A Convenient Method for the Synthesis of P^1-(7-Methylguanosine-5') P^2-(Ribonucleoside-5') Diphosphates

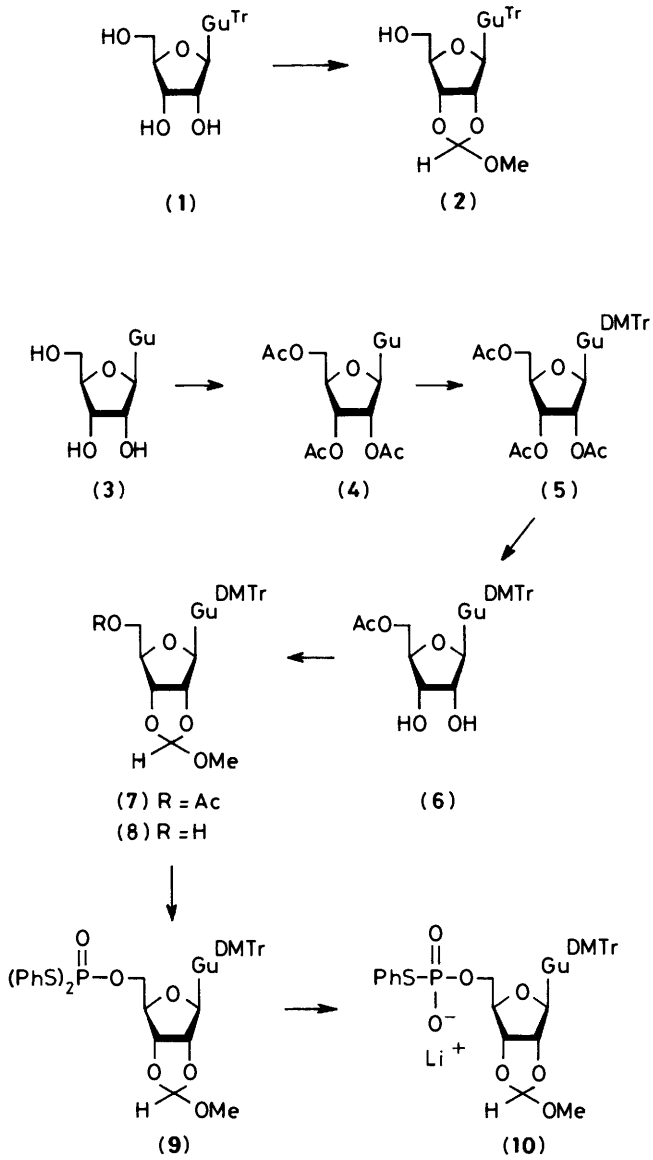
Mitsuo Sekine, Takashi Kamimura, and Tsujiaki Hata*
Department of Life Chemistry, Tokyo Institute of Technology, Nagatsuta, Midoriku, Yokohama 227, Japan

Chemical synthesis of m^{7}G^5'ppNu (Nu = A, G, C, and U) has been achieved by condensation of the protected capping agent (**11**) with the four common stannylated ribonucleotides (**12a—d**) in the presence of iodine as activating agent, followed by removal of all the acid-labile protecting groups with 80% acetic acid.

