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SYNTHESIS OF OLIGORIBONUCLEOTIDES BY USE OF S,S-DIPHENYL N-MONOMETHOXYTRITYL RIBONUCLEOSIDE 3′-PHOSPHORODITHIOATES

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Abstract—The fully protected ribonucleotide units (**6a–d**) have been synthesized in 42–62% overall yields by the 5- or 6-step reactions. The dimethoxtrityl, monomethoxytrityl, tetrahydropyran-2-yl, and phenylthio groups were introduced onto the 5′-OH, *exo*-amino, 2′-OH, and 3′-phosphoryl functions, respectively. The units were converted to the OH or phosphodiester components by treatment with trifluoroacetic acid or with phosphinic acid–triethylamine. Both the components were appropriately coupled by means of mesitylenedisulphonyl chloride 3-nitro-1,2,4-triazole to give dimers in high yields. This method was successfully applied to the synthesis of GpUpApUpUpApApUpAp, i.e. the 5′-terminal base sequence of brome mosaic virus mRNA No. 4 filament.

In oligoribonucleotide synthesis, several research groups[1] have proposed appropriately protected mononucleotide units as key intermediates, from which a 5′-OH or 3′-phosphate protecting group was selectively removed for chain elongation. In the phosphotriester approach, a combination of the 2′- and 5′-protecting groups should be chosen carefully because selection of the 5′-OH protecting group depends on the stability of the 2′-OH protecting group. The dimethoxytrityl group has been used with the tetrahydropyran-2-yl,[2] 4 - methoxytetrahydropyran - 4-yl,[3] o-nitrobenzyl,[4] t-butyldimethylsilyl,[5] methoxybenzyl[6] groups. Since Markiewicz[7] reported that the tetraisopropyldisiloxan - 1,3 - diyl (TIPS) group was useful for the simultaneous protection of ribonucleoside 3′,5′-diols, the tetrahydropyranyl-type of protecting groups became more practical because they could easily be introduced on the 2′-OH under the usual conditions. From economical and practical points of view, our attention was focused on commercially available reagents for construction of the four common protected ribonucleotide units. In a previous communication,[8] we reported briefly a new method for the synthesis of oligoribonucleotides by use of S,S-diphenyl N - monomethoxytrityl - 2′ - O-(tetrahydropyran - 2 - yl) - 5′ - O - dimethoxytritylribonucleoside 3′ - phosphorodithioates.

In this paper, we report the details of the new approach and its application to the synthesis of a nonaribonucleotide, GUAUUAAUA,which appears in the 5′-terminal structure of brome mosaic virus mRNA No. 4 filament.[6]

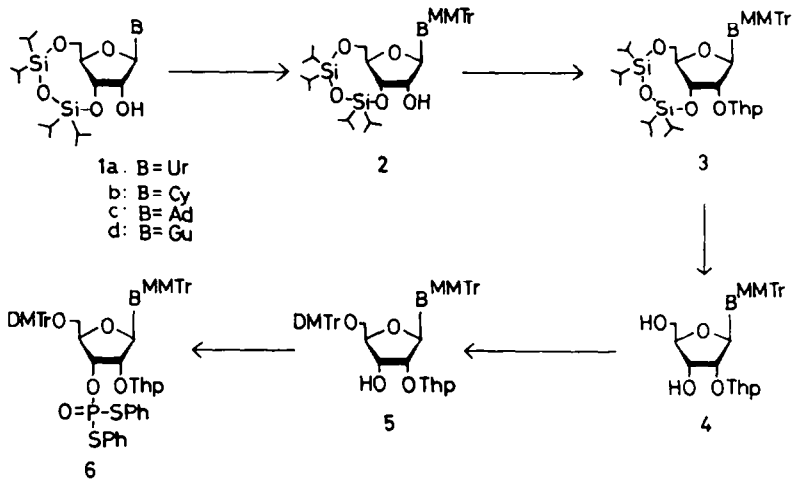
RESULTS AND DISCUSSION

The outline of synthesis of the four common units (**6a–d**) is shown in Scheme I. For the synthesis of intermediates **3b–d** containing an *exo*-amino function, an alternative way *via* pyranylation of **1b–d** followed by tritylation is possible. However, the pyranylation of **1d** led to a bis(tetrahydropyranyl) derivative (**7**). All attempts to obtain selectively the 3′ - O - pyranylated product have failed. Therefore, we chose the generally applicable method as depicted in Scheme I whereupon highly lipophilic N - monomethoxytrityl - 5′ - O - dimethoxytrityl - nucleosides **3b–d** were obtained in high yields.

DMAP-catalyzed reactions proceed very rapidly compared with the usual tritylation in pyridine. Even at the initial stage (after 10 min) *ca* 80% of conversion was attained. In the tritylation no 2′ - O - monomethoxytritylated product was detected. The highly selective introduction of the MMTr group was rationalized in terms of a steric bulk of the neighbouring 3′,5′-TIPS group. No isomerization of the TIPS group during the reaction and workup was observed. The introduction of the MMTr group at the early stage facilitated isolation of a series of compounds **2–6** which could be eluted usually with only methylene chloride from a silica gel column. Sometimes co-solvents such as hexane were added to the solvent for the satisfactory separation of the product from excess reagents.

The reaction of **2a–d** with 2,3-dihydropyran in the presence of *p*-toluenesulphonic acid in dioxan was carried out to obtain **3**. However, large amounts of polymeric materials were always present with higher R_F's than the desired products and caused difficult separations. The problem was not severe in the case of **3b** and **3d** which were located separately from the byproducts and were isolated. In the case of **3a** and **3c**, purification was performed after the desilylation. The pyranylation of **2a** resulted in a complex mixture containing large amounts of colored byproducts. Therefore, pyridinium p-toluenesulphonate[11] was employed to avoid the coloration. At this stage, a pair of diasteroisomers due to the THP group were observed on TLC in the case of **3a** and **3c**. (Table 10). In all the pyranylations, the MMTr groups on the base residues were found to be stable.

Previously, we reported that a combined reagent of triethylammonium chloride and potassium fluoride, a modification of Carpino's procedure,[12] was effective for removal of the TIPS group from 3. We found that tetraethylammonium bromide could be used as well as the chloride. The desilylation was accelerated a



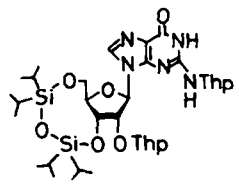
Scheme 1.

little by use of the former. This seems to be due to more rapid salt exchange so that the reactive species, tetraethylammonium fluoride, was produced. For the purification of the uridine derivative **4a**, pretreatment with Dowex 50 W X2 was required for removal of the excess ammonium salt. If this treatment was eliminated, the THP was lost to a considerable extent during chromatography. In other cases, the products were extracted with methylene chloride and then purified by chromatography. At this stage, the diasteroisomers of **4a** and **4b** can be separated by chromatography but they were isolated as the mixture for the reason given in a later section. The usual dimethoxytritylation of **4** and **5** in high yield.

