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Studies on Chemical Synthesis of mRNAs. I. Synthesis and Properties of N^2-Tritylguanosine Derivatives and Application to Synthesis of pGpUpU

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Trityl (Tr), 4-methoxytrityl (MMTr), and 4,4′-dimethoxytrityl (DMTr) groups were introduced into the 2-amino group of 2′,3′,5′-tri-*O*-acetylguanosine by treatment with the corresponding trityl chlorides in pyridine to afford the N^2-tritylated guanosine derivatives in high yields. Similarly, the N^6-tritylated adenosine derivatives were synthesized. The stability of the three kinds of trityl groups under acidic conditions were described. The MMTr and Tr groups were found to be suitable for the protection of the 2-amino group of guanosine in oligonucleotide synthesis. An appropriately protected N^2-tritylguanosine 5′-phosphorodithioate derivative was synthesized and utilized for the synthesis of pGpUpU.

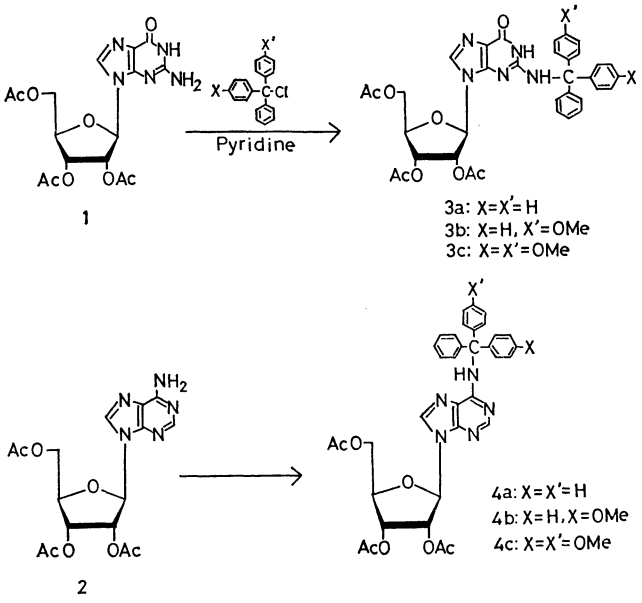
In 1975, Miura[1] discovered the so-called "cap" structure in the 5′-terminal region of cytoplasmic polyhedrosis virus mRNA which was represented as m^{7}G$^{5'}$pppAmpGpU···. Since then, the cap structure has been found from several viruses, eukaryotic cells, and mammarian cells.[2] The cap structure is now well recognized as the common structure of eukaryotic mRNAs. Therefore, much attention has been paid to the correlation between the structure and functions of mRNAs in peptide synthesis.[3]

In connection with our study on the chemical synthesis of 5′-terminal structures of eukaryotic mRNAs, especially, the common sequence of mosaic viruses represented as m^{7}G$^{5'}$pppGpUpU···, we wish to report here a basic study on the protection of the 2-amino group of guanosine and also describe the synthesis of pGpUpU.

Results and Discussion

Since 7-methylguanosine (m^{7}G), contained in the cap structure, is quite unstable under alkaline conditions and decomposes to a ring-opening product,[4] the chemical synthesis of capped oligoribonucleotides should be designed to perform under neutral and mildly acidic conditions. In general,[5] the amino groups of nucleoside bases were masked with acyl groups. Only a few have been reported of the acid labile protecting groups for the amino groups. Among them, Holy[6] reported the dimethylaminomethylene group as the protecting group of the 2-amino function of guanine moiety. However, this group is unstable under both acidic and alkaline conditions. On the other hand, 4,4′-dimethoxytrityl (DMTr) group has been used as the acid labile protecting group of the 2-amino group of guanosine by Khorana[7] and Shimidzu.[8] In this study, we tried to use the latter type of protecting groups since introduction of trityl groups enhanced the lipophilicity of the product and the groups could serve as the markers which were easily detected by spraying 10% perchloric acid solution on a chromatogram.

First, the three kinds of trityl groups (Tr, MMTr, and DMTr), were introduced to the 2-amino group of 2′,3′,5′-tri-*O*-acetylguanosine (**1**). In a similar manner, 2′,3′,5′-tri-*O*-acetyladenosine (**2**) was also tritylated. The conditions and results are summarized in Table 1. It was found that the tritylations of **2** proceeded more readily than those of **1**. All the products **3** and **4** could be isolated in pure form as the stable compounds. The N^2-tritylguanosine derivatives **3a**—**c** were proved to be less polar than the N^6-trityladenosine derivatives **4a**—**c** from their R_f values.



The stability of the Tr, MMTr, and DMTr groups introduced into **1** and **2** was examined. Compounds **3a**—**c** and **4a**—**c** were respectively stirred with silica gel (Wakogel C-200) in dichloromethane at room temperature for one day. The amounts of the detritylated compound **1** and **2** were estimated spectrophotometrically after thin layer chromatography. Table 4 shows that the adenosine derivatives are less stable than the guanosine derivatives on silica gel.

Similar results were obtained by treatment of **3a**—**c** and **4a**—**c** with 80% acetic acid as summarized in Table 5. Complete removal of each trityl group from **3** or **4** could be performed within one day at room

TABLE 1. CONDITIONS AND RESULTS OF N^2- AND N^6-TRITYLATION OF **1** AND **2**

Trityl chloride	(equiv.)	Temp/°C	Time/h	Product	Yields of **3** and **4**/%
TrCl	1.6	100	2	**3a**	91
MMTrCl	2.0	r. t.	24	**3b**	92
DMTrCl	1.6	r. t.	12	**3c**	96
TrCl	4.0	100	12	**4a**	80
MMTrCl	3.0	60	24	**4b**	91
DMTrCl	4.0	60	12	**4c**	95

TABLE 2. MELTING POINTS AND ELEMENTAL ANALYSES OF **3a—c** AND **4a—c**

Compound	Mp θ_m/°C	Formula	Found (%)			Calcd (%)		
			C	H	N	C	H	N
3a	155 (decomp)	$C_{35}H_{33}N_5O_8$	64.10	5.09	9.99	64.51	5.10	10.75
3b	156—157 (decomp)	$C_{36}H_{35}N_5O_9 \cdot 1/2H_2O$	63.27	5.24	9.77	63.52	5.33	10.29
3c	142—145	$C_{37}H_{37}N_5O_{10} \cdot H_2O$	61.21	5.29	9.36	60.90	5.39	9.60
4a	123—124	$C_{35}H_{33}N_5O_7$	66.11	5.34	10.53	66.13	5.43	10.78
4b	116—118	$C_{36}H_{35}N_5O_8$	64.48	5.34	10.12	64.95	5.30	10.52
4c	113—118	$C_{37}H_{37}N_5O_9$	63.20	5.48	9.40	63.06	5.43	9.94