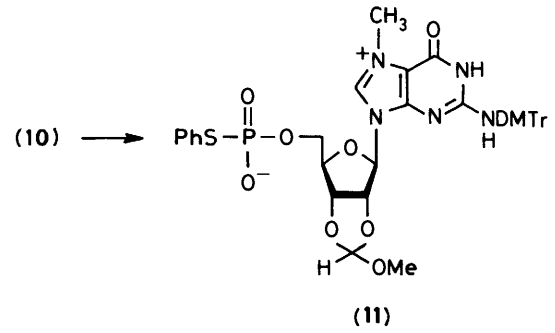
Eukaryotic mRNAs are known to possess a common 'cap' structure of m^{7}G^5'pppNupNupNup ... at their 5'-end. The biological function of the cap structure has been studied in a number of laboratories.[1–12] Recently, the correlation between the cap structure and the number of bridged phosphates has been discussed with respect to the *in vitro* methylation and inhibitory effects on peptide synthesis.[13,14] In connection with these studies, we needed m^{7}G^5'ppNu (Nu = ribonucleoside) lacking one phosphate group compared with the natural cap structure. Although we have previously reported a method for the synthesis of m^{7}G^5'pppG and m^{7}G^5'pppA by using unprotected nucleotide derivatives,[15,16] the method was not reproducible owing to the extreme instability of the unprotected capping reagent, m^{7}G^5'ppSPh, which often decomposed during purification. Therefore, we have studied a new synthetic route to m^{7}G^5'ppNu, which can be prepared through the usual chromatographic separation on silica gel except for the final deprotection and isolation stages.

In order to monitor the reactions easily, the 2-amino group of guanosine was first masked with a trityl group.[17] Our synthetic strategy for obtaining the protected m^{7}G^5'pSPh is shown in Scheme 1. For the protection of the amino group, the 4,4'-dimethoxytrityl (DMTr) group was finally chosen since the methylation at the last stage greatly enhanced the stability of the trityl groups under acidic conditions. A preliminary study showed that, once the 7-position was methylated, the trityl (Tr) and 4-monomethoxytrityl (MMTr) groups attached to the 2-amino function were resistant to acidic treatment. Since the methoxymethylenation of 2-*N*-tritylguanosine (**1**) resulted in a poor yield of 2',3'-*O*-methoxymethylene-2-N-tritylguanosine (**2**) because of the simultaneous orthoesterification of the 5'-hydroxy group, we chose an alternative route to compound (**6**) from guanosine (**3**) by employing the DMTr group as a better N-blocking group. The selective deacetylation of the triacetate (**5**), obtained from compound (**4**), by a modification of the procedure of Ishido *et al.*[18] gave the 5'-*O*-acetyl derivative (**6**) in high yield; the product was converted *in situ* into the 5'-free guanosine derivative (**8**) in an overall yield of 55% from *the acetate* (**6**). Phosphorylation of compound (**8**) with cyclohexylammonium *S,S*-diphenyl dithiophosphate (PSS)[19,20] in the presence of 4,5-dimethoxybenzene-1,3-disulphonyl dichloride (DMS)[21] gave an 81% yield of the dithiophosphate (**9**). Since the conditions employed in this phosphorylation were very mild, side reactions associated with the O^6-guanine moiety did not occur to any significant extent.[22]

Prior to methylation, one of the two phenylthio groups was removed from the dithiophosphate (**9**) by treatment with lithium hydroxide. The diester (**10**) was then extracted with CH_2Cl_2–BuOH and purified by repeated extractive work-up. The final methylation was carried out in the presence of sodium dihydrogen phosphate (Scheme 2). If the salt was absent, the DMTr group was lost to a considerable extent. The fully protected m^{7}G^5'pSPh (**11**) could be easily purified by t.l.c. on silica gel since this compound has relatively high R_F values on t.l.c. because of its neutral structure. In addition, the zwitterion (**11**) was found to be very stable since, on storage at room temperature for two years, it did not decompose at all.



Scheme 1.