Bifunctional condensing agents have been proved to be useful for the synthesis of oligonucleotides in the liquid phase since they play also a role of separation of desired products from sulphonylated byproducts.[13] Phosphorylation of 5 with cyclohexylammonium S,S-diphenyl phosphorodithioate (PSS)[14] was conducted by the use of two kinds of bifunctional condensing agents, 1,3-dimethoxy-benzene-(sulphonyl)-2,4-disulfonyl chloride (DMS)[13] and mesitylenedisulphonyl chloride (MDS).[13] The use of DMS resulted in prolonged reaction times although the reagent was very mild. On the other hand, the 3′-free nucleosides **5a–c** underwent facile phosphorylation with PSS in the presence of MDS to afford the fully protected nucleotide units (**a–c**) in high yields. In the case of the guanosine derivative **6d**, base modification was observed during the phosphorylation.[15,16] As the reaction proceeded, an O^6-phosphorylated byproduct accumulated to a considerable extent and appeared a little higher than the desired product **6d**. TLC analysis indicates that the side reaction occurred at a rate similar to that of the 3′-O-phosphorylation. Therefore, **4** equiv of PSS and 1.5 equiv of MDS were employed. Upon subsequent treatment with 0.25 M triethylammonium bicarbonate (TEAB), the diphosphorylated species was converted to **6d**. Generally, the phosphorylation of **5** was complete in 1 hr. All the phosphorylated nucleotide units **6a–d** appeared as single spots on TLC. Therefore, it is not necessary to separate the diastereomers during the course of a series of reactions. It was also confirmed that when each of the isomers **5a** and **5a′** was phosphorylated separately, there was no difference in reactivity between them. Thus, the fully protected units could be obtained in 42–62% overall yields.

Selective deprotection of the DMTr Group from **6**

The combination of the 5′-DMTr and 2′-THP groups has previously been used by Smrt[17] and



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Takaku,[18] who chose the conditions of 80% acetic acid at 0° and 2% toluenesulphonic acid in CH_2Cl_2–MeOH (7:3, v/v) at 0° for selective removal of the DMTr group. Initially, we employed the latter since the workup was easier and obtained the hydroxyl derivatives **8a** and **8b** in 70–85% yields. However, a significant loss of the THP group was often observed before the complete removal of the DMTr group. Since the partial loss of the THP group became severe for condensation of longer oligomers, we have searched for alternative procedures. First, we have attempted to use zinc bromide in nitromethane[19] and ferric chloride in CH_2Cl_2.[20] However, the former resulted in heterogeneous mixtures owing to the insolubility of the units **6** in this medium. The latter led to the simultaneous deprotection of the MMTr group. Markiewicz[3] reported that 0.1% trifluoroacetic acid in $CHCl_3$ at 0° was useful for the selective removal of the DMTr group from 2′ - O - (4 - methoxytetrahydropyran - 4-yl) - 5′ - O - dimethoxytritylribonucleosides with a 3′-protected phosphate. Therefore, we applied this

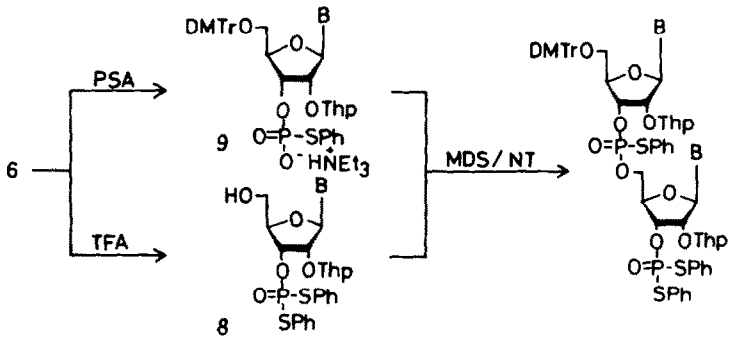


Table 1. Synthesis of 2

Unit	Solv. Base-cat.	Time (h)	Product	Yield (%)
C	Py	48	2c	75
	CH_2Cl_2-Et_3N-DMAP	12	2c	80
A	Py	48	2b	96
	CH_2Cl_2-Et_2N-DMAP	3	2b	95
G	Py	48	2d	92
	CH_2Cl_2-Et_2N-DMAP	3	2d	92

Table 2. Synthesis of 3

Unit	Catalyst	Product	Yield (%)
U	PPTS	3a	—
C	TsOH	3b	97
A	TsOH	3c	—
G	TsOH	3d	86

Table 3. Synthesis of 4

Unit	Agent	Time (h)	Temp (°C)	Product	Yield (%)
U	Et_4NBr-KF	1	55	4a	81[a] from 2a
C	Et_4NBr-KF	4	50	4c	75[b]
A	Et_4NBr-KF	1.5	53	4b	84[b] from 2c
G	Et_4NCl-KF	20	r.t.	4d	93

Table 4. Synthesis of 5

Unit	DMTrCl (equiv)	Time (h)	Product	Yield (%)
U	1.46	3	5a	93
C	1.03	4	5b	97
A	1.5	3	5c	89
G	1.5	1.5	5d	91

Table 5. Synthesis of 6

Unit	Condensing Agent	Time (h)	Product	Yield (%)
U	DMS (1.5)	15	5a	89
C	DMS (2.0)	5	5c	70
C	MDS (2.0)	1	5c	89
A	DMS (1.5)	5	5b	86
A	MDS (2.0)	1	5b	88
G	DMS (1.5)	5	5d	77
G	MDS (2.0)	1	5d	90