TABLE 3. SPECTRAL DATA OF **3a—c** AND **4a—c**

Compound	UV (EtOH) $\lambda_{max}(\varepsilon \times 10^{-3})$	$\lambda_{min}(\varepsilon \times 10^{-3})$	$\lambda_{sh}(\varepsilon \times 10^{-3})$	^{1}H NMR($CDCl_3$) δ
3a	260 (17.4)	237 (10.8)	274 (14.4)	1.96(3H, s, $CH_3C(O)$), 2.06(6H, s, $CH_3C(O)$), 4.20(3H, s, H-4′ and 5′), 5.20(2H, m, H-2′ and 3′), 5.36(1H, d, $J_{1',2'}$=7.5 Hz, H-1′), 7.20(15H, m, Tr), 7.89(1H, s, H-8)
3b	260 (17.2)	247 (14.8)	277 (15.6)	1.96(3H, s, $CH_3C(O)$), 2.07(6H, s, $CH_3C(O)$), 3.64(3H, s, CH_3O), 4.20(3H, s, H-4′ and 5′), 5.24(2H, m, H-2′ and 3′), 5.40(1H, d, $J_{1',2'}$=7.5 Hz, H-1′), 6.68(2H, d, J=9 Hz, *m*-protons of MMTr), 7.20(13H, m, MMTr), 7.82(1H, s, H-8)
3c	262 (19.0)	249 (18.0)	277 (17.8)	1.96(3H, s, $CH_3C(O)$), 2.07(6H, s, $CH_3C(O)$), 3.68(6H, s, CH_3O), 4.20(3H, s, H-4′ and 5′), 5.32(2H, m, H-2′ and 3′), 5.46(1H, d, $J_{1',2'}$=7.5 Hz, H-1), 6.70(4H, d, J=9 Hz, *m*-protons of DMTr), 7.24(15H, m, DMTr), 7.55(1H, s, H-8)
4a	274 (17.8)	241 (5.2)	284 (12.0)	2.08(9H, m, $CH_3C(O)$), 4.39(3H, s, H-4′,5′), 5.68(1H, m, H-3′), 5.92(1H, t, $J_{1',2'}$=5 Hz, $J_{2',3'}$=5.5 Hz, H-2′), 6.14(1H, d, $J_{1',2'}$=5 Hz, H-1′), 6.98(1H, s, NH), 7.28 (15H, m, Tr), 7.91(1H, s, H-2), 8.04(1H, s, H-8)
4b	274 (18.4)	245 (8.0)	284 (12.0)	2.10(9H, m, $CH_3C(O)$), 3.78(3H, s, CH_3O), 4.40(3H, s, H-4′,5′), 5.70(1H, m, H-3′), 5.93(1H, t, $J_{1',2'}$=5 Hz, $J_{2',3'}$=5.5 Hz, H-2′), 6.15(1H, d, $J_{1',2'}$=5, H-1′), 6.78 (2H, d, J=9 Hz, *m*-protons of MMTr), 6.94(1H, s, NH), 7.30(13H, m, MMTr), 7.91(1H, s, H-2), 8.07(1H, s, H-8)
4c	274 (18.6)	248 (10.7)	284 (14.2)	2.08(9H, m, $CH_3C(O)$), 3.76(6H, s, CH_3O), 4.40(3H, s, H-4′,5′) 5.70(1H, m, H-3′), 5.93(1H, t, $J_{1',2'}$=5 Hz, $J_{2',3'}$=5.5 Hz, H-2′), 6.14(1H, d, $J_{1',2'}$=5 Hz, H-1′), 6.76 (4H, d, 9 Hz, *m*-protons of DMTr), 6.91(1H, s, NH), 7.30(1H, m, DMTr), 7.90(1H, s, H-2), 8.08(1H, s, H-8)

Table 4. Stability of trityl groups of **3** and **4** in CH_2Cl_2 in the presence of silica gel (Wakogel C-200)

Compound	**3a**	**3b**	**3c**	**4a**	**4b**	**4c**
Detritylation after 24 h (%)	3	26	75	17	64	84

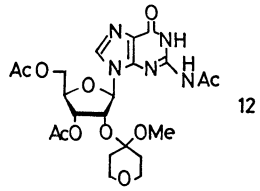
Table 5. Deprotection of trityl groups from **3** and **4**

Conditions	Yield of **1**/%			Yield of **2**/%		
	3a	**3b**	**3c**	**4a**	**4b**	**4c**
R. t., 1 h	48	58	90	64	91	92
R. t., 6 h	70	94	95	94	96	96
R. t., 24 h	81	100	100	100	100	100
100 °C, 10 min	100	—	—	—	—	—

temperature except for **3a**. In the case of **3a**, the trityl group was removed completely at 100 °C for 10 min.

Recently, the 5′-terminal structure of brome mosaic virus mRNA has been determined by Kaesberg.[9] It was described as $m^7G^{5'}$ pppGpUpU···. From biological interest of the 5′-terminal structure of brome mosaic virus mRNA, we aimed to synthesize the terminal trimer pGpUpU utilizing the trityl group. In order to prepare a N^2-tritylguanosine derivative having the unmasked 2′-hydroxyl group as the starting material for the synthesis of pGpUpU, the selective deacetylation of **3a** by means of hydrazine hydrate was first examined to obtain 3′,5′-di-*O*-acetyl-N^2-tritylguanosine (**5**). However, **5** and the 2′,5′-diacetate (**6**) were not easily separated, and **5** could not be purified by recrystallization. Repeated chromatography was needed for the complete purification of **5**,[10] since the difference in R_f value between **5** and **6** was unappreciable. From the synthetic point of view this approach could not be applied to the preparation of **5** on a large scale. Therefore, the synthesis of a 2′-protected N^2-tritylguanosine derivative (**7**) was carried out by an alternative route outlined in Scheme 1.

