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Studies on Transfer Ribonucleic Acids and Related Compounds. XXVII.[1] Linear and Cyclic Oligonucleotides obtained by Polymerization of Protected Ribonucleoside 3′-Phosphates

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An improved method for the dicyclohexylcarbodiimide-catalyzed polymerization of protected ribonucleoside 3′-phosphates (Cp, Ap, Up or Gp) is described. The formation of 5′-O-pyridinium compounds was eliminated and various 5′-O-monomethoxytritylated protected homooligonucleotides could be rapidly isolated in reasonable yields. The isolation and purification of 3′,5′-cyclized oligonucleotides (cCpCp, cUp(Up)$_n$ or cGpGp) are described.

Keywords——linear and cyclic oligoadenylates; linear and cyclic oligocytidylates; linear and cyclic oligoguanylates; linear and cyclic oligouridylates; UV

Among the earliest studies directed towards the synthesis of oligoribonucleotides with natural 3′—5′ phosphodiester linkages were chemical polymerizations of suitably protected ribonucleotide 3′-phosphates. At this stage only the polymerizations of protected Up[3] and Ap were examined.[4] Characterizations of the two series of linear oligomers, as well as the range of side products generated, were performed after breakdown of pyrophosphates, removal of protecting groups and column chromatography on DEAE-cellulose. A drawback in these syntheses was the ready formation of 5′-C-pyridinium compounds by attack of the pyridine solvent.[5] More recently,[6] DCC-catalyzed polymerization of N-benzoyl-2′-O-tetrahydropyranylguanosine 5′-phosphate in the presence of N-benzoyl-2′,3′-di-O-tetrahydropyranylguanosine 5′-phosphate as a chain terminator afforded impressive yields of oligoguanylates with 5′-phosphates. In this reaction, only tetramers and higher chain length material could be detected after deprotection, in marked contrast to the earlier studies where dimers and trimers were the major products. We have also examined the DCC-catalyzed polymerization of ibG(Ib)p in the presence of (Ib)ibG(Ib)p as a chain terminator.[7a] In this case blocked oligomers with 5′-terminal protection and a 3′-phosphate group were separated and purified by column chromatography. Again, the appearance of 5′-C-pyridinium side-products caused complication.

1) Paper XXVI: A.F. Markham, E. Nakagawa, E. Ohtsuka, and M. Ikehara, Biopolymers, in press.
2) Location: *133-1 Yamadakami, Suita, Osaka, 565, Japan.*
3) Abbreviations are as recommended by the IUPAC-IUB Commission, *J. Biol. Chem.*, **245**, 5171 (1970); DCC, dicyclohexylcarbodiimide; DCU, dicyclohexylurea; TEAE-cellulose, triethylaminoethyl-cellulose; TEAA, triethylammonium acetate; TEAB, triethylammonium bicarbonate. Other abbreviations are as described previously.[1,8]
4) *a*) D.H. Rammler, Y. Lapidot, and H.G. Khorana, *J. Am. Chem. Soc.*, **85**, 1989 (1963); *b*) C. Coutsogeorgopoulos and H.G. Khorana, *ibid.*, **86**, 2926 (1964); *c*) Y. Lapidot and H.G. Khorana, *ibid.*, **85**, 3857 (1963).
5) G. Weimann and H.G. Khorana, *J. Am. Chem. Soc.*, **84**, 419 (1962).
6) D.G. Straus and J.R. Fresco, *J. Am. Chem. Soc.*, **95**, 5025 (1973).
7) *a*) E. Ohtsuka, M. Ubasawa, S. Morioka, and M. Ikehara, *J. Am. Chem. Soc.*, **95**, 4725 (1973); *b*) E. Ohtsuka, H. Tsuji, and M. Ikehara, *Chem. Pharm. Bull.* (Tokyo), **22**, 1022 (1974).
8) E. Ohtsuka, K. Murao, M. Ubasawa, and M. Ikehara, *J. Am. Chem. Soc.*, **92**, 3441 (1970).