Table 6. Physical Properties of Compounds 2–6

Compound	Rf Value (Solv.)[a]	M.P.[b] (°C)	Elemental analysis formula Calcd(%) Found(%) C C H H N N	UV (EtOH) λ max (ε x 10^{-4}) λ min (ε x 10^{-4})	^{1}H NMR ($CDCl_3$) δ (ppm)
2b	0.94(A) 0.85(C)		$C_{41}H_{55}N_3O_7Si_2$ 64.96 64.80 7.31 7.27 5.54 5.55	285(1.68) 249(1.16)	0.95(m, 28H, CH_3CSi), 3.76(s, 3H, CH_3O-), 3.93-4.24(m, 5H, H-2',3',4', 5'), 5.07(d, 1H, J=7.5Hz, H-5), 5.77 (s, 1H, H-1'), 6.81(m, 2H, ArH), 7.2 (m, 12H, ArH), 7.68(m, 1H, H-6)
2c	0.96(A) 0.83(C)		$C_{42}H_{55}N_5O_6Si_2$ 64.51 64.88 7.08 7.05 8.95 9.04	273(2.65) 240(1.17)	1.05(d, 28H, CH_3CSi), 3.67(s, 3H, CH_3O-), 4.06(br s, 3H, H-4',5'), 4.5 (br s, 1H, H-2'), 5.00(br s, 1H, H-3'),5.83(s, 1H, H-1'), 6.71(d, 2H, J 8Hz, m-H of MMTr), 6.93-7.24(m, 12H, ArH), 7.87(s, 1H, H-2), 7.97(s, 1H, H-8).
2d	0.69(A) 0.59(B)		$C_{42}H_{55}N_5O_7Si_2\ H_2O$ 62.11 61.81 6.76 6.76	260(1.94) 249(1.44)	1.05(m, 28H, CH_3CSi), 3.64(5, 3H, CH_3O-), 3.94(m, 3H, H-4',5'), 4.17(m 2H, H-2',3'), 5.22(s, 1H, H-1'), 6.6 (d, 2H, H=8Hz, ArH), 6.94-7.44(m, 12H, ArH), 7.76(s, 1H, H-8)
3a	0.62(A) 0.51(B)				
3b	0.87(A) 0.77(B)		$C_{46}H_{63}N_3O_8Si_2\ 0.5H_2O$ 64.90 65.02 7.58 7.19 4.98 4.78	278(2.10) 247(1.56)	0.92(m, 28H, CH_3C-Si), 1.62(H, 6H, C- methylene of Thp), 3.52(m, 2H, O- methylene of Thp), 3.62(s, 3H, CH_3O) 3.92-4.29(m, 5H, H-2',3',4',5'), 5.0 (d, J=7.5Hz, 1H, H-5), 5.92(s, 1H, H- 1'), 6.74(d, J=8Hz, 2H, ArH), 7.19(m 13H, ArH), 7.68(dd, J=7.5Hz, 1H, H-6
3c	0.97(A) 0.67, 0.59(D)				
3d	0.73(A) 0.64(B)		$C_{47}H_{63}N_5O_8Si_2\ H_2O$ 62.71 62.72 7.29 7.13 7.78 7.77	264(1.96) 244(1.62)	1.06(m, 28H, CH_3CSi), 1.55(m, 6H-C- methylene of Thp), 3.44(m, 2H, O- methylene of Thp), 3.72(s, 3H, CH_3O) 3.96(m, 3H, H-4',5'), 4.17(m, 2H, H- 2',3'), 4.79(br s, 1H, acetal proton of Thp), 5.43(br s, 1H, H-1'), 6.74(dd, J=8Hz, 2H, ArH), 7.24(m, 12H, ArH), 7.65(s, 1H, H-8)
4a	0.35, 0.42(A) 0.22, 0.29(B)				
4b	0.73(A) 0.64(B)	135-137		277(1.52) 247(1.06)	1.54(m, 6H, C-methylene of Thp), 3.3 (m, 2H, O-methylene of Thp), 3.77(s, 3H, CH_3O), 4.01(m, 2H, H-5'), 4.40(m 2H, H-3',4'), 4.58(m, 1H, H-2'), 4.7 (br s, 1H, acetal proton of Thp), 5.07(d, J=7.5Hz, 1H, H-5), 5.61(dd, J=7.5Hz, 1H,H-1'), 6.77(d, J=8Hz, 2H ArH), 7.58(d, J=7.5Hz, 1H, H-6)
4c	0.77, 0.89(A) 0.66, 0.81(B)	116-118	$C_{35}H_{37}N_5O_6\ 0.5H_2O$ 66.44 66.53	275(2.23) 246(1.01)	1.52(m, 6H, C-methyl of Thp), 3.54(r 2H, O-methylene), 3.76(s, 3H, CH_3O-) 3.86(m, 2H, H-5'), 4.27(m, 1H, H-4' 4.52(m, 1H, H-2'), 4.82(m, 1H, aceti proton), 5.06(m, 1H, H-3'), 5.89(d, J=6Hz, H-1'), 6.75(d, 2H, J=8Hz, Ar 7.27(m, 12H, MMTr), 7.81(s, 1H, H-2 8.00(s, 1H, H-8)
4d	0.47, 0.51(A) 0.27, 0.34(B)	156-158	$C_{35}H_{37}N_5O_7$ 65.71 66.02 5.83 6.04 10.95 10.61	260(1.42) 247(1.22)	1.45(m, 6H, C-methylene of Thp), 3, m, 2H, O-methylene of Thp), 3.67(s, CH_3O), 3.91(m, 3H, H-4',5'), 4.17(m 2H, H-2',3'), 5.50(bs, 1H, H-1'), 6 (d, J=8Hz, 2H, ArH), 7.24(m, 12H, A 7.74(s, 1H, H-8)
5a	0.67, 0.76(A) 0.57, 0.67(B)	135-137	$C_{34}H_{34}N_3O_7$ 68.10 67.71 6.22 6.41 7.01 6.95	277(1.52) 247(1.06)	1.65(m, 6H, C-methylene of Thp), 3. m, 4H, H-5' amd O-methylene of Thp) 3.77(s, 9H, CH_3O), 4.11(m, 1H, H-4 4.42(m, 2H, H-2',2'), 4.81(br s, 1H Acetal proton of Thp), 5.33(d, J=8H 1H, H-5), 5.98(s, 1H, H-1'), 6.83(8Hz, 2H, ArH), 7.32(m, 13H, ArH), d, J=8Hz, 1H, H-6),

Table 6. (*Contd*)