We have chosen 4-methoxytetrahydropyranyl group[11] as the 2′-hydroxyl protecting group, since it could be removed under acidic conditions and had no chiral center in contrast with tetrahydropyranyl group. A convenient method for the synthesis of 3′,5′-di-*O*-acetyl-N^2-benzoylguanosine (**8**) has been reported recently.[12] The methoxytetrahydropyranylation of **8** gave the 2′-protected guanosine derivative (**9**) in 71% yield. Treatment of **9** with butylamine–methanol (1:1, v/v) gave 2′-(4-methoxytetrahydropyran-4-nyl)guanosine (**10**) in 76% yield. Acetylation of **10** with 20 equiv. of acetic anhydride in pyridine gave selectively the 3′,5′-di-*O*-acetylated derivative (**11**) in 90% yield. When more than 20 equiv. of acetic anhydride was used, N^2,$O^{3'}$,$O^{5'}$-triacetylated product (**12**) was formed to an appreciable extent. N^6-tritylation of **11** in pyridine at 100 °C for 1 h gave the fully protected guanosine derivative (**13**) in 87% yield. Deacylation of **13** by means of butylamine–methanol (1:1, v/v) gave **7** in 90% yield.



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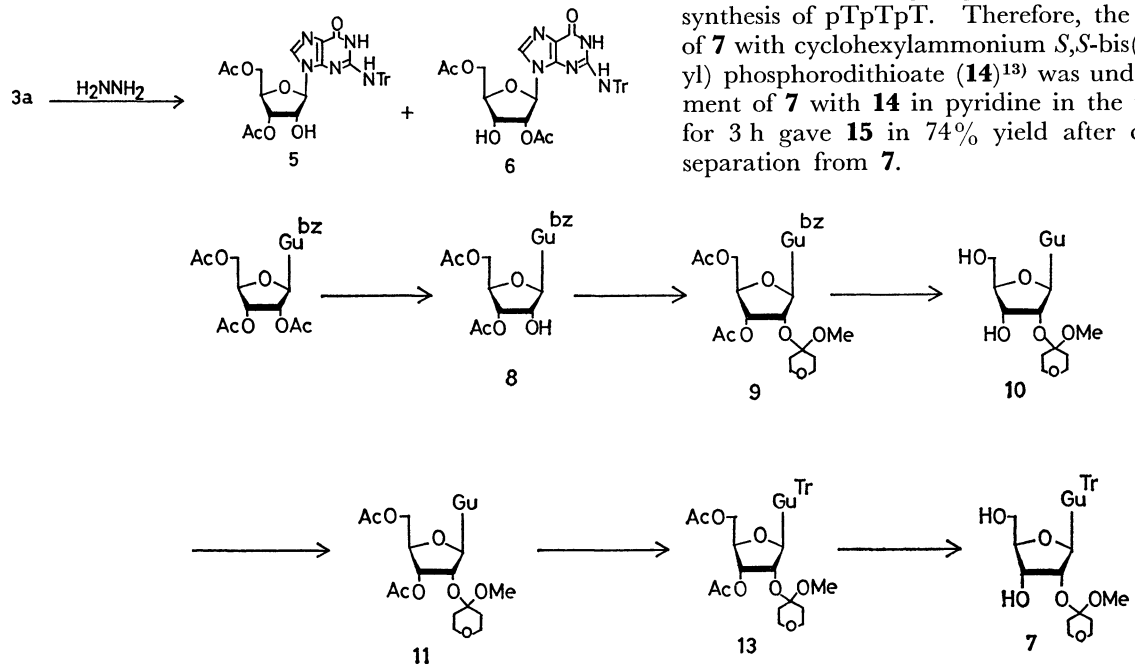
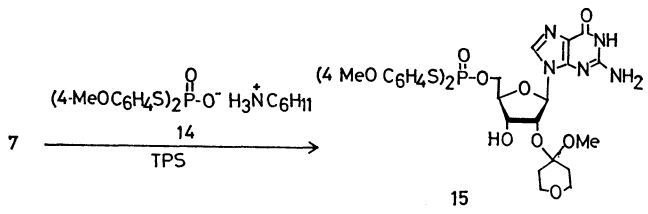
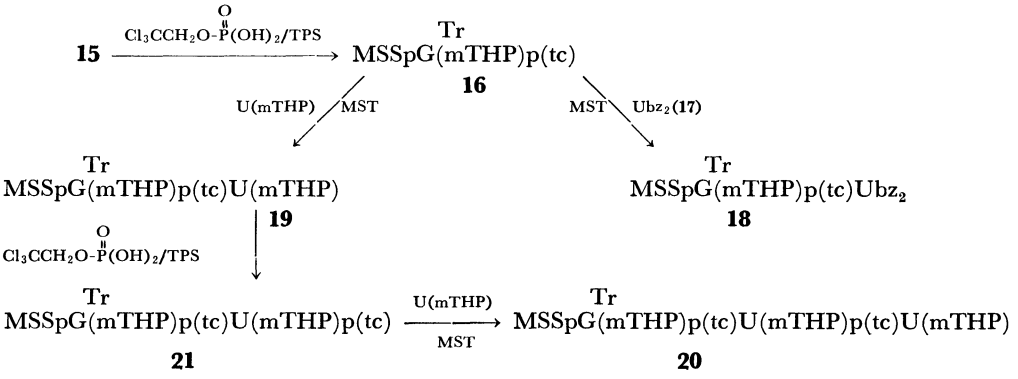
For the construction of the triphosphate bridge involved in the cap structure of the mRNA, a suitable protected phosphate group should be introduced into the 5′-hydroxyl group of **7**. In a previous paper,[13] we described the utility of 4-methoxyphenylthio group as the 5′-terminal phosphate protecting group in the synthesis of pTpTpT. Therefore, the phosphorylation of **7** with cyclohexylammonium *S*,*S*-bis(4-methoxyphenyl) phosphorodithioate (**14**)[13] was undertaken. Treatment of **7** with **14** in pyridine in the presence of TPS for 3 h gave **15** in 74% yield after chromatographic separation from **7**.



Scheme 1.