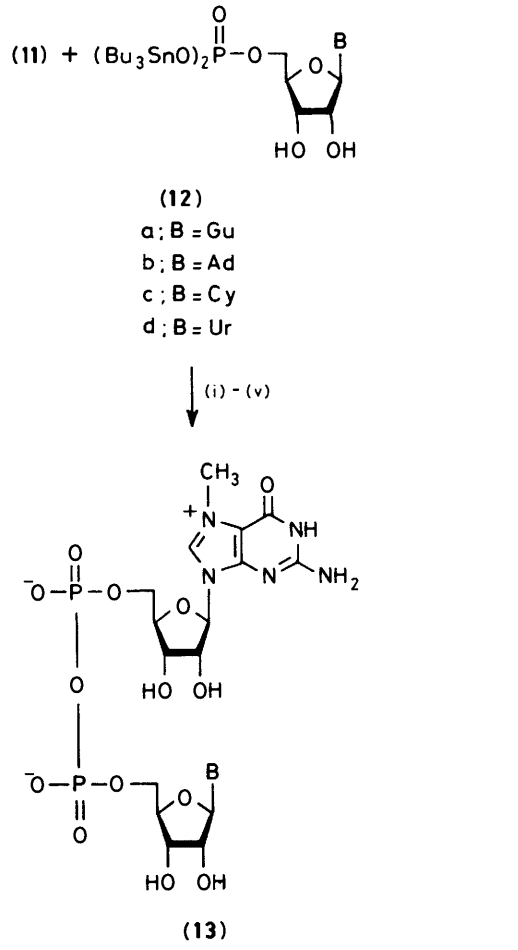
Scheme 2. *Reagents*: MeI–NaH_2PO_4, Me_2NCOMe

Although the ^1H n.m.r. spectrum of compound (**2**) exhibited two types of diastereoisomeric peaks due to the methoxymethylene group, those of the methoxymethylene derivatives (**7**)—(**11**) derived from the acetate (**6**) showed no evidence of diastereoisomeric mixtures, suggesting exclusive formation of one isomer or superposition of two isomers' resonances.

In a previous paper,[23] we reported that bis-(tributylstannyl) nucleoside 5′-phosphates[24] could be used as pyridine-soluble acceptors of activated *S*-phenyl tributylstannyl butyl thiophosphate for the synthesis of unsymmetrical nucleoside butyl diphosphates. Therefore, the pyrophosphate bond formation between reagents (**11**) and (**12**) in the presence of iodine as activating agent has been examined. Excess of the metallo phosphate (**12**) was required for satisfactory yields (Figure) because of the relatively poor nucleophilicity of the stannyl esters. However, the reactions could be carried out homogeneously even in the case of guanylic acid [guanosine 5′-(dihydrogen phosphate)]. The excess of nucleotides did not hamper the isolation of the coupling product (**13**) since the latter could be easily separated into the organic phase during extraction and the former became water-soluble after exchange from the stannyl ester to the pyridinium salt by addition of pyridine hydrochloride to the mixture prior to the extraction. The crude extracted material was further treated *in situ* with 80% acetic acid at room temperature for 16 h and the resulting mixture was separated by preparative paper electrophoresis (Scheme 3).

Since the 7-methylguanosine derivatives tend to decompose during chromatography and work-up even under neutral conditions,[25] the final separation of the product was best performed by paper electrophoresis. The desired diphosphate derivatives, separated as major bands, were eluted with water and the yields were estimated by u.v. spectrophotometry. Two minor products were also separated and identified as pm^7G and its symmetric pyrophosphate. An independent experiment in the absence of the stannylated ribonucleotides gave pm^7G and $m^7G^5'ppm^7G$ in 54 and 16% yield, respectively. The mobilities of the products were consistent with the expected values as shown in the Table. During electrophoresis and extraction with water, the products were found to be stable. By this method we could obtain $m^7G^5'ppNu$ with 10—200 O.D. units which are sufficient for biological studies. Enzymic degradation of each sample by incubation with snake venom phosphodiesterase gave pm^7G and the corresponding nucleotide in a 1:1 ratio.

Compound (**11**) might also be utilized for the synthesis of other types of m^7G-containing nucleotides.