Compound	Rf Value (Solv.)[a]	M.P.[b] (°C)	Elemental analysis formula Calcd(%) Found(%) C H N / C H N	UV (EtOH) λ max (ε x10^{-4}) λ min (ε x10^{-4})	^{1}H NMR ($CDCl_3$) δ (ppm)
5b	0.90(A) 0.84(B)	138-140	$C_{55}H_{55}N_3O_9$ H_2O 71.80 72.10 6.24 6.20 4.57 4.37	278(1.66) 258(1.40)	1.58(m,, C-methylene of Thp), 3.32(m, 4H, H-5'and O-methylene of Thp), 3.72(s, 9H, CH_3O), 3.95(m, 1H, H-4'), 4.11(m, 1H, H-2'), 4.53(m, 1H, H-3'), 4.68(d, J=7.5Hz, 1H, H-5), 4.92(br s, 1H(acetal-proton of Thp), 6.19(d, J=4.5Hz, 1H, H-1'), 6.72(m, 6H, ArH), 7.22(m, 31H, ArH, of MMTr and PSS), 7.62(m, 1H, H-6)
5c	0.92(A) 0.62, 0.71(C)	123-125	$C_{56}H_{55}N_5O_8$ $0.5H_2O$ 71.93 72.11 6.14 6.04 7.49 7.44	275(3.47) 251(2.38)	1.51(m, 6H, C-methyleme of Thp), 3.20-3.54(m, 3H, O-methyleme of Thp and H-5'), 3.70(s, 9H, CH_3O), 4.23(m, 1H, H-4'), 4.47(m, 1H, H-2'), 4.69(br s, 1H, acetal proton of Thp), 4.97(m, 1H, H-3'), 6.15(dd, 1H, J=6Hz, H-1'), 6.76(d, 6H, J=8Hz, m-proton of MMTr and DMTr), 7.25(m, 21H, ArH), 7.96(s, 1H, H-2), 8.00(s, 1H, H-8), 8.56(1H, NH)
5d	0.66, 0.69(A) 0.58, 0.62(B)	135-137	$C_{56}H_{55}N_5O_9$ $0.5H_2O$ 70.72 70.43 5.93 5.99 7.36 7.09	262(2.52) 251(2.38)	1.50(m, 6H, C-methylene of Thp), 3.35(m, 2H, O-methylene of Thp), 3.62(s, 3H, CH_3O of MMTr), 3.72(s, 6H, CH_3O of DMTr), 4.04(m, 4H, H-3',4',5'), 4.30(m, 1H, H-2'), 5.52(d, J=4.5Hz, 1H, H-1'), 6.64(d, J=8Hz, 2H, ArH of MMTr), 6.73(d, J=8Hz, 4H, ArH of DMTr), 7.21(m, 21H, ArH), 7.47(s, 1H, H-8)
6a	0.90(A) 0.80(B)	95-98.5	$C_{47}H_{47}N_2O_{10}PS_2$ 63.07 63.24 5.29 5.33 3.12 3.21 S: 7.16 7.36	sh 253(2.18)	1.60(m, 6H, C-methylene of Thp), 3.37-3.53(m, 4H, H-5' and O-methylene of Thp), 3.77(s, 6H, CH_3O), 4.03(m, 1H, H-4'), 4.72(m, 1H, H-2'), 4.91(br s, 1H, acetal proton of Thp), 5.21(m, 1H, H-3'), 5.31(m, 1H, H-5), 6.22(dd, J=7.5 Hz, 1H, H-1'), 6.80(d, J=8Hz, 4H, ArH), 7.29(m, 19H, ArH), 7.62(d, J=8Hz, H-6)
6b	0.94(A) 0.84, 0.88(B)	112-115	$C_{67}H_{64}N_3O_{10}PS_2$ 69.00 69.36 5.53 5.16 3.25 3.60 S: 5.39 5.50	274(1.52) 263(1.47)	1.56(m, 6H, C-methylene of Thp), 3.32(m, 4H, H-5' and O-methyleme of Thp), 3.72(s, 9H, CH_3O-), 3.95(m, 1H, H-4'), 4.11(m, 1H, H-2'), 4.53(m, 1H, H-3'), 4.68(d, J=7.5Hz, 1H, H-5), 4.92(br s, 1H acetal proton of Thp), 6.19(d, J=4.5Hz, 1H, H-1'), 6.72(m, 6H, ArH), 7.22(m, 31H, ArH of MMTr, DMTr and PSS), 7.62(m, 1H, H-6)
6c	0.96(A) 0.79(C)	100-103	$C_{68}H_{64}N_5O_9PS_2$ 70.19 68.61 5.42 6.60 5.88 5.37 S: 5.39 5.21	271(6.85) 246(3.57)	1.61(m, 6H, C-methylene of Thp), 3.00-3.59(m, 4H, H-5' and O-methylene of Thp), 3.75(s, 9H, CH_3O), 4.17(m, 1H, H-4'), 4.53(br s, 1H, H-2'), 4.83(m, 1H, acetal proton of Thp), 5.35(m, 1H, H-3'), 6.18(d, 1H, J=6Hz, H-1'), 6.78(d, 6H, J=8Hz, m-proton of MMTr and DMTr), 7.31(m, 31H, ArH), 8.00(s, 1H, H-2), 8.08(s, 1H, H-8)
6d	0.71(A) 0.67(B)	133(dec)	$C_{68}H_{64}N_5O_{10}PS_2$ 67.00 67.81 5.35 5.35 5.80 5.85 S: 5.32 4.56	261(3.32) 251(3.26)	1.52(m, 6H, C-methylene of Thp), 3.20(m, 2H, O-methylene of Thp), 3.58(s, 9H, CH_3O), 4.02(m, 3H, H-4',5'), 4.27(m, 1H H-2'), 4.76(m, 1H, acetal proton of Thp), 5.06(m, 1H, H-3'), 5.59(dd, J=7.5 Hz, 1H, H-1'), 6.75(d, J=8Hz, 6H, ArH), 6.94-7.71(m, 33H, ArH of DMTr, MMTr, PSS and H-8)

CH_2Cl_2-MeOH (9:1, v/v), B: CH_2Cl_2-MeOH (12:1, v/v), C: CH_2Cl_2-MeOH(20:1, v/v), D: CH_2Cl_2-MeOH(80:1, v/v)

npounds 2b-d and 3a-d did not have clear melting points. All the melting points of 4a-b were measured after reprecipitation from r CH_2Cl_2 solutions into hexane-ether (9:1, v/v).

Table 7. Concentration of TFA required for complete removal of the DMTr group from oligomers within 1 hr

Fully protected oligomer	Concentration of TFA in $CHCl_3$
U	0.2–0.3%
A	
AA	0.3–0.4%
UAA	0.5%
AUUA	0.55%

Table 8. Conditions and results of removal of the DMTr group from oligomers

Fully protected oligomer	concentration of TFA in CH_2Cl_2[a]	Time (min)	Rf value[b] (silica gel)	
U	2% TsOH/0°C (CH_2Cl_2- MeOH, 7:3)	30	0.53, 0.55 (A) 0.38, 0.42 (B)	79
A	0.15	120		91
	0.5	12	0.92 (A)	84
	0.4	50	0.79 (B)	92
G	0.5	15	0.67 (A)	94
AA	0.4	80	0.50 (B)	77
AU	0.5	45	0.44 (B)	77
UAA	0.4	70	0.56 (B)	74
AUUA	0.55	18	0.54 (A) 0.46 (B)	72
UAAUA	0.55	38	0.54 (A)	82
UAAUAA	0.55	65	0.55 (A) 0.45 (B)	81

[a] The deprotection was carried out at 0°C
[b] A: CH_2Cl_2-MeOH (9:1, v/v), B: CH_2Cl_2-MeOH (12:1, v/v)

system to the conversion of **6c** to **8c**. Under the same conditions (0.1% TFA–$CHCl_3$ 100 mL/mmol) as in the case of the above units, **8c** was obtained in 71% yield after 3 hr. However, after 1.5 hr, a spot which had lost the THP group began to appear on TLC. We found that the use of a more concentrated solution of TFA resulted in more rapid elimination and no loss of the THP group. Upon treatment of **6c** with 0.15–0.5% TFA within 1 hr, **8c** could be obtained in 91–92% yield. These conditions were successfully applied to the other nucleotide derivatives.

Selective deprotection of one phenylthio group from **6a–d**

Our previous papers[8,13,21] showed that one of two phenylthio groups could be removed from S,S-diphenyl nucleoside phosphorodithioates of the triester-type by means of phosphinic acid (PSA) in pyridine under very mild conditions. The latter reagent is capable of the selective removal of a phenylthio group without damage of the acid-labile DMTr and alkali-labile acyl groups. The reaction was also accelerated at elevated temperatures or by use of an excess of PSA. In the ribo series, the phenylthio group was found to be a little more stable than in the deoxyribo series because of the steric hindrance of the neighbouring THP group. Therefore, we attempted the removal of one phenylthio group using 15 equiv of PSA at 40° for 4 hr. However, these conditions caused the simultaneous deprotection of the DMTr group to some degree (*ca.* 5%). Since the removal of the phenylthio group is known to be independent of the kind of salt of phosphinic acid,[22] we added triethylamine to the PSA solution in pyridine. As a result, a 4 M solution of triethyl- ammonium phosphinate in pyridine gave **9** in high yield without any loss of the DMTr group.