Next, the synthesis of the protected pGpUpU was performed according to Neilson's procedure.[14] Phosphorylation of **15** with 2,2,2-trichloroethyl dihydrogenphosphate was examined by using TPS, 1-(4-nitrophenylsulfonyl)-1*H*-1,2,4-triazole,[15] or dicyclohexylcarbodiimide (DCC). Among those condensing agents, TPS was found to be the most effective. When **15** was phosphorylated by using TPS, TLC of the reaction mixture showed more than 9 spots. However, the mixture was hydrolyzed with aqueous pyridine at room temperature for one day, the desired 3'-phosphorylated product (**16**) appeared as main spot on TLC. Subsequent coupling of **16** with 2',3'-di-*O*-benzoyluridine (**17**) in the presence of 1-(mesitylsulfonyl)-1*H*-1,2,4-triazole (MST)[15] gave the fully protected guanylyl(3'→5') uridine derivative (**18**) in 80% yield.

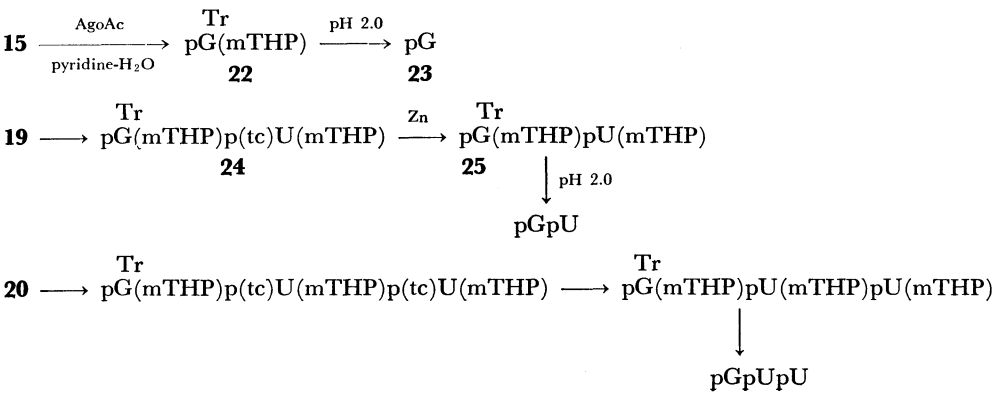


In a similar manner, the dinucleotide (**19**) was obtained in 60% yield from **16** and 2'-*O*-(4-methoxytetrahydropyranyl)uridine [U(mTHP)]. The trimer (**20**) was synthesized by a similar procedure: When a dinucleotide derivative obtained by the phosphorylation of **19** with 2,2,2-trichloroethyl phosphate in the presence of TPS was condensed with U(mTHP) by means of MST, the desired trinucleotide (**20**) was obtained in 54% yield.

During the coupling reactions by-products having blue fluorescence were detected on TLC. They appeared just on the desired products and decomposed to the starting material (**21**) and the desired product during workup. Since O^6-dialkoxyphosphinyl derivatives of **3a** has blue fluorescence,[16] the present by-products might be the O^6-phosphorylated derivatives concomitantly formed from the reaction of **18** or **20** with **16** or **21**.

Deprotection of the Protected Oligonucleotides. We first tested the deprotection of mononucleotide **15**. Treatment of **15** with silver acetate in pyridine–water (2:1, v/v) at room temperature for 24 h followed by chromatography on Whatman 3 MM papers gave 2'-*O*-(4-methoxytetrahydropyranyl)-N^2-tritylguanosine 5'-phosphate (**22**). Subsequent treatment of **22** with 0.01 M (1 M=1 mol dm^{-3}) HCl (pH 2.0) with vigorous stirring for 2 d gave guanosine 5'-phosphate (**23**). The overall yield of **23** from **15** was almost quantitative (99%).

The removal of the protecting groups from the dinucleotide **19** was carried out as follows. As described above, treatment of **19** with silver acetate[13] gave pG^{Tr}(mTHP)p(tc)U(mTHP) (**24**). The 2,2,2-trichloroethyl group was removed from **24** by treatment with zinc/acetylacetone according to the method reported by Adamiak.[17] Finally, the resulting product (**25**) was converted to pGpU in an overall yield of 50% by treatment with 0.01 M HCl for 2 d. Finally, in a similar manner, from the protected trinucleotide **20** pGpUpU was obtained in 41% yield. Both the dimer and the trimer were degraded by snake venom phosphodiesterase to give pG and pU in the theoretical ratios. Usually, 2,2,2-trichloroethyl group can also be removed by treatment with tetra-



butylammonium fluoride (TBAF). However, attempts to remove the 2,2,2-trichloroethyl group from **24** by means of TBAF were unsuccessful. This result was consistent with that reported by Ogilvie[18] that the presence of a charged phosphate one unit removed from the triester phosphate was sufficient to prevent removal of the internucleotidic phenyl group by TBAF. On the other hand, treatment of **19** with TBAF gave the 5′-terminal phosphorofluoridate derivative, FpG^{Tr}-(mTHP)pU(mTHP), which remained unchanged upon treatment with silver acetate in aqueous pyridine. The decrease of the yields of the deprotected oligomers might be mainly due to side reactions or adsorption of the nucleotidic substances on the zinc surface on the removal of the 2,2,2-trichloroethyl group, although the latter group was rapidly deprotected. While improvement of the yields in the coupling and deblocking processes remained as a problem to be investigated, the present approach provides oligomers with the acid labile protecting groups, which may be useful for the joining with the 'cap' part. Further extention of this wark is now in progress.

Experimental

Melting points were determined on a Thomas-Hoover apparatus and are uncorrected. ^1H NMR spectra were recorded at 100 MHz on a JEOL ST-100 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 systems used were Solvent I: isopropyl alcohol–concentrated aqueous ammonia–water (7:1:2, v/v/v); Solvent II: isopropyl alcohol–concentrated aqueous ammonia–water (6:1:3, v/v/v). Paper electrophoresis was carried out with an apparatus similar to that described by Markham and Smith.[19] The buffer used were Buffer I: 0.05 M phosphate (pH 8.0); Buffer II: 0.873 M acetic acid (50 ml)–morpholine (3.3 ml) (pH 3.5) solution (1 l).