Scheme 3. *Reagents/operations*: (i) I_2–pyridine; (ii) Na_2SO_4; (iii) pyridinium hydrochloride; (iv) extraction; (v) 80% AcOH

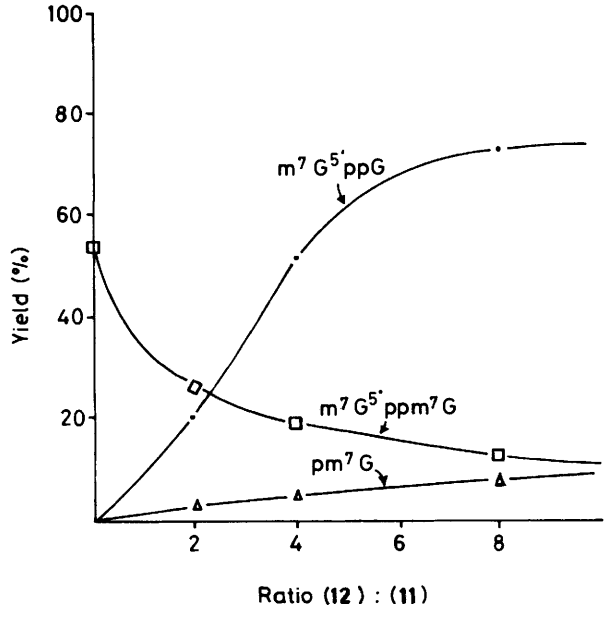


Figure. Reaction of the stannylated nucleotides (**12**) with the protected capping agent (**11**)

Experimental

M.p.s were determined on a Thomas-Hoover apparatus and are uncorrected. ^1H n.m.r. spectra were recorded at 100 MHz on a JNM-PS-100 spectrometer. U.v. spectra were obtained on a

Table. Physical properties of $m^7G^{5'}$ ppNu

(12)	Base	Product	λ (nm) pH 7.0 max	λ (nm) pH 7.0 min	λ (nm) pH 7.0 sh	Electrophoresis mobility (relative to pm^7G, pH 8.0)	Yield %
a	Gu	$m^7G^{5'}$ppG (**13a**)	253	231	271	0.74	73
b	Ad	$m^7G^{5'}$ppA (**13b**)	260	233	287	0.70	66
c	Cy	$m^7G^{5'}$ppC (**13c**)	260	237	271	0.94	69
d	Ur	$m^7G^{5'}$ppU (**13d**)	257	232		0.63	61
None		$m^7G^{5'}ppm^7G$	257 280	239 272		0.59	16

Hitachi 124 spectrophotometer. Column chromatography was performed with silica gel C-200 purchased from Wako Co. Ltd., and a minipump for a goldfish bowl was conveniently used to attain sufficient pressure for rapid chromatographic separation. T.l.c. was performed on precoated t.l.c. plates of silica gel 60 F-254 (Merck). The R_F values of the protected nucleoside derivatives were measured after development with CH_2Cl_2–MeOH (9:1, v/v) unless otherwise noted. *N-N*-Dimethylacetamide was distilled and stored over molecular sieves (3A). Elemental analyses were performed by the Microanalytical Laboratory, Tokyo Institute of Technology, at Nagatsuta.

2′,3′-O-*Methoxymethylene*-2-N-*tritylguanosine* (**2**).—To a suspension of compound (**1**)[25] (7.37 g, 14 mmol) in dry dioxane (40 ml) was added trimethyl orthoformate (20 ml) and molecular sieves 3A (0.5 g). Toluene-*p*-sulphonic acid monohydrate (TpSA·H_2O) (5.33 g, 28 mmol) was added and the mixture was stirred for 1 h. Then the reaction was quenched by addition of a saturated solution of ammonia in methanol (20 ml). The resulting precipitate was filtered off and the filtrate was evaporated under reduced pressure. The residue was chromatographed on a column of silica gel (200 g) with CH_2Cl_2–MeOH to give the *product* (**2**) (2.75 g, 35%) (Found: C, 63.1; H, 5.2; N, 11.45. $C_{31}H_{29}N_5O_6$·H_2O requires C, 63.58; H, 5.33; N, 5.33%); R_F 0.39; δ_H ($CDCl_3$) 3.21 and 3.23 (3 H, s, diastereoisomeric CH_3O), 3.42 and 3.64 (2 H, m, diastereoisomeric 5′-H), 4.13 (2 H, m, 3′- and 4′-H), 4.40 and 4.77 (1 H, m, diastereoisomeric 2′-H), 5.35 and 5.54 (1 H, d, *J* 2 Hz, diastereoisomeric 1′-H), 6.80—7.42 (15 H, m, Ph), 7.56 (1 H, br s, 8-H), and 8.49 (1 H, m, NH).