Synthesis of oligoribonucleotides

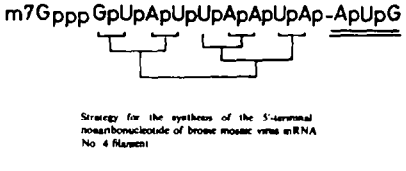
In order to demonstrate the utility of the new type of ribonucleotide units we decided to synthesize a nonaribonucleotide of GUAUUAAUA which is present in the 5′-terminal leader sequence of brome mosaic virus mRNA No. 4 filament. This RNA fragment will be useful for the biological study on the leader sequence of eukaryotic mRNA between the 5′-end and the initiation codon AUG. Therefore, some dinucleotides were prepared by the condensation of **8** with **9** in the presence of bifunctional condensing agents. Since the combination of mesitylenedisulphonyl chloride (MDS) and tetrazole, reported previously, was not applicable to the synthesis of oligonucleotides with relatively high contents of U and G owing to their side reactions,[15,16] we chose a combined reagent of MDS and 3-nitro-1,2,4-triazole (NT), which resulted in less side reactions. The conditions and results are summarised in Tables 9–10. The rate of removal of the DMTr group decreased with an increase in the chain length of oligomers. On the contrary, the selective deprotection

Table 9. Synthesis of dimers

Diester component (mmol)		Hydroxyl component	Ratio of diester/ hydroxyl	Condensing agent MDS (equiv)	Azole[a] (equiv)		Time (min)	Rf[b] A	B	C	Yield (%)
A	0.5	U	0.97	3	Te	6	2	0.91	0.85		74
A	0.5	U	1.20	3	NT	3	0.75				76
U		A	1.19	3	Te	6	1				68
U	0.3	A	1.25	1.5	NT	1.5	0.75				77
A		A	1.25	3	Te	3	1.5		0.66	0.53	80
A	0.48	A	1.20	3	NT	3	0.75				85
G	0.32	U	1.24	3	NT	3	0.75	0.51	0.44		57
U	0.11	U	1.10	3	NT	3	0.25	0.61	0.47		84

[a] Te and NT refer to 1H-tetrazole and 3-nitro-1,2,4-triazole.
[b] A: CH_2Cl_2-MeOH (9:1, v/v), B: CH_2Cl_2-MeOH (12:1, v/v), C: CH_2Cl_2-MeOH (20:1, v/v)

of a 3′-terminal phenylthio group from oligomers by PSA seemed to be independent of the chain length. The rate was increased by use of a higher concentration of PSA. The problem is that oligomers longer than dimers have internal phenylthio groups that must not be removed simultaneously by the PSA treatment. In fact, the use of a 5 M solution of triethylammonium phosphinate (25 equiv) in pyridine led to the internal P–S bond cleavage to a significant extent. However, the unwanted reaction could be avoided by use of smaller amounts of triethylamine. Finally, we found that a 4 M solution of phosphinic acid (25 equiv) containing 5 equiv of triethylamine in pyridine was suitable for the selective removal of the 3′-terminal phenylthio group. Thus, the protected oligomers listed in Table 10 were synthesized in good yields by the condensation between the hydroxyl and diester components. Table 10 implies that the guanosine containing oligomers were obtained in relatively low yields.



Strategy for the synthesis of the 5′-terminal nonanbonucleotide of brome mosaic virus mRNA No 4 Rna segment

Deprotection of the protecting groups

There are several methods for removal of the phenylthio group. It can be removed by the silver ion catalyzed hydrolysis[21] or by the oximate-promoted hydrolysis.[23] For removal of the acid labile protecting groups, 0.01 M HCl in dioxan–water (1:1) adjusted to pH2.0 by addition of 0.1 M HCl was employed whereby the DMTr and MMTr groups could be removed effectively in homogeneous solutions. In order to ascertain the complete removal at each step, the fully protected pentamer UAAUA was treated subsequently with PSA, tetramethylguanidium *o*-nitrobenzaldoximate,[23] silver acetate and finally 0.01 M HCl. Thus, UpApApUpAp was obtained in 40% yield. The pentamer was completely degraded by incubation with spleen phosphodiesterase to give Up and Ap in the correct ratio of 2:3. The relatively low yields seem to be ascribable to considerable loss during the isolation procedure, since TLC analysis at each stage showed that each treatment gave a major spot. Therefore, we applied carefully the above procedure to the nonamer and obtained crude unprotected nonamer in 25% yield by paper chromatography. The crude nonamer was further purified by DEAE Sephadex ion exchange chromatography and gel electrophoresis after labelling the 5′-terminal hydroxyl group with γ-^{32}P ATP by using polynucleotide kinase. The nonamer was obtained as the compound having a 3′ - terminal 2′,3′ - cyclic phosphate group. This is probably because the 3′-terminal phenylthio group remaining after the oximate treatment could not be removed effectively by silver acetate and the final acid treatment led to the 2′,3′-cyclization with concomitant elimination of the phenylthio group. The labelled nonamer was completely digested by nuclease P1 and RNase T2 to give ^{32}pG and ^{32}pGp, respectively. Its alkaline hydrolysis also gave only ^{32}pG (2′ or 3′)p. The sequence of the nonamer was determined by standard methods.

EXPERIMENTAL

Mps were determined on a Thomas–Hoover apparatus and are uncorrected. ^{1}H NMR spectra were recorded at 100 MHz on a JNM-PS-100 spectrometer. UV spectra were obtained on a Hitachi 124 spectrophotometer. Paper chromatography was performed by descending technique with Whatman 3 MM papers using solvent I (2 - propanol - concentrated ammonia–water, 7:1:2, v/v/v) and solvent II (1-propanol-concentrated amonia–water, 55:10:35, v/v/v). Column chromatography was performed with silica gel C-200 purchased from Wako Co. Ltd., and a minipump for a goldfish basin was conveniently used to gain a medium pressure for rapid chromatographic separation. HPLC was performed on a IEX-540 column (Toyo soda) using 0.7 M ammonium acetate (pH 7.0) at the flow rate of 0.7 ml/min. Thin layer chromatography was performed on precoated TLC plates silica gel 60 F-254 (Merck). Pyridine was distilled twice from *p*-toluenesulphonyl chloride and from CaH_2 and then stored over molecular sieves 4A. CH_2Cl_2 was dried over P_4O_{10} overnight, decanted, distilled from K_2CO_3, and stored over molecular sieves 4A. TIPSCl was prepared according to the lit procedure.[7] In this preparation the chlorination of 1,1,3,3-tetraisopropyldisiloxane with Cl_2 was carried out at 0°. Elemental analyses were performed by the Microanalytical Laboratory, Tokyo Institute of Technology, at Nagatsuta.

General procedure for synthesis of **2**. For the synthesis of **2**, compounds **1a–c** prepared according to the lit were used. The guanosine derivative **1d** was prepared as follows. A mixture of well pulverised guanosine (2.84 g, 10 mmol) and imidazole (2.72 g, 40 mmol) was coevaporated several times with dry pyridine and suspended in dry DMF (15 mL). TIPSCl (3.32 g, 10.5 mmol) was added with vigorous stirring. After 4 hr, the mixture was poured into water (1 L) and the

Table 10. Synthesis of oligomers

Diester component (mmol)		Hydroxyl component	Phosphinic acid (equiv)	Removal of PhS group: Et_3N (equiv)	Pyridine (ml)	Temp (°C)	Time (min)	Ratio of diester/hydroxyl	Condensing agent: MDS (equiv)	NT (equiv)	Time min	Rf[a] A	Rf[a] B	Yield (%)
U	0.23	AA	18	8	2.3	35	90	1.3	3	3	80		0.60	83
AU	0.1	A	25	2	0.6	35	120	1.1	3	3	40	0.63		85
AU	0.29	UA	25	5	1.8	35	90	1.2	3	3	90		0.50	84
GU	0.12	AU	20	2	0.6	35	210	1.3	3	3	120	0.52	0.43	63
UA	0.39	AUA	25	5	2.5	40	60	1.1	3	3	60	0.50		74
GUAU	0.064	UAAUA	30	15	0.5	38	70	1.4	5	5	120		0.44	59

[a] A: CH_2Cl_2–MeOH (9:1, v/v), B: CH_2Cl_2–MeOH (12:1, v/v), C: CH_2Cl_2–MeOH (20:1, v/v)

ppt was collected by filtration, washed with water and ether, and dried over P_4O_{10} under reduced pressure to give crude **1d** (93%). This was used for the next reaction without further purification.