Pyridine was distilled from *p*-toluenesulfonyl chloride and stored over calcium hydride. Dichloromethane (CH_2Cl_2) was distilled after being kept over P_4O_{10} for 1 d, redistilled from potassium carbonate, and stored over molecular sievers (3A). Dioxane (1 l) was purified by passing nitrogen gas into the refluxing mixture which contained concentrated hydrochloric acid (13 ml) and water (10 ml) followed by neutralization with potassium hydroxide, extraction, and distillation over sodium. 2′,3′,5′-Tri-*O*-acetylguanosine (**1**) and 2′,3′,5′-tri-*O*-acetyladenosine (**2**) were prepared according to the literature method.[20]

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

Typical Procedure for the Synthesis of ***3*** *and* ***4***. Compound **1** (1.02 g, 2.5 mmol) was rendered anhydrous by repeated coevaporation with dry pyridine and dissolved in dry pyridine (25 ml) trityl chloride (1.12 g, 4 mmol) was added and the mixture was stirred at 100 °C for 2 h. The mixture was cooled to room temperature and ice (5 g) was added. After being kept for 30 min, the mixture was extracted with CH_2Cl_2 (3×30 ml). The combined organic extracts were dried over Na_2SO_4 and evaporated *in vacuo*. The residue was chromatographed on a column of silica gel using CH_2Cl_2–MeOH to give **3a** (1.49 g, 91%). The conditions and results are summarized in Table 1. Physical properties and elemental analysis of **3** and **4** are listed in Table 2.

*3′,5′-Di-*O*-acetyl-2′-*O*-(4-methoxytetrahydropyran-4-yl)-*N^2*-benzoylguanosine (****9****).* To a solution of *p*-toluenesulfonic acid monohydrate (570 mg, 3 mmol) in dioxane (120 ml) was added **8** (5.64 g, 12 mmol). The mixture was stirred for 1 h, and then 5,6-dihydro-4-methoxy-2*H*-pyran (30 mmol)[11] was added. After 3 h, the resulting mixture was neutralized with 1 M methanolic sodium methoxide and evaporated to dryness. The residue was chromatographed on a column of silica gel (98:2—97:3 CH_2Cl_2–MeOH) to give **9** (5.61 g, 71%): Mp 129 °C; NMR ($CDCl_3$) δ 1.76 (4H, m, pyranyl ring protons), 2.06 (3H, s, $CH_3C(O)$), 2.16 (3H, s, $CH_3C(O)$), 2.56 (3H, s, CH_3–O), 3.63 (4H, m, pyranyl ring protons), 4.50 (3H, m, H-4′ and 5′), 5.06 (1H, m, H-3′), 5.30 (1H, m, H-2′), 5.90 (1H, d, $J_{1',2'}$= 7.5 Hz, H-1′), 7.20—8.20 (6H, m, ArH and H-8). Found: C, 54.94; H, 5.35; N, 11.10%. Calcd for $C_{27}H_{31}O_{10}N_5 \cdot 1/2H_2O$: C, 54.56; H, 5.33; N, 11.78%.

*2′-O-(4-Methoxytetrahydropyran-4-yl)guanosine (****10****).* Compound **9** (7.51 g, 12.9 mmol) was dissolved in 1:1 butylamine–methanol (100 ml), and the solution was allowed to stand at room temperature. After 1 d, the solvent was evaporated *in vacuo*. The residue was washed with benzene and recrystallized from methanol to give **10** (3.91 g, 76%): Mp 226 °C (decomp); $UV^{H_2O}_{max}$ 252 nm ($\varepsilon=14.4\times10^3$), $UV^{H_2O}_{min}$ 222 nm ($\varepsilon=3\times10^3$), $UV^{H_2O}_{sh}$ 269 nm ($\varepsilon=3\times10^3$); NMR ($CDCl_3$) δ 1.63 (4H, m, Pyranyl ring protons), 2.67 (3H, s, CH_3–O), 3.35 (4H, m, pyranyl ring protons), 3.58 (2H, m, H-5′), 3.94 (1H, m, H-4′), 4.07 (1H, m, H-3′), 4.70 (1H, dd, $J_{1',2'}=7.4$ Hz, $J_{2',3'}=5$ Hz, H-2′), 5.13 (2H, m, OH), 5.87 (1H, d, $J_{1',2'}=7.4$ Hz, H-1′), 6.45 (1H, br.s, NH_2), 7.98 (1H, s, H-8). Found: C, 48.05; H, 5.85; N, 17.68%. Calcd for $C_{16}H_{23}N_5O_7$: C, 48.36; H, 5.83; N, 17.62%.

*3′,5′-Di-*O*-acetyl-2′-*O*-(4-methoxytetrahydropyran-4-yl)guanosine (****11****).* To a mixture of acetic anhydride (20 ml) and pyridine (23 ml) was added **10** (4.03 g, 10.2 mmol). The suspension was vigorously stirred at 60 °C for 2 h. The resulting homogeneous solution was cooled to room temperature. The crystalline precipitate was collected by filtration, washed with benzene, and recrystallized from methanol to give **11** (4.39 g, 90%): Mp 263 °C (decomp); $UV^{H_2O}_{max}$ 253 nm ($\varepsilon=14.5\times10^3$), $UV^{H_2O}_{min}$ 222 nm ($\varepsilon=2.9\times10^3$), $UV^{H_2O}_{sh}$ 270 nm ($\varepsilon=10.4\times10^3$); NMR ($CDCl_3$) δ 2.06 (4H, m, pyranyl ring protons), 2.06 (3H, s, $CH_3C(O)$), 2.16 (3H, s, $CH_3C(O)$), 2.62 (3H, s, CH_3–O), 3.42 (4H, m, pyranyl ring protons), 4.30 (3H, br.s, H-4′ and 5′), 5.11 (1H, dd, $J_{1',2'}=7.6$ Hz, $J_{2',3'}=4.6$ Hz, H-2′), 5.22 (1H, t, $J_{2',3'}=$ 4.6 Hz, $J_{3',4'}=4.6$ Hz, H-3′), 5.90 (1H, d, $J_{1',2'}=7.6$ Hz, H-1′), 6.55 (1H, br.s, NH_2), 8.00 (1H, s, H-8). Found: C, 49.52; H, 5.74; N, 14.00%. Calcd for $C_{20}H_{27}N_5O_9$: C, 49.81; H, 5.65; N, 14.55%.