5′-O-*Acetyl*-2-N-(4,4′-*dimethoxytrityl*)*guanosine* (**6**).—To a solution of compound (**5**)[17] (16.4 g, 24.1 mmol) in methanol (200 ml) was added hydroxylamine sulphate (8.7 g, 53 mmol) and triethylamine (7.4 ml, 53 mmol). The resulting mixture was vigorously stirred for 15 h. The clear solution was evaporated, treated with water (100 ml), extracted with CH_2Cl_2 (3 × 50 ml), and evaporated under reduced pressure. The residue was chromatographed on a column of silica gel (350 g) with CH_2Cl_2–MeOH to give *compound* (**6**) (12.9 g, 85%) (Found: C, 61.1; H, 5.2; N, 10.45. $C_{33}H_{33}N_5O_8$·H_2O requires C, 61.39; H, 5.46; N, 10.85%); R_F 0.35; δ_H ($CDCl_3$) 3.36 (6 H, s, CH_3O), 3.68—4.20 (4 H, m, 3′- and 4′-H and 5′-H), 4.67 (1 H, m, 2′-H), 5.34 (1 H, d, *J* 4 Hz, 1′-H), 6.68 (4 H, d, *J* 8 Hz, ArH), 6.96—7.46 (10-H m, 8-H and ArH), and 10.47 (1 H, br s, NH).

2-N-(4,4′-*Dimethoxytrityl*)-2′,3′-O-*methoxymethyleneguanosine* (**8**).—TpSA·H_2O (3.66 g, 19.2 mmol) was dissolved in dry dioxane (50 ml) and molecular sieves 3A (1 g) were added. After the mixture had been stirred for 30 min, trimethyl orthoformate (19 ml) and compound (**6**) (6.05 g, 9.63 mmol) were successively added to the vigorously stirred mixture. After being stirred for 1 min, the mixture was treated with pyridine (5 ml) and the filtrate was poured into stirred ether (500 ml). The supernatant was decanted and the oily precipitate was dissolved in a mixture of CH_2Cl_2 and pyridine. The solution was evaporated under reduced pressure and coevaporated several times with toluene. The residue was chromatographed on a column of silica gel (100 g) with CH_2Cl_2–MeOH to give the crude acetate (**7**), which was treated with butylamine–MeOH (200 ml; 1:1, v/v) for 3 h. The amine and solvent were removed under reduced pressure and the residue was chromatographed in the usual manner to give *compound* (**8**) (5.3 g, 55%) (Found: C, 61.2; H, 5.2; N, 10.7. $C_{33}H_{33}N_5O_8$·H_2O requires C, 61.39; H, 5.46; N, 10.85%); R_F 0.37; δ_H ($CDCl_3$) 3.30 (3 H, s, CH_3OCH), 3.42 (2 H, m, 5′-H_2), 4.00—4.27 (2 H, m, 3′- and 4′-H), 4.60 (1 H, m, 2′-H), 5.64 (1 H, d, *J* 3 Hz, 1′-H), 5.79 (1 H, s, $CHOCH_3$), 6.67 (2 H, d, *J* 9 Hz, ArH), 6.69 (2 H, d, *J* 9 Hz, ArH), 6.92—7.46 (10 H, m, 8-H and ArH), and 10.79 (1 H, br s, NH).

