = 4.9 Hz, 2 H), 4.44 (t, J = 4.3 Hz, 2 H), 4.20-4.36 (m, 6 H). ^{31}P NMR (D_2O) -10.84 (d, J = 19.4 Hz, P^1 and P^3), -22.32 (t, J = 19.4 Hz, P^2).

N^7-Methylation of $G^{5'}$pppG using methyl methanesulfonate. $G^{5'}$pppG (114 mg, 0.14 mmol) was dissolved in 1.0 M glycine - HCl buffer (pH 3.5, 3.6 ml). To a solution was added methyl methanesulfonate (35 μl, 0.41 mmol) at room temperature. After being stirred for 4 days, pyridine was added to the solution and then the solution was concentrated. The residue was applied to a DEAE Sephadex A-25 column. The elution was performed with a linear gradient of 0.10 - 0.30 M NH_4HCO_3 for 9 h. Fractions containing $m^7G^{5'}$pppG were collected and concentrated to dryness. Ammonium salt of $m^7G^{5'}$pppG was obtained in 37% yield (43 mg) as a white powder.

Synthesis of $m^7G^{5'}$pppG. The pyridinium salt of pG (200 mg, 0.50 mmol) was coevaporated twice with dry pyridine and then once with dry toluene and finally dissolved in dry MPD (10 ml). To the solution was added methyl iodide (124 μl, 2.00 mmol). After being stirred for 8 h, methyl iodide was removed under reduced pressure and then a small amount of pyridine (500 μl) was added to the solution. pm^7G was formed quantitatively. The solution was then used without further purification.

A mixture of **2** (100 mg, 0.22 mmol) and the pyridinium salt of pG (89 mg, 0.22 mmol) was coevaporated three times with dry pyridine and finally dissolved in dry MPD - HMPA (3:1, v/v, 15 ml). To the solution was added dry triethylamine (60 μl, 0.44 mmol) and then a solution of silver nitrate (45 mg, 0.26 mmol) in dry pyridine (1 ml) was added at room temperature. After being stirred for 30 min, the solution of pm^7G prepared above was added. A solution of anhydrous $CuCl_2$ (150 mg, 1.12 mmol) in MPD (2 ml) was then added. The mixture was stirred at room temperature for 24 h. 4-Chlorobenzenethiol (630

mg, 4.36 mmol) was added to the solution to scavenge any excess Ag^+ and Cu^{2+}. After the addition of water, the precipitate was filtered off and the filtrate was washed with chloroform. A slight amount of copper(II) ion was completely removed by Dowex 50W-X2 (H^+ form). The eluent was concentrated and the residue was applied to a DEAE Sephadex A-25 column. The elution was performed with 0.10 M NH_4HCO_3 for 1 h and then with a linear gradient of 0.10 - 0.25 M NH_4HCO_3 for 7 h. Fractions containing $m^7G^{5'}pppG$ were collected and concentrated to dryness. Ammonium salt of $m^7G^{5'}pppG$ was obtained in 57% yield (108 mg) as a white powder. ^1H NMR (D_2O) 7.99 (s, 1 H), 5.88 (d, J = 3.3 Hz, 1 H), 5.78 (d, J = 6.3 Hz, 1 H), 4.65 (t, J = 5.6 Hz, 1 H), 4.52 (t, J = 3.6 Hz, 1 H), 4.20-4.48 (m, 8 H), 4.03 (s, 3 H). ^{31}P NMR (D_2O) -10.93 (d, J = 18.4 Hz, P^1 and P^3), -22.50 (br s, P^2).

Synthesis of $m^7G^{5'}pppA$. A mixture of **2** (100 mg, 0.22 mmol) and the pyridinium salt of pA (85 mg, 0.22 mmol) was coevaporated three times with dry pyridine and finally dissolved in dry MPD - HMPA (3:1, v/v, 15 ml). To the solution was added dry triethylamine (60 µl, 0.44 mmol) and then a solution of silver nitrate (45.0 mg, 0.26 mmol) in dry pyridine (1 ml) was added at room temperature. After being stirred for 30 min, the solution of previously prepared pm^7G was added. A solution of anhydrous $CuCl_2$ (150 mg, 1.12 mmol) in MPD (2 ml) was then added. The mixture was stirred at room temperature for 24 h. 4-Chlorobenzenethiol (630 mg, 4.36 mmol) was added to the solution. After the addition of water, the precipitate was filtered off and the filtrate was washed with chloroform. A slight amount of copper(II) ion was completely removed by Dowex 50W-X2 (H^+ form). The eluent was concentrated and the residue was applied to a DEAE Sephadex A-25 column. The elution was performed with 0.10 M NH_4HCO_3 for 1 h and then with a linear gradient of 0.10 - 0.30 M NH_4HCO_3 for 9 h. Fractions containing $m^7G^{5'}pppA$ were collected and concentrated to dryness. Ammonium salt of $m^7G^{5'}pppA$ was obtained in 53% yield (98 mg) as a white powder. ^1H NMR (D_2O) 8.42 (s, 1 H), 8.19 (s, 1 H), 6.01 (d, J = 5.9 Hz, 1 H), 5.87 (d, J = 3.6 Hz, 1 H), 4.66 (t, J = 5.6 Hz, 1 H), 4.22-4.54 (m, 9 H), 4.00 (m, 3 H). ^{31}P NMR (D_2O, 109.25 MHz) -10.88 (d, J = 17.0 Hz, P^1 and P^3), -22.46 (t, J = 19.4 Hz, P^2).

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