*3′,5′-Di-*O*-acetyl-2′-*O*-(4-methoxytetrahydropyran-4-yl)-*N^2*-tritylguanosine (****13****).* To a solution of **11** (482 mg, 1 mmol) in pyridine (10 ml) was added trityl chloride (557 mg, 2 mmol) and the mixture was kept at 80 °C for 6 h. After being cooled to room temperature, the mixture was poured onto crushed ice. The resulting aqueous mixture was extracted with dichloromethane (4×10 ml). The combined organic extracts were dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. The residue was chromatographed on a column of silica gel (99:1 CH_2Cl_2–MeOH) to give crude **7** (610 mg, 87%): NMR ($CDCl_3$) δ 1.55 (4H, m, pyranyl ring protons), 2.10 (3H, s, $CH_3C(O)$), 2.19 (3H, s, $CH_3C(O)$), 2.50 (3H, s, CH_3–O), 3.51 (4H, m, pyranyl ring protons), 4.18 (3H, br.s, H-4′ and 5′), 4.52 (1H, dd, $J_{1',2'}=7.6$ Hz, $J_{2',3'}=6.0$ Hz, H-2′), 5.16 (1H, d, $J_{2',3'}=$ 6 Hz, H-3′), 5.40 (1H, d, $J_{1',2'}=7.6$ Hz, H-1′), 7.04—7.60

(15H, m, Tr), 7.99 (1H, s, H-8). Since **11** could not be purified by recrystallization and repeated chromatography, the crude material was used for the next reaction.

2'-O-(4-Methoxytetrahydropyran-4-yl)-N²-tritylguanosine (7). The crude **11** (96 mg, 0.137 mmol) was dissolved in 1:1 butylamine–methanol (80 ml) and the mixture was kept at room temperature for one day. The solvent was removed *in vacuo* and the residue chromatographed on a column of silica gel (97:3, CH_2Cl_2–MeOH) to give **7** (79 mg, 90%): Mp 168 °C; UV_{max}^{EtOH} 261 nm ($\varepsilon=16.4\times10^3$), UV_{min}^{EtOH} 238 nm ($\varepsilon=10.8\times10^3$), UV_{sh}^{EtOH} 275 nm ($\varepsilon=14.5\times10^3$); NMR ($CDCl_3$) δ 1.62 (4H, m, pyranyl ring protons), 2.62 (3H, s, $3H_3$–O), 3.10—3.95 (6H, m, pyranyl ring protons and H-5'), 4.05—4.29 (2H, m, H-3',4'), 4.84 (1H, dd, $J_{1',2'}=7.5$ Hz, $J_{2',3'}=5.4$ Hz, H-2'), 5.54 (1H, d, $J_{1',2'}=7.5$ Hz, H-1'), 7.26 (15H, m, Tr), 7.76 (1H, s, H-8). Found: C, 62.57; H, 5.73; N, 10.23%. Calcd for $C_{35}H_{37}N_5O_7$: C, 62.21; H, 6.21; N, 10.36%.

S,S-Bis(4-methoxyphenyl)-2'-O-(4-methoxytetrahydropyran-4-yl)-N²-tritylguanosine-5'-phosphorodithioate (15). To a solution of **7** (303 mg, 0.47 mmol) in dry pyridine (1 ml) was added **14** (230 mg, 0.52 mmol) and TPS (171 mg, 0.56 mmol), successively. After the solution was stirred for 3 h, 1:1 pyridine–water (2 ml) was added. The mixture was extracted with dichloromethane (4×2 ml). The combined organic extracts were dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue was chromatographed on a column of silica gel (99:1 CH_2Cl_2–MeOH) to give **15** (335 mg, 74%) as a foam: UV_{max}^{EtOH} 256 nm ($\varepsilon=33.0\times10^3$), UV_{min}^{EtOH} 227 nm ($\varepsilon=28.0\times10^3$), UV_{sh}^{EtOH} 242 nm ($\varepsilon=29.7\times10^3$); NMR ($CDCl_3$) δ 1.60 (4H, m, pyranyl ring protons), 2.71 (3H, s, CH_3–O), 3.58 (4H, m, pyranyl ring protons), 3.71 (6H, s, CH_3–O), 4.11 (2H, m, H-5'), 4.22—4.58 (3H, m, H-2',3' and 4'), 5.37 (1H, d, $J_{1',2'}=5.7$ Hz, H-1'), 6.78 (4H, d, $J=9$ Hz, *m*-protons of CH_3O–C_6H_4–S), 7.08—7.52 (19H, m, Tr and *o*-protons of CH_3O–C_6H_4–S). Found: C, 58.69: H, 5.20; N, 6.94%. Calcd for $C_{49}H_{50}N_5O_{10}PS_2\cdot2H_2O$: C, 58.85; H, 5.44; N, 7.00%.

Synthesis of 18. A mixture of **15** (34 mg, 0.035 mmol) and 2,2,2-trichloroethyl dihydrogenphosphate (16.1 mg, 0.07 mmol) was rendered anhydrous by repeated coevaporation with dry pyridine and finally dissolved in dry pyridine (0.5 ml). TPS (43 mg, 0.14 mmol) was added and the mixture was stirred at room temperature. After 1 d, the mixture was treated with 1:1 pyridine–water (10 ml) overnight. The main spot of R_f 0.1 (CH_2Cl_2:MeOH, 12:1, v/v) was observed on silica gel plate. The mixture was extracted with CH_2Cl_2 (4×5 ml). The organic exeracts were combined washed water (2×5 ml), and evaporated to dryness. The residue was mixed with **17** (48 mg, 0.105 mmol) and rendered anhydrous by repeated coevaporation with dry pyridine. The mixture was dissolved in dry pyridine (0.5 ml) and 1-(*p*-nitrobenzenesulfonyl)-1*H*-1,2,4-triazole (NBST) (53 mg, 0.21 mmol) was added. The mixture was stirred at room temperature for 3 d and then pyridine–water (1:1, v/v) (2 ml) was added. After being kept for 30 min, the mixture was extracted with CH_2Cl_2 (4×5 ml). The organic extracts were combined, dried over Na_2SO_4, evaporated to dryness, and chromatographed on a column of silica gel (99:1→99:2 CH_2Cl_2–MeOH) to give **18** (45 mg, 80%): UV_{max}^{EtOH} 256 nm ($\varepsilon=19200$), UV_{max}^{EtOH} 233 nm ($\varepsilon=21600$), UV_{min}^{EtOH} 247 nm ($\varepsilon=16000$), UV_{min}^{EtOH} 221 nm ($\varepsilon=19500$); NMR ($CDCl_3$) δ 1.68 (4H, m, pyranyl ring protons), 2.60 (3H, s, OCH_3), 3.60 (4H, m, pyranyl ring protons), 3.76 (6H, s, CH_3O–C_6H_4), 4.60 (6H, m, H-4',5', of Ura and Guo), 4.72 (2H, d, $J=7.8$ Hz, Cl_3CCH_2O), 5.12 (1H, m, H-2' of Guo), 5.80 (3H, m, H-2',3' of Ura and H-3' of Guo), 6.06 (1H, d, $J_{1',2'}=7.4$ Hz, H-1' of Guo), 6.22 (1H, d, $J_{1',2'}=8$ Hz, H-1' of Ura), 6.82 (4H, d, $J=9$ Hz, *m*-protons of CH_3O–C_6H_4–S), 7.28 (25H, m, Tr, *o*-protons of CH_3O–C_6H_4–S, *m,p*-protons of benzoyl), 7.96 (4H, m, *o*-protons of benzoyl). Found: C, 54.25; H, 4.44; N, 6.18%. Calcd for $C_{74}H_{70}Cl_3N_7O_{20}P_2S_2\cdot H_2O$: C, 54.60; H, 4.46; N, 6.18%.