2-N-(4,4′-*Dimethoxytrityl*)-2′,3′-O-*methoxymethyleneguanosine*-5′ S,S-*Diphenyl Dithiophosphate* (**9**).—PSS[19,20] (2.06 g, 5.4 mmol) was co-evaporated three times with dry pyridine and dissolved in dry pyridine (20 ml). To the solution was added DMS (1.64 g, 5.4 mmol). After being stirred for 2 h, the solution was mixed with a pyridine solution (25 ml) of compound (**8**) (2.85 g, 4.5 mmol) predried by repeated coevaporation with dry pyridine. The solution was concentrated to *ca.* 30 ml and stirred for 2 h. Then ice–water was added and the solution was extracted with CH_2Cl_2 (3 × 200 ml). The combined extracts were dried over Na_2SO_4, evaporated under reduced pressure, coevaporated with toluene, and chromatographed to give the *dithiophosphate* (**9**) (3.24 g, 81%) (Found: C, 59.9; H, 4.65; N, 7.65. $C_{45}H_{42}N_5O_9PS$ requires C, 60.60; H, 4.75; N, 7.85%); R_F 0.47; δ_H ($CDCL_3$) 3.29 (3 H, s, CH_3OCH), 3.68 (6 H, s, CH_3O), 4.12 (2 H, m, 5′-H_2), 4.20—4.40 (2 H, m, 3′- and 4′-H), 4.71 (1 H, m, 2′-H), 5.64 (1 H, d, *J* 2 Hz, 1′-H), 6.70 (4 H, d, *J* 8 Hz, ArH), 7.00—7.60 (20 H, m, ArH), and 10.56 (1 H, br s, NH).

Lithium 2-N-(4,4′-*Dimethoxytrityl*)-2′,3′-O-*methoxymethyleneguanosine*-5′ S-*Phenylthiophosphate* (**10**).—To a solution of compound (**9**) (892 mg, 1 mol) in dioxane (15 ml) was added 1M aqueous LiOH (5 ml). After being stirred for 20 min, the mixture was diluted with water (25 ml) and washed with CH_2Cl_2–BuOH (25 ml; 5:1, v/v) to remove some by-products. An aqueous solution (10 ml) of LiCl (2 g) was added to the aqueous layer and the mixture was extracted with CH_2Cl_2–BuOH (4 × 20 ml; 5:1, v/v). The organic layers were combined, dried over Na_2SO_4, concentrated to *ca.* 15 ml, and poured into vigorously stirred diethyl ether (300 ml). The resulting precipitate was collected by filtration, washed with ether (50 ml), and dried over P_4O_{10} to give the thiophosphate (**10**) (734 mg, 91%). However, this material contained a large amount of basic inorganic material. If this crude (**10**) was used in the next methylation, a considerable amount of by-product was formed. Therefore, the crude material was further dissolved in water (16 ml) and brine (55 ml). The solution was extracted with CH_2Cl_2–BuOH (3 × 70 ml; 5:2, v/v). The same work-up as described above

gave pure *thiophosphate* (**10**) (703 mg, 87%) (Found: C, 54.2; H, 5.3; N, 7.9. $C_{39}H_{37}LiN_5O_{10}PS{\cdot}2H_2O$ requires C, 54.61; H, 4.82; N, 8.16%); R_F 0.15.