Compound **1b**, **1c** or **1d** (10 mmol) was coevaporated several times with dry pyridine, and then toluene until the pyridine had been removed completely. The dried material was dissolved or suspended in dry CH_2Cl_2 (40 mL) and treated with MMTrCl (6 g, 19.4 mmol) and DMAP (44 mg, 0.4 mmol). The mixture was stirred until it had become homogeneous, and then Et_3N (2.8 mL, 20 mmol) was added. After the mixture being stirred for 3 hr (in the case of **1c** and **1d**) or 12 hr (**1b**), the reaction was quenched by addition with pyridine–water (1:1, v/v, 30 mL). After the mixture was extracted with CH_2Cl_2 (2 × 30 mL), the extracts were combined, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was chromatographed on a column of silica gel with CH_2Cl_2–hexane (3:7, v/v)→CH_2Cl_2 or CH_2Cl_2–MeOH (9:1, v/v) to give **2**.

General procedure for synthesis of **3**. To a soln of **2** (12 mmol) in dry dioxan (100 ml) or dry CH_2Cl_2 (30 ml) (in the case of **2a**) were added molecular sieves 4A (1 g), *p*-toluenesulphonic acid monohydrate (2.51 g, 13.2 mmol) or pyridinium *p*-toluenesulphonate (3.81 g, 13.2 mmol) and 2,3-dihydropyran (21.9 ml, 240 mmol). After being stirred vigorously for 1 hr or 48 hr, the mixture was hydrolysed by addition of concentrated ammonia (5.2 ml), and the ppt of ammonium *p*-toluenesulphonate was filtered off. The filtrate was evaporated under reduced pressure and then co-evaporated several times with ethanol for removal of the excess dihydropyran. Chromatography of the residue gave **3** as listed in Table 2.

General procedure for synthesis of **4**. A mixture of **3** (12 mmol), a tetraethylammonium halide (72 mmol) and potassium fluoride (4.35 g, 72 mmol) in acetonitrile–water (60 ml–1.3 ml) was stirred with vigorous stirring at room temp or at 50–53° for the times listed in Table 3. Then the supernatant was evaporated under reduced pressure and the residue was chromatographed on a column of silica gel to give **4**. In the case of **4a**, the residue was dissolved in pyridine–MeOH–water (3:1:1, v/v/v, 100 ml) through a column of Dowex 50 W X2 (pyridinium form, 120 ml) and the resin was washed with the above mixed solvent (1 L). The eluent was evaporated and chromatography was performed as described previously.

General procedure for synthesis of **5**. Compound **4** (18 mmol) was coevaporated several times with dry pyridine and dissolved in dry pyridine (40 ml) and DMTrCl (8.5 g, 27 mmol) was added. After being kept at room temp for 1.5–5 hr, the mixture was quenched by addition of water and extracted with CH_2Cl_2. The usual workup gave **5**.

General procedure for synthesis of **6**. To a soln of PSS (1.15 g, 3 mmol), dried by coevaporation with dry pyridine, in dry pyridine (20 ml) was added MDS (1.27 g, 4 mmol). The soln was kept for 30 min and then added to **5** (2.0 mmol) dried by coevaporation with dry pyridine. After being stirred for 1 hr, the mixture was quenched by addition of water and extracted with CH_2Cl_2 (3 × 30 ml). The organic extracts were combined, washed with 0.25 M TEAB (2X 20 ml) and water (2X 20 ml), and dried over na_2SO_4. After removal of the solvet under reduced pressure, the residue was coevaporated several times with toluene and chromatographed to give **6**. In the case of **5d**, 4.56 g (12 mmol) of PSS and 1.43 g (4.51 mmol) of MDS were used and the residue obtained after removal of the solvent was treated with pyridine (20 ml)-0.25 M TEAB (20 ml) for 30 min. Extraction with CH_2Cl_2 followed by chromatography gave **6d**

General procedure for removal of the DMTr group. To a soln of an appropriate monomer unit or oligomer (0.5 mmol) in dry $CHCl_3$ (25 ml) at 0° was added 1% TFA in $CHCl_3$ (25 ml). The soln was kept at 0° for 12 min and then a mixture of pyridine–water (1:1, v/v, 40 ml) was added. The usual extractive workup followed by chromatography gave the hydroxyl component as listed in Table 8.

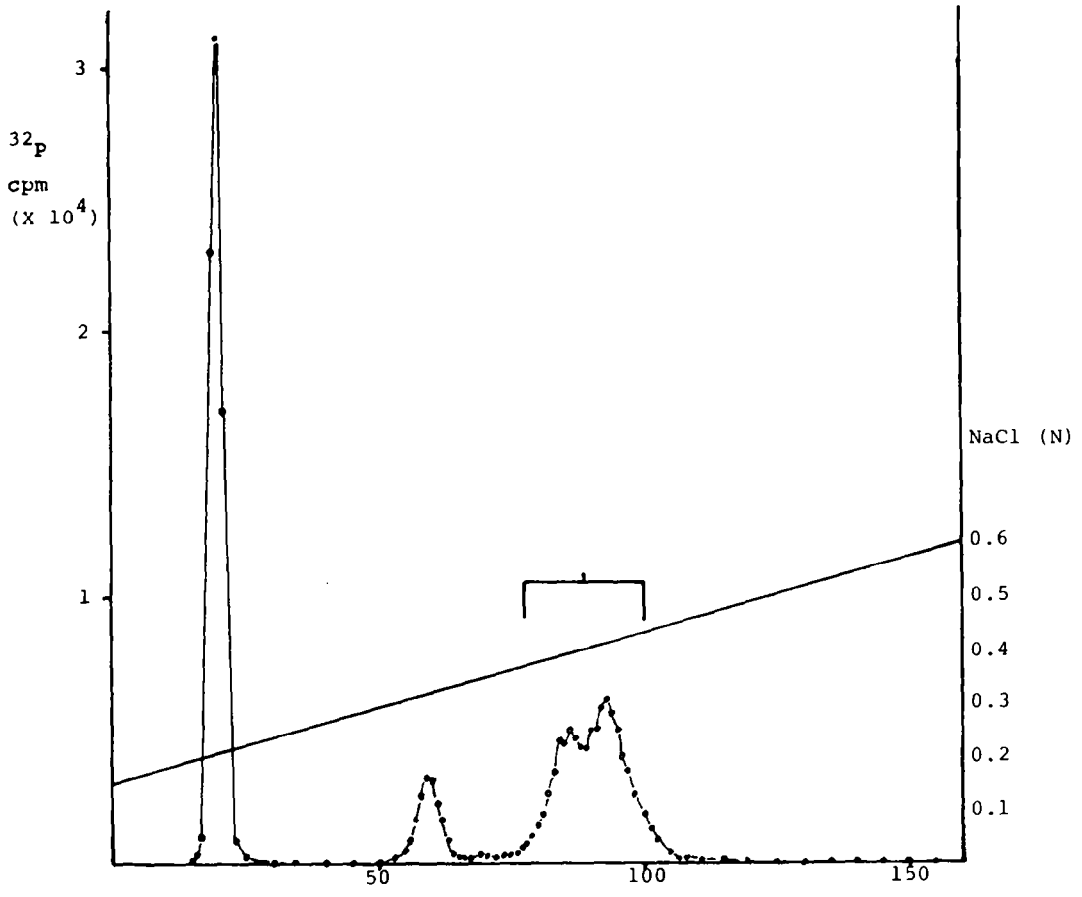


Fig. 1. Separation of the labeled nonamer by DEAE A52 ion exchange chromatography.