Synthesis of 19. A mixture of **15** (70 mg, 0.073 mmol) and 2,2,2-trichloroethyl dihydrogenphosphate (33.5 mg, 0.146 mmol) was rendered anhydrous as described above and dissolved in dry pyridine (1 ml). TPS (88 mg, 0.292 mmol) was added and the mixture was stirred at room temperature in the dark for 1 d. Then, the mixture was treated with 1:1 pyridine–water (10 ml) and the resulting solution was kept overnight. Extraction with CH_2Cl_2 (4×10 ml) followed by evaporation gave **16**, which was mixed with 2'-*O*-(4-methoxytetrahydropyran-4-yl)uridine (52 mg, 0.146 mmol).

The mixture was rendered anhydrous by repeated coevaporation with dry pyridine and finally dissolved in dry pyridine (0.2 ml). 1-(Mesitylenesulfonyl)-1*H*-1,2,4-triazole (MST) (73 mg, 0.29 mmol) was added and the mixture was stirred continuously or 4 d. The usual workup gave **19** (66 mg, 60%): UV_{max}^{EtOH} 256 nm ($\varepsilon=45000$), UV_{min}^{EtOH} 227 nm ($\varepsilon=33000$), UV_{sh}^{EtOH} 243 nm ($\varepsilon=39000$); NMR ($CDCl_3$) δ 1.40—1.90 (8H, m, pyranyl ring protons), 2.59 (3H, s, CH_3O), 3.16 (3H, s, CH_3O), 3.60 (8H, m, pyranyl ring protons), 3.81 (6H, s, CH_3O–C_6H_4), 4.36 (8H, m, H-2', 3',4',5' of Ura and H-4',5' of Guo), 4.62 (2H, d, $J=7.8$ Hz, Cl_3CCH_2O), 4.76 (1H, m, H-2' of Guo), 5.00 (1H, m, H-3' of Guo), 5.41 (1H, d, $J_{1',2'}=8$ Hz, H-1' of Ura), 5.72 (1H, d, $J=8$ Hz, H-4 of Ura), 5.88 (1H, d, $J_{1',2'}=7.4$ Hz, H-1', of Guo), 6.88 (4H, d, $J=9$ Hz, *m*-protons of CH_3O–C_6H_4S–), 7.28 (20H, m, Tr, *o*-protons of CH_3O–C_6H_4S–, and H-5 of Ura). Found: C, 50.69; H, 4.86; N, 6.20. Calcd for $C_{66}H_{72}Cl_3N_7O_{20}P_2S_2\cdot2H_2O$: C, 51.08; H, 4.94; N, 6.32%.

Synthesis of 21. A mixture of **19** (104 mg, 0.069 mmol) and 2,2,2-trichloroethyl dihydrogenphosphate (31.4 mg, 0.137 mmol) was rendered anhydrous as described before and dissolved in dry pyridine (1 ml). TPS (83 mg, 0.274 mmol) was added and the mixture was stirred at room temperature for 1 d. The mixture was treated with 1:1 pyridine–water (10 ml) at room temperature overnight and then extracted with CH_2Cl_2 (4×5 ml). The organic extracts were combined, evaporated to dryness, and mixed with 2'-*O*-(4-methoxytetrahydropyran-4-yl)uridine (73.6 mg, 0.206 mmol). After being dried by coevaporation with dry pyridine several times, the mixture was treated with MST (103.4 mg, 0.412 mmol) in dry pyridine (0.2 ml) at room temperature for 5 d. The usual workup gave **21** (76 mg, 54%): UV_{max}^{EtOH} 257 nm ($\varepsilon=55000$); UV_{min}^{EtOH} 228 nm ($\varepsilon=33000$); NMR ($CDCl_3$) δ 1.40—1.98 (12H, m, pyranyl ring protons), 2.60 (3H, s, CH_3O of Guo), 3.10 (6H, s, CH_3O of Ura), 3.68 (12H, m, pyranyl ring protons), 3.80 (6H, s, CH_3O–C_6H_4), 4.40 (13H, m, H-2',3',4',5' of Guo), 4.64 (2H, d, $J=7.8$ Hz, Cl_3CCH_2O), 4.70 (2H, d, $J=7.8$ Hz, Cl_3CCH_2O), 5.04 (2H, m, H-2',3' of Guo), 5.48—6.12 (5H, m, H-1',4 of Ura and H-1' of Guo), 6.86 (4H, d, $J=9$ Hz, *m*-protons of $CH_3OC_6H_4S$–), 7.28 (20H, m, Tr *o*-protons of CH_3O–C_6H_4S–, and H-5 of the first Ura), 7.68 (1H, d, $J=8.2$ Hz, H-5 of the second Ura). Found: C, 47.15; H, 4.72, N, 6.25%. Calcd for $C_{83}H_{94}Cl_3N_9O_{30}P_3S_2\cdot2H_2O$: C, 47.39; H, 4.70; N, 6.00%.

Removal of Two 4-Methoxyphenylthio Groups from 15. To a solution of **15** (8.5 mg, 8.5 μmol) in 2:1 pyridine–water (1 ml) was added silver acetate (28 mg, 0.17 mmol). The solution was vigorously stirred at room temperature for 24 h. The resulting suspension was diluted with 2:1 pyridine–

water (10 ml) and bubbled by hydrogen sulfide at 0 °C until a clear supernatant solution had been obtained (*ca.* 5 min). The black precipitate was removed by centrifugation and the supernatant was passed through a column (1×2 cm) of Dowex 50 W×2 (pyridinium form). The column was washed with 1:5 pyridine–water (50 ml). The eluents were collected and concentrated to dryness. The residue was chromatographed on Whatman 3 MM papers (Solvent I). A band of R_f 0.44 was cut and eluted with 1:1 water–ethanol (200 ml) to give pG^{Tr}(mTHP) as ammonium salt (145 OD): UV_{max}^{EtOH} 256 nm, UV_{min}^{EtOH} 245 nm, UV_{sh}^{EtOH} 270 nm.