2-N-(4,4'-*Dimethoxytrityl*)-2',3'-*methoxymethylene*-7-*methylguanosine*-5' S-*Phenylthiophosphate Inner Salt* (**11**).—Compound (**10**) (548 mg, 0.68 mmol) was dissolved in *N*-*N*-dimethylacetamide (4 ml) and well pulverized $NaH_2PO_4{\cdot}H_2O$ (106 mg, 0.68 mmol) was added. To the mixture was added methyl iodide (0.68 ml). After being stirred vigorously for 2.5 h, the mixture was filtered, washed with CH_2Cl_2, and transferred to a separating funnel. Water (50 ml) and CH_2Cl_2 (50 ml) were added. After being shaken, the CH_2Cl_2 layer was collected and the aqueous layer was further extracted with CH_2Cl_2 (3 × 50 ml). The organic extracts were combined, dried over Na_2SO_4, filtered, concentrated to *ca.* 80 ml, and transferred again to a separating funnel. The CH_2Cl_2 solution was washed with water (100 ml) and the washings were further extracted with CH_2Cl_2 (3 × 50 ml). The CH_2Cl_2 extracts were combined, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was dissolved in CH_2Cl_2–MeOH (5 l; 2:1, v/v) and poured into ether (300 ml). The precipitate (284 mg) appeared immediately and was collected by filtration. When the filtrate was kept at room temperature for 1 h, white crystals (201 mg, 36%) precipitated out and were collected by filtration. This material was found to be chromatographically pure and had m.p. 199—203 °C. The first precipitate was subjected to preparative t.l.c. on silica gel using CH_2Cl_2–MeOH as developer to give the pure zwitterion (**11**) (151 mg, 27%). The total yield was 63% (Found: C, 58.0; H, 5.0; N, 8.55. $C_{40}H_{40}N_5O_{10}PS{\cdot}H_2O$ requires C, 57.76; H, 5.09; N, 8.42%); R_F 0.47; δ_H ($CDCl_3$) 3.21 (3 H, s, CH_3OCH), 3.71 (6 H, s, CH_3O), 3.92 (3 H, s, CH_3N), 3.90—4.33 (3 H, m, 4'-H and 5'-H_2), 4.52—4.92 (2 H, m, 2'- and 3'-H), 5.60 (1 H, s, 1'-H), 5.67 (1 H, s, 1, CH_3OCH), 6.74 (4 H, d, *J* 8 Hz, ArH), 6.86—7.55 (4 H, m, ArH), 8.52 (1 H, br s, 8-H), and 9.10 (1 H, br s, NH).

General Procedure for Synthesis of $m^7G^{5'}ppNu$.—A mixture of the zwitterion (**11**) (20.4 mg, 25 μmol) and an appropriate stannylated ribonucleotide (**12a—d**) (0.2 mmol) was coevaporated several times with dry pyridine and was finally dissolved in dry pyridine (0.5 ml). To the mixture was added iodine (127 mg, 0.5 mmol). The solution was stirred vigorously for 30 min and then 1M HCl in water–pyridine (0.1 ml; 9:1, v/v) was added. The solution was transferred with pyridine–water (20 ml; 2:1, v/v) to a separating funnel containing CH_2Cl_2–BuOH (20 ml; 5:2, v/v), brine (10 ml), water (20 ml), 1M Na_2SO_4 (2 ml), and 1M HCl in pyridine–water (1 ml; 9:1, v/v). After the mixture had been shaken, the lower layer was collected and the aqueous phase was further extracted with CH_2Cl_2–BuOH (5 × 20 ml; 5:2, v/v). The combined organic extracts were passed through a column of Na_2SO_4, which was then washed with CH_2Cl_2–BuOH (20 ml; 5:2, v/v). The eluant was evaporated to dryness and the residue was dissolved in pyridine–water (10 ml; 49:1, v/v) in a measuring flask. One-tenth of the solution was taken, evaporated to dryness, and coevaporated twice with toluene. The residue was dissolved in 80% AcOH (10 ml). After being kept for 16 h, the solution was evaporated under reduced pressure. The residue was dissolved in pyridine–water (1 ml; 2:1, v/v) and half of the solution was applied to strips of Toyo Roshi No 51A paper. After the solvent had been removed from the papers by a drier, electrophoresis was performed at 1 500 V for 1.5 h using a 0.05M phosphate buffer (pH 8.0) or a morpholine–acetic acid buffer (pH 3.5). The fluorescent band corresponding to the diphosphate (**13**) was eluted with water and the yield was determined spectrophotometrically using the values at 258 nm which were estimated to have 10% hypochromicity: (**13a**): 1.82×10^4; (**13b**): 2.16×10^4; (**13c**): 1.42×10^4; (**13d**): 1.68×10^4.

Each sample (10 OD) obtained was incubated with snake venom phosphodiesterase (Boehringer, 10 μg) in a Tris–HCl buffer (pH 8.0) at 37 °C for 3 h. This enzymic degradation gave a 1:1 mixture of pm^7G and the corresponding 5'-ribonucleotide which was analysed by paper electrophoresis as described above.

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