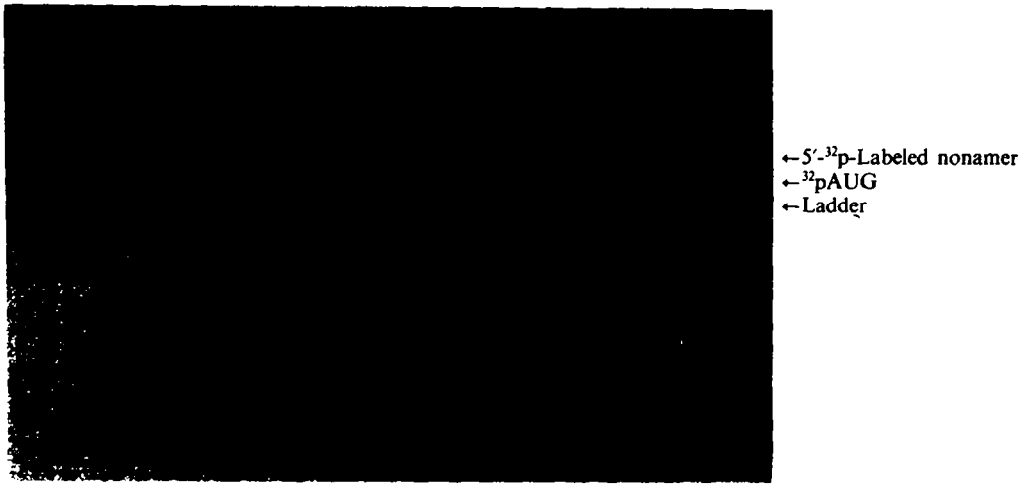


Fig. 2. 20% Acrylamide gel electrophoresis in 7 M urea of the 5′-^{32}P-labeled nonamer. A and B mark the bromophenol blue and xylene cyanol FF dye markers. C is the 5′-^{32}P-labeled nonamer which has a 3′-terminal 2′, 3′-cyclic phosphate group. D may be the 5′-^{32}P-labeled nonamer with a 3′-terminal 3′-phosphate group. Since the amount of D was small, the sequencing of D was not tried. The ladder was obtained by heating poly A in 80% formamide followed by enzymatic 5′-phosphorylation. Since ^{32}pAUG appeared at position 4, ^{32}pAUGp would be expected to appear at position 2. Therefore, C and D were determined as mentioned above. The fastest moving band of the ladder is ^{32}pA (2′, 3′-cyclic)p.

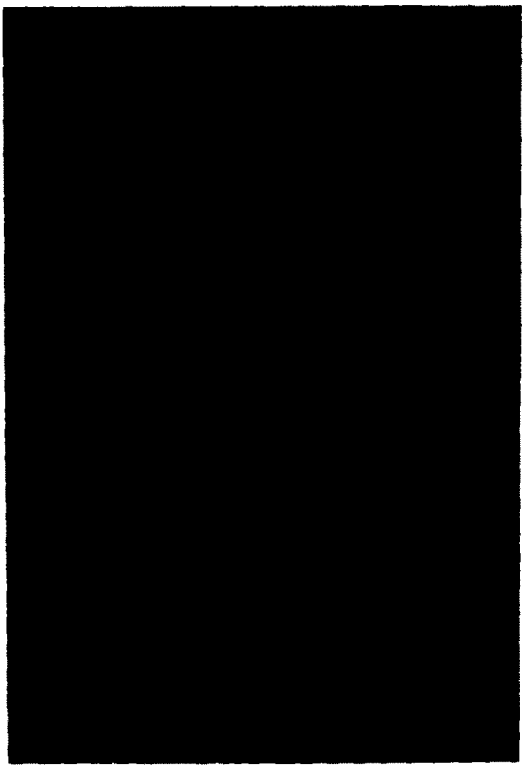


Fig. 3. Sequencing analysis of the 5'-labeled nonamer. B: Untreated nonamer; T_1: RNase T_1; U_2: RNase U_2; A: RrNase A.

General procedure for synthesis of oligomers. To a soln of phosphinic acid in pyridine as listed in Tables 9 and 10 was added a fully protected monomer or oligomer and Et_3N. The mixture was kept at 30–35°. After the substrate was converted completely to the diester, pyridine–water (1:1. v/v,) was added. The resulting soln was first extracted two times with hexane–ether (3:1, v/v) for removal of DMTrOH and benzenethiol, and then several times with CH_2Cl_2. The CH_2Cl_2 extracts were combined, washed two times with 2 M TEAB, and dried over $MgSO_4$. The solvent was removed under reduced pressure and the residual syrup was mixed with an appropriate hydroxyl component and 3 - nitro - 1,2,4 - triazole. The mixture was coevaporated several times with dry pyridine and dissolved in dry pyridine. MDS was added and the solution was stirred vigorously. After the reaction was complete, the mixture was treated with 0.5 M TEAB (pH 7.5) and extracted several times with CH_2Cl_2. After the combined CH_2Cl_2 extracts were washed a few times with 0.5 M TEAB, the solvent was removed *in vacuo* and the residue was coevaporated three times with toluene. The crude product extracted was purified by chromatography on a column of silica gel (10–20 fold). Elution was performed using 0.5–1% pyridine-containing CH_2Cl_2–MeOH. The final concentrations of MeOH in CH_2Cl_2 were 0.5–1, 0.3–1.2, 1.1–1.4, 1.5–2.1 and 2.3% in the case of **2**, **3**, **4**, **6** and **9** mers, respectively.