Complete Deprotection of pG^{Tr}(mTHP). The pG^{Tr}-(mTHP), (60 OD) obtained in the above experiment was concentrated to dryness and suspended in 0.01 M hydrochloric acid (pH 2.0) (5 ml). The heterogeneous mixture was vigorously stirred at room temperature for 2 d. The resulting emulsion was neutralized with pyridine (1 ml) and the solution was evaporated to dryness. The residue was analyzed by paper electrophoresis using Buffer I to give pG (48 OD) at 252.5 nm (λ_{max}), 99% from **15**, which was identified with the authentic sample.

*Removal of Bis(4-methoxyphenylthio) Group from **19**.* To a solution of **19** (9.3 mg, 6 μmol) in 2:1 pyridine–water (1 ml) was added silver acetate (20 mg, 0.12 mmol). The solution was worked up in the same manner as described in the case of **15**, which gave pG^{Tr}(mTHP)p(tc)U(mTHP) (**24**) (90 OD): R_f 0.4 (Solvent I); UV_{max}^{EtOH} 255 nm, UV_{min}^{EtOH} 228 nm.

*Removal of 2,2,2-Trichloroethyl Group from **24**.* The above-mentioned product (36 OD) was evaporated to dryness and dissolved in 1:2 *N,N*-dimethylformamide–water (0.6 ml). To the solution were added zinc power (3.1 mg, 48 μatom) and acetylacetone (0.06 ml). The resulting mixture was stirred vigorously at room temperature for 12 h. The mixture was diluted with 1:1 pyridine–water (10 ml) and passed through a column (1×3 cm) of Dowex 50 W×2 (pyridinium form). The column was washed with 1:1 pyridine–water (50 ml). The eluents were concentrated, applied to Whatman 3 MM papers, and chromatographed by using Solvent I. A band of R_f 0.27 was cut and eluted with 1:1 water–ethanol (200 ml) to give pG^{Tr}(mTHP)pU(mTHP) (**25**) (30 OD): $UV_{max}^{EtOH/H_2O\ (1:1)}$ 256 nm, $UV_{min}^{EtOH/H_2O\ (1:1)}$ 226 nm.

*Complete Deprotection of **25**.* Compound **25** (15 OD) obtained in the above experiment was evaporated to dryness and suspended in 0.01 M hydrochloric acid (pH 2.0) (2 ml). The suspension was stirred vigorously at room temperature for 2 d. The resulting emulsion was neutralized with pyridine (1 ml). The solution was concentrated, applied to Whatman 3 MM papers, and chromatographed (Solvent II) for 2 d to give pGpU (a band moved 2 cm from the origin, 12 OD) which was eluted with 0.05 M phosphate buffer (pH 7.0) (50 ml): $UV_{max}^{pH\ 7.0*}$ 256 nm, $UV_{max}^{pH\ 7.0*}$ 228 nm (*0.05 M phosphate buffer).

Enzyme Assay of pGpU. The dimer, pGpU (10 OD), obtained in the above experiment was incubated with snake venom phosphodiesterase (10 μg) in 0.1 M Tris–HCl buffer (pH 8.0, 0.12 ml) at 37 °C for 12 h. Then, the mixture was treated with pyridine (0.5 ml), applied to Toyo Roshi No. 51 A paper, and analyzed by paper electrophoresis (pH 3.5, 1500 V, 3 h), which showed the two bands of pG and pU eluted with water in the ratio of 0.89:1.00.

*Removal of Bis(4-methoxyphenylthio) Group from **20**.* To a solution of **20** (18.3 mg, 8.7 μmol) in 2:1 pyridine–water (0.5 ml) was added silver acetate (29 mg, 0.174 mmol). The solution was vigorously stirred at room temperature for 14 h. The same workup as described in the case of **15** gave pG^{Tr}(mTHP)p(tc)U(mTHP)p(tc)U(mTHP) (150 OD): R_f 0.41 (Solvent I) R_f 0.67 (Solvent D); UV_{max}^{EtOH} 250 nm.

Removal of 2,2,2-Trichloroethyl Group from pG^{Tr}(mTHP)p(tc)-U(mTHP)p(tc)U(mTHP). An aqueous ethanol solution of the product (60 OD) obtained above was evaporated and dissolved in 2:1 pyridine-*N,N*-dimethylacetamide (1 ml). To the solution was added zinc powder (9.2 mg, 0.14 mmol) and acetylacetone (0.1 ml). The resulting mixture was vigorously stirred at room temperature for 16 h. The same workup as described in the case of **24** gave a band of R_f 0.58 (Solvent II), which was eluted with 1:1 water–ethanol (100 ml) to give pG^{Tr}(mTHP)pU(mTHP)pU(mTHP) (30 OD): $UV_{max}^{EtOH\text{-}H_2O\ (1:1)}$ 252 nm.

Complete Deprotection of pG^{Tr}(mTHP)pU(mTHP)pU(mTHP). An aqueous ethanol solution of pG^{Tr}(mTHP)pU(mTHP) (30 OD) was evaporated to dryness and suspended in 0.01 M hydrochloric acid (3 ml). The suspension was vigorously stirred at room temperature for 4 d. The resulting emulsion was quenched with pyridine (1 ml). The solution was applied to Whatman 3 MM papers and chromatographed by using Solvent II to give pGpUpU (20 OD, *ca.* 40% from **20**): R_f 0.33 (Solvent II): $UV_{max}^{pH\ 7.0}$ 255 nm, $UV_{max}^{pH\ 7.0}$ 237 nm.

Enzyme Assay of pGpUpU. The trimer, pGpUpU (15 OD), was incubated with snake venom phosphodiestrase (15 μg) in 0.1 M Tris–HCl buffer (pH 8.0, 0.12 ml) at 37 °C for 12 h. Paper electrohoresis (Buffer II) showed the two bands corresponding to pG and pU, which was eluted in the ratio of 1.00:2.06.

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