Chemical polymerization of protected cytidine 3′- or 5′-phosphates has not previously been performed. In this paper we describe the polymerization of protected mononucleotides derived from the four major nucleosides [bzC(Bz)p, bzA(Bz)p U(Bz)p and ibG(Bz)p] (Chart 1), and a method for eliminating the formation of 5′-pyridinium compounds is described. Linear homooligomers protected with 5′-O-monomethoxytrityl groups were obtained in good yields. Cyclic oligonucleotides are obtained as by-products in the polymerization of mononucleotides, and we have previously studied the circular dichroism (CD) properties of cApAp and cApApAp as compared to ApAp and ApApAp.[7b] It seems probable that, in tRNA the nucleotides on the 5′-side of the anticodon must have a more folded relative orientation than is usually observed in oligonucleotides.[9] Normally these nucleotides have pyrimidine bases[10,11] and we were therefore interested in examining cCpCp and cUpUp. CD studies on these compounds and cGpGp are reported, so data of this type are now available for cyclic homodinucleotides containing the four commonly occurring ribonucleic acid (RNA) bases.

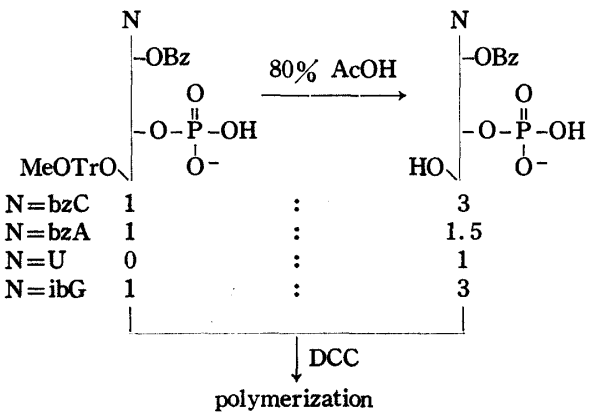

Chart 1

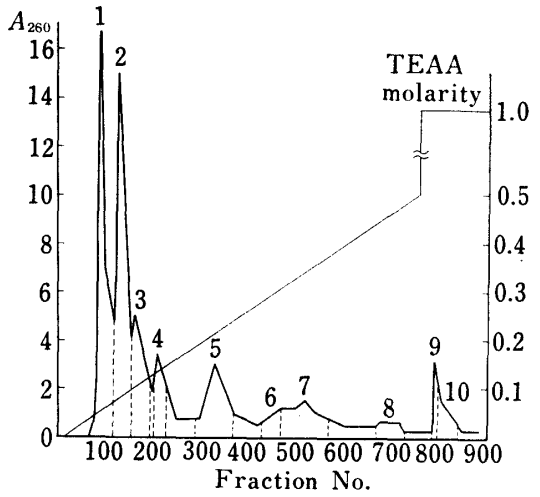

Fig. 1. Chromatography of the Product obtained by the Polymerization of protected Cpon a Column (3.4 × 62 cm) of TEAE-Cellulose (acetate form)

Elution was performed with a linear gradient of 0—0.5 M (TEAA) in 80% ethanol (total volume 16.6 l. This was followed by 1.0 M TEAA in 80% ethanol (3 l). The peaks are identified in Table II.

Compounds obtained in these polymerizations have also been used as substrates in joining reactions catalyzed by RNA ligases[12] and in CD studies on the effects of terminal 2′,3′-cyclic phosphates on mono- and oligoribonucleotide conformations.[1,13]

Polymerization of bzC(Bz)p

Initially we sought to confirm that protected cytidine 3′-phosphate could be polymerized in the established manner,[7] and to this end one equivalent of (MeOTr) bzC (Bz)p[8] as a chain terminator plus 4.33 equivalents of bzC(Bz)p were treated with 5.1 equivalents of DCC in anhydrous pyridine. Fig. 1 illustrates the elution profile obtained on a column of TEAE-

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10) B.G. Barrell and B.F.C. Clark, "Handbook of Nucleic Acid Sequences," Joyson-Bruwers, Oxford, 1974.
11) D. Gauss and M. Sprinzl, *Nucleic Acids Res.*, Special Supplement, rl (1977).
12) *a*) E. Ohtsuka, S. Nishikawa, R. Fukumoto, S. Tanaka, A.F. Markham, M. Ikehara, and M. Sugiura, *Eur. J. Biochem.*, **81**, 285 (1977); *b*) E. Ohtsuka, S. Nishikawa, M. Sugiura, and M. Ikehara, *Nucleic Acides Res.*, **3**, 1613 (1976).
13) A.F. Markham, S. Uesugi, E. Ohtsuka, and M. Ikehara, *Biochemistry*, **18**, 4936 (1979).

cellulose (acetate form) after a work-up involving solvent extraction of excess DCC and acidic treatment to remove 5′-monomethoxytrityl protecting groups. The acidic treatment was included here in order to increase the ease of chromatographic separation by avoiding the possible presence of two homologous series of protected cytidylate oligomers with and without 5′-protection. Materials in the various peaks were characterized by paper chromatography and paper electrophoresis before and after removal of the protecting groups (Table I). Table II lists contents of the various peaks. It was anticipated that after purification of the various

Table I. Paper Chromatography and Paper Electrophoresis of Cytidylates

	Paper chromatography *Rf* in solvent			Paper electrophoresis *Rm* at pH 7.5
	A	B	C	
(MeOTr)bzC(Bz)p		0.72	0.87	0.74
bzC(Bz)p		0.72	0.76	0.78
(MeOTr)Cp	0.73			0.80
(MeOTr)C > p				0.42
c[bzC(Bz)p]		0.90	0.89	0.42
C > p	0.65		0.57	0.65
Cp	0.51	0.20	0.25	1.00
cCp	0.64			0.65
cCpCp	0.58			0.80
cCpCpCp	0.46			0.86
CpCp	0.42			1.01
$(Cp)_3$	0.33			1.05
$(Cp)_4$	0.26			1.05
$(Cp)_5$	0.18			1.05
5′-Pyr–Cp	0.42			0.54
CpC	0.54			0.50
CpCpC	0.45			0.71
CpCpCpC	0.29			0.84
CpCpCpCpC	0.20			0.90
$[bzC(Bz)p]_2$		0.92		0.72
$[bzC(Bz)p]_3$		0.91		0.75
$[bzC(Bz)p]_4$		0.94		0.75
$c[bzC(Bz)p]_2$		0.90	0.89	0.51
(MeOTr)CpCp	0.64			0.75
(MeOTr)CpCpCp	0.53			0.75
(MeOTr)CpCpCpCp	0.38			0.78

Table II. Ion-exchange Chromatography of Polymerized Products of CP (Fig. 1)

Peak	Fractions pooled	Total A_{260}	% of eluted[a] A_{260}	Identification (purity)
1	10—120	1949	19.9	5′-Pyridinium bzC(Bz)p
2	121—160	8332	20.9	c[bzC(Bz)p] (60%)
3	161—195	3067	7.7	bzC(Bz)p
4	206—230	1703	4.3	$c[bzC(Bz)p]_2$ (71%)
5	291—370	4244	10.6	bzC(Bz)pbzC(Bz)p (76%)
6	431—470	1102	2.8	Some $c[bzC(Bz)p]_3$ plus unidentified material
7	471—570	3286	8.2	$[bzC(Bz)p]_3$ (70%)
8	671—730	1147	2.9	$[bzC(Bz)p]_4$ (74%)
9	786—797	605	1.5	$[bzC(Bz)p]_5$ (45%)
10	798—840	1306	3.2	Higher oligomers plus unidentified material

a) Calculated based on 39855 A_{260}, 1.48 mmol, 84% recovery.

linear acylated oligocytidylates, monomethoxytritylation could be performed selectively at 5′-termini to furnish fully protected blocks for further condensation. Synthesis of some *E. coli* $tRNA_f^{Met}$[14] sequences using these homooligomers has been reported.[15] However this approach meant that acetolysis to cleave pyrophosphates could not be performed before column chromatography, as 5′-O-acetylated species might be generated which would not lend themselves to selective 5′-deprotection in subsequent stages. The result of this is that the materials in the various peaks are rather inhomogeneous. The large amount of 5′-C-pyridinium bzC(Bz)p generated in the absence of steps to prevent this and the absence of measures to eliminate pyrophosphates mean that the homologous series of 5′-C-pyridinium oligomers as well as the possible pyrophosphate permutations (with and without the 5′-C-pyridinium substituent) are present as contaminants in the major peaks. In deprotection before analysis, these pyrophosphate species generate oligomers and monomers with all the possible permutations of 5′-hydroxyl or C-pyridinium groups and 3′-phosphates or 2′,3′-cyclic phosphates from the same column peaks. Material corresponding to many of these compounds was tentatively identified by paper chromatography, paper electrophoresis and ultraviolet (UV) spectroscopy.

Analysis of linear oligocytidylates after deprotection was performed by removal of 3′-terminal phosphate with *E. coli* alkaline phosphatase and complete digestion of the resulting oligomers from the second polymerization below. The expected ratio of cytidine 3′-phosphate to cytidine was obtained for the dimer, trimer and tetramer.

Polymerization of bzA(Bz)p in DMF

As an alternative to the above procedure, we attempted to eliminate 5′-C-pyridinium compound formation in the polymerization of (MeOTr)bzA(Bz)P with 1.5 equivalents of bzA(Bz)P. After careful removal of any amine by passage through Dowex 50×2 pyridinium resin, the condensation reaction was performed with DMF as a solvent in the presence of a trace of pyridine to maintain 5′-O-protection and more of the pyridinium resin as an amine

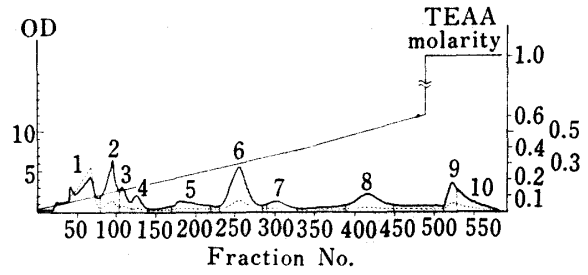

Fig. 2. Chromatography of the Product obtained by the Polymerization of protected Ap on a Column (3.4×57 cm) of TEAE-Cellulose (acetate form)

Elution was performed with a linear gradient of 0.03—0.4 M TEAA in 95% ethanol (7 l total volume) then 1.0 M TEAA (1.0 l) in 95% ethanol. The peaks are identified in Table IV. {——280 nm. {·····300 nm.

Table III. Paper Chromatography and Paper Electrophoresis of Adenylates

	Paper chromatography *Rf* in solvent A	Paper electrophoresis *Rm* at pH 7.5
Ap	0.32	1.00
A > p	0.59	0.54
cAp	0.61	0.54
cApAp	0.44	0.76
cApApAp	0.38	0.89
ApAp	0.20	1.00
ApApAp	0.10	1.01
ApApApAp	0.04	1.03
ApApApApAp	0.01	1.04
(MeOTr)Ap	0.82	0.73
(MeOTr)ApAp	0.69	0.82
(MeOTr)ApApAp	0.52	0.87
(MeOTr)ApApApAp	0.36	0.92
(MeOTr)ApApApApAp	0.23	0.98
ApA	0.45	0.42
ApApA	0.31	0.62
ApApApA	0.15	0.74

14) S.K. Dube, K.A. Marcker, B.F.C. Clark, and S. Corey, *Nature* (London), **218**, 232 (1968).
15) E. Ohtsuka, T. Tanaka, S. Tanaka, A.F. Markham, T. Miyake, E. Nakagawa, S. Nishikawa, and M. Ikehara, *Nucleic Acids Res.*, Spec. Pub. No. 3, s117 (1977).

Table IV. Ion-exchange Chromatography of Polymerized Products of Ap (Fig. 2)

Peak	Fractions pooled	A_{280}	% of eluted A_{280}	Identification
1	20— 80	—	—	Non-nucleotidic
2	81—104	1752	13.3	(MeOTr)bzA(Bz)p
3	106—119	626	4.8	c[bzA(Bz)p] (70%) (MeOTr)bzA(Bz)p (30%)
4	120—240	536	4.1	$c[bzA(Bz)p]_2$
5	170—220	1009	7.7	Unidentified
6	230—285	2972	22.6	$(MeOTr)[bzA(Bz)p]_2$ (85%)
7	290—330	905	6.9	Mainly unidentified some $c[bzA(Bz)]_3$
8	390—450	1606	12.2	$(MeOTr)[bzA(Bz)p]_3$ (85%)
9	515—530	856	6.5	$(MeOTr)[bzA(Bz)p]_4$ (74%)
10	531—580	1144	8.7	Mainly $(MeOTr)[bzA(Bz)p]_5$ plus longer oligomers

scavenger. After 5 days a considerable amount of oligomeric material was formed and excess DCC, DCU and Dowex resin were removed. The relative amounts of oligomeric materials were determined as a check for gross decomposition in the stages between this work-up and the final chromatographic separation. Decomposition was apparently not extensive. The entire reaction mixture was treated with monomethoxytrityl chloride in anhydrous pyridine. Next, small aliquots were treated with methanolic ammonia. The reaction was considered complete when no material could be detected with a relative mobility similar to that of an Ap marker in paper electrophoresis. The mixture was treated with acetic anhydride without work-up, followed by aqueous pyridine treatment to cleave acetylphosphate, and was then applied directly to a column of TEAE-cellulose. Fig. 2 shows the elution profile and Table III shows the chromatographic properties of the adenylates. The peaks are identified in Table IV. The material in the peaks was much more homogeneous, and no 5′-C-pyridinium bzA(Bz)p was detected. Enzymatic analysis of purified linear adenylates as described above gave the expected yields of Ap and A after *E. coli* alkaline phosphatase treatment followed by ribonuclease M digestion; no resistant material was detected.

Table V. Paper Chromatography and Paper Electrophoresis of Uridylates

Compound	Paper chromatography *Rf* in solvent A	Paper electrophoresis *Rm* at pH 7.5
Up	0.30	1.00
U > p	0.56	0.73
5′-Pyr–Up	0.78	0.54
cUp	0.56	0.72
cUpUp	0.31	0.80
cUpUpUp	0.71	0.85
cUpUpUpUp	0.08	0.95
UpUp	0.18	1.01
$(Up)_3$	0.11	1.04
$(Up)_4$	0.05	1.04
$(Up)_5$	0.02	1.04
UpU	0.36	0.59
$(Up)_2U$	0.22	0.80
$(Up)_3U$	0.12	0.85
$(Up)_4U$	0.05	0.88

Table VI. Ion-exchange Chromatography of Polymerized Products of Up

Peak	Fractions pooled	A_{260}	% of eluted A_{260}	TEAB molarity	Identification
1	10— 30	—	—	H_2O	Non-nucleotidic
2	109—116	138	4.0	0.05	5′-pyridinium Up
3	122—134	874	25.5	0.065	cUp
4	170—182	177	5.2	0.10	Up
5	194—208	356	10.4	0.12	cUpUp
6	257—268	148	4.3	0.165	UpUp
7	291—308	149	4.3	0.195	cUpUpUp
8	328—340	114	3.3	0.22	UpUpUp
9	355—366	53	1.5	0.24	cUpUpUpUp
10	391—407	92	2.7	0.27	UpUpUpUp
11	449—456	154	4.5	1.00	Higher oligomers

Polymerization of bzC(Bz)p in DMF

A polymerization of bzC(Bz)p in the presence of 0.38 equivalents of (MeOTr)bzC(Bz)p was performed using this modified procedure and again, after 7 days reaction, no 5′-O-pyridinium compounds could be detected amongst the products. After treatment of the entire reaction mixture with monomethoxytrityl chloride in pyridine the products were acetolyzed to cleave pyrophosphates. As in the case of adenylate, polymerization by this method gave relatively pure protected oligomers, although again traces of by-products were present after chromatography.

Polymerization of U(Bz)p

Pyridinium U(Bz)p was polymerized with DMF as a solvent in the presence of Dowex 50×2 (pyridinium form) resin. The reaction was performed in the absence of fully protected (MeOTr)U(Bz)P as a chain terminator because the cyclized compounds were required for CD studies. As before, no 5′-C-pyridinium Up was detected when an aliquot was deprotected after 3 days reaction. However, the ratio of linear to cyclic oligomers was high, so the polymerization mixture was diluted with pyridine in an attempt to induce cyclization. After a further 5 days the reaction mixture was completely deprotected with methanolic ammonia after cleavage of pyrophosphates and applied to a column of DEAE-cellulose. The elution positions of various peaks are given in Table V and the chromatographic properties of the various components in Table VI. The elution profile was almost exactly superimposable

Table VII. Identification of Peaks eluted on TEAE-cellulose (acetate form) Chromatography of Protected Oligoguanylates with 5′-*O*-Monomethoxytrityl Groups

Peak	Fractions pooled	A_{260}	% of eluted	Identification
1	60— 82	985	10.5	c[ibG(Bz)p]
2	87—100	1115	11.9	(MeOTr)ibG(Bz)p
3	170—194	582	6.2	Pyrophosphate of (MeOTr)ibG(Bz)p
4	185—200	568	6.0	Unidentified
5	201—209	357	3.8	Unidentified
6	210—219	632	6.7	$c[ibG(Bz)p]_2$
7	235—255	445	4.7	Unidentified
8	284—304	426	4.6	$(MeOTr)[ibG(Bz)p]_3$
9	312—360	808	8.6	$c[ibG(Bz)]_3$
10	381—401	207	2.2	$(MeOTr)[ibG(Bz)p]_4$
11	402—440	431	4.6	$c[ibG(Bz)p]_4$
12	553—560	91	1.0	Unidentified

on that obtained[4b] after the polymerization of U(Ac)p in the presence of a chain terminator, except that the relative amounts of the cyclic oligomers were increased. However, the addition of pyridine also led to the formation of 5′-C-pyridinium U(Bz)p (Table V). This supports the previously suggested involvement of neighboring phosphodiester linkages in the formation of such materials.[5] The relative amount of the 5′-C-pyridinium species encountered here was *ca.* 30% of that encountered previously.[4b] Enzymatic characterization of $(Up)_2$, $(Up)_3$, $(Up)_4$ and $(Up)_5$ was performed by digestion with phosphatase to generate UpU, $(Up)_2U$, $(Up)_3U$ and $(Up)_4U$. These compounds were fully digested with ribonuclease A to yield Up/U ratios of 1.00, 1.97, 3.01 and 3.97 respectively.

Polymerization of ibG(Bz)p

To improve the yields of oligonucleotides, we have replaced the 2′-O-isobutyryl group with benzoyl.[16] Polymerization was performed using ibG(Bz)p and (MeOTr)ibG(Bz)p in a ratio of 3 to 1 under essentially the same conditions as described previously; free 5′-hydroxyl groups were tritylated and treated with acetic anhydride to cleave pyrophosphates. The distribution of the products (Table VII) was essentially the same as that obtained previously using the 2′-O-isobutyryl derivative.[8] However the tritylated compounds were eluted earlier than the corresponding cyclic oligomers. *Rf* values of the products are shown in Table VIII.

Table VIII. Chromatography and Electrophoretic Mobilities of Guanylic Acids

Compound	Paper chromatography in solvent A relative *Rf* to Gp	Paper electrophoresis relative mobility (G–Gp)
G	1.7	0
Gp	1.0	1.0
cGp	1.1	0.61
(MeOTr)Gp	3.5	0.58
GpGp	0.36	1.0
(MeOTr)GpGp	2.2	
(MeOTr)GpGp > p	2.6	
cGpGp	0.62	0.70
GpG	0.92	0.39
GpGpGp	0.28	
(MeOTr)GpGpGp	1.5	
cGpGpGp	0.28	0.74
GpGpGp		0.61

Experimental

General Methods——Paper chromatography (PPC) was performed by the descending technique using Whatman 3MM paper (preparative scale) or Toyo 51A paper (analytical scale) in the following solvent systems: A, propan-1-ol: conc. ammonia: water (6: 1: 3, v/v); B, propan-2-ol: conc. ammonia: water (7: 1: 2, v/v): C, ethanol: 1m ammonium acetate, pH 7.5 (7: 3, v/v). Paper electrophoresis (PEP) was performed on the same materials in 50mm triethylammonium bicarbonate (TEAB) buffer, pH 7.5, for 1 hr with a potential gradient of 23 V/cm. The relative mobilities (*Rm*) of oligonucleotides are expressed relative to the mobilities of the particular nucleoside (*Rm* 0.00) and nucleoside 3′-phosphate (*Rm* 1.00). Enzymatic digestions were performed as described previously.[7a]

The methods of preparation of fully protected nucleoside 3′-phosphates and of purification of solvents and reagents are described elsewhere.[7a,8,16]

The usual work-up of aliquots of the polymerization reactions involved dissolving a small sample (*ca.* 10 μl) of the reaction mixture in 50% aqueous pyridine (2 ml), filtration, and extraction (4 times) with pentane or hexane (2 ml). The aqueous phase was then kept at room temperature for *ca.* 12 hr. Where removal of

16) E. Ohtsuka, E. Nakagawa, T. Tanaka, A.F. Markham, and M. Ikehara, *Chem. Pharm. Bull.* (Tokyo), **26**, 2998 (1978).

acid-labile monomethoxytrityl groups was necessary, the solution was evaporated, then coevaporated with aqueous butan-1-ol (1 : 1, v/v) (3 times). The residue was then treated with 80% aqueous acetic acid (2 ml) for 1 hr at room temperature unless otherwise stated. Acetic acid was removed by evaporation and the residue was coevaporated with aqueous butan-1-ol as above. For removal of acyl protecting groups the above residue (or that from the aqueous pyridine treatment) was dried by coevaporation with pyridine (3 times) then treated with 15 N methanolic ammonia for 24 hr at 30°. Solvents were removed by evaporation and the resulting deprotected compounds were usually subjected to PPC or PEP as solutions in 50% aqueous pyridine. The above conditions for acetic acid or ammoniacal treatments are standard unless otherwise noted in the text. Precipitation of protected nucleotides was performed, after drying by evaporation with added pyridine (3 times), by dissolving the material in pyridine and dropping the solution into rapidly stirred mixtures of ether–pentane (3 : 2, v/v) unless otherwise stated. Precipitated material was collected by centrifugation, washed (3 times) with the same mixture and dried *in vacuo* over P_2O_5.

Polymerization of bzC(Bz)p——(i) In Pyridine: Pyridinium (MeOTr)bzC(Bz)p (0.33 mmol, 291 mg, 9570 A_{260}) and pyridinium bzC(Bz)p (1.43 mmol, 874 mg, 38610 A_{260}) were dried by coevaporation with pyridine (5 times) then treated with DCC (9.0 mmol, 1.857 g) in pyridine (6 ml). After 15 min at room temperature the solution was evaporated to a syrup and kept at 30°. An aliquot was worked up after 3 days in the usual way then fully deprotected as described above. PPC in solvent A and PEP indicated the formation of linear oligomers up to the pentamer plus the cyclic monomer, dimer and trimer and 5′-C-pyridinium cytidine 3′-phosphate. A similar pattern was obtained after 6 days reaction. At this stage 60% aqueous pyridine (50 ml) was added at 0° and the solution extracted with pentane (40 ml, 3 times). After 6 hr at room temperature, DCU was filtered off, then the solvents were evaporated. Traces of pyridine were removed by coevaporation twice with toluene then the residue was treated with 80% aqueous acetic acid (50 ml) at room temperature for 2 hr. After removal of acetic acid, the residue was dissolved in 80% aqueous ethanol (200 ml) containing pyridine (40 ml) and applied to a column (3.4×62 cm) of TEAE-cellulose (acetate form). The column was washed with 4 column bed volumes of 80% ethanol then eluted with a linear gradient of 0—0.5 M TEAA in 80% ethanol (total volume 16.6 l). This was followed by 1.0 M TEAA in 80% ethanol (3 l). Fractions of 21.6 ml were collected every 6 min. The elution profile is illustrated in Fig. 1 and the various peaks are identified in Table II. Pooled material from the peaks was recovered by the removal of ethanol, then by dissolving the residue in butan-1-ol and extracting repeatedly with water. The organic phases were evaporated with added aqueous pyridine, converted to pyridinium salts by passage through Dowex 50×2 (pyridinium form) in 50% aqueous pyridine, dried by coevaporation with pyridine and precipitated. Samples for analysis were deprotected with 15 N methanolic ammonia as described above. This method of recovery of material after elution in ethanolic TEAA is standard, except that in the presence of monomethoxytrityl protecting groups the butan-1-ol phase was washed with 1% aqueous pyridine.

(ii) In DMF: Pyridinium (MeOTr)bzC(Bz)p (0.7 mmol, 7800 A_{304}) and pyridinium bzC(Bz)p (1.87 mmol, 20600 