Deprotection of the fully protected UpApApUpAp. To the fully protected UpApApUpAp (37.7 mg, 10.2 μmol) were added 4 M pyridinium phosphinate in pyridine (250 μl) and Et_2N (70 μl). The mixture was set aside at 38° for 1.5 hr. This treatment gave a new spot with R_f 0.62 (solvent I, silica gel plate). Then, pyridine–water (2:1, v/v, 5 ml) was added and the soln was extracted first with hexane–ether (5:1, v/v, 10 ml). The aqueous layer was then extracted several times with CH_2Cl_2. The CH_2Cl_2 extracts were combined and

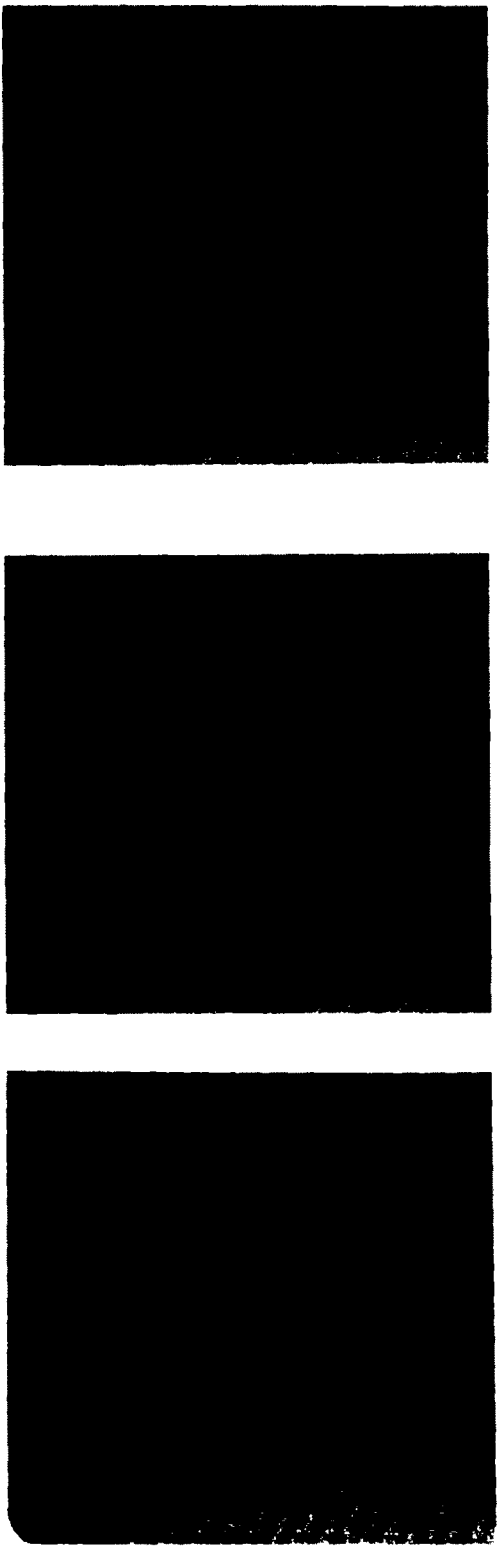


Fig. 4. Alkaline and enzymatic hydrolysis of the 5'-^{32}p labeled nonamer. The mixture obtained after each treatment was analysed by two-dimension chromatography on Avicel-SF cellulose plate (10 × 10, Funakoshi): 1st-dimension—isobutyric acid-0.5 N ammonium hydroxide (5:3, v/v); 2nd-2-propanol-concentrated HCl–water (70:15:15, v/v/v). A: Alkaline hydrolysis; B: Nuclease P_1 digestion; C: RNase T_2 digestion.

evaporated under reduced pressure. The residual syrup was dissolved in dioxan–water (7:1, v/v, 1.22 ml) containing 0.5 M tetramethylguanidium *o*-nitrobenzaldoximate and the mixture was stirred for 44 hr, whereupon the spot was changed to a new spot with R_f 0.56. The mixture was treated with pyridine–water (1:1, v/v, 10 ml) and extracted with ether–AcOEt (3:1, v/v) for removal of the excess oximate. The aqueous phase was extracted with CH_2Cl_2 (4 × 10 ml). The CH_2Cl_2 extracts were combined and evaporated under reduced pressure. The residue was dissolved in a soln of silver acetate (69.1 mg, 0.41 mmol) in pyridine–water (2:1, v/v, 0.82 ml) and the mixture was vigorously stirred for 45 hr. (The reaction was monitored by TLC, which was exposed to a H_2S gas and then developed with solvent II. If this pretreatment was omitted, TLC did not give clear spots.) Then H_2S was bubbled into the soln at 0° until a clear supernatant had been obtained. It took *ca* 15 min. The resulting Ag_2S was removed by centrifugation and the supernatant was passed through a column of Dowex 50W × 2 (pyridinium form, 5 ml). The resin was washed with pyridine–water (2:1, v/v, 10 ml). The eluent and washings were combined, evaporated under reduced pressure and coevaporated several times with benzene. At this stage, a DMTr-containing major spot with R_f 0.29 was observed. The pyridine-free residue was dissolved in 0.01 M HCl (dioxan–water, 1:1, v/v, 20 ml) and the mixture was stirred vigorously. After 40 hr, 5 ml of 0.01 M HCl was added and the soln was stirred for an additional 8 hr. The soln were extracted with ether–hexane (2:1, v/v, 15 ml). The aqueous layer was evaporated *in vacuo* always in the presence of pyridine. The acid treatment gave a new spot with R_f values of 0.15 (solvent I) and 0.47 (solvent II). The residue was dissolved in water and applied to Whatman 3 MM papers. Development with solvent II for 3 days gave a main band of the pentamer, which moved 10 cm from the origin. The band was cut and eluted with water to give UpApApUpAp (177OD, λmax 257 nm, λmin 229 nm, 40%): retention time = 7.0 min.

This pentamer (39 OD) was incubated with spleen phosphodeisterase in 0.05 M ammonium acetate (pH 6.5, 300 μl) at 37° for 15 hr. The incubation mixture was analyzed by HPLC, which showed two peaks of 2Up and 3Ap at 4.8 and 5.1 min, respectively.

Deprotection of the fully protected GpUpApUpUpApApUpAp. In a manner similar to that described in the above experiment, the fully protected nonamer (29 mg, 4.64 μmol) was deprotected. The following conditions were employed: (1) 4 M PSA (230 μl) in pyridine and triethylamine (50 μl), 38°, 4 hr; (2) tetramethylguanidium *o*-nitrobenzaldoximate in dioxan–water (7:1, v/v, 1.25 ml); (3) AgOAc (212 μmol) in pyridine–water (1:1, v/v, 0.37 ml), r.t., 44 h; (4) 0.01 M HCl (pH 2.0) in dioxan–water (1:1, 20 ml), r.t., 90 h; (5) paper chromatography on Whatman 3 MM papers developed with solvent II for 3 days. The band that moved 1–5 cm from the origin was eluted with water to give crude unprotected nonamer (109 OD, λmax 257 nm, λmin 230 nm, 25%).

A portion of this crude material was ^{21}P-labeled at the 5-OH group by incubation with T4 polynucleotide kinase by the lit method.[25] After removal of the excess [γ-^{32}P] ATP by gel filtration using Sephadex G75, the mixture was further purified by DEAE A25 ion exchange chromatography as shown in Fig. 1. The 5′-laveled nonamer was finally purified by 20% polyacrylamide gel electrophoresis as shown in Fig. 2. The sequencing of the labeled nonamer was conducted according to the published procedure.[24] This result is shown in Fig. 3.

For the enzymatic and alkaline hydrolysis of the nonamer, the following conditions were employed: (1) Nuclease P1 (5 μl), 0.1 M acetate buffer (pH 5.8)–H_2O (2 μl–13 μl), 37°, 30 min; (2) Ribonuclease T2 (1 μl, 0.5 μ unit/10 μl), 0.1 M acetate buffer (pH 4.8)–H_2O (4.5 μl–17 μl), 37°, 15 h; (3) 1 M KOH–H_2O (6.7 μl-13.3 μl), 37°, 18 hr. These results are shown in Fig. 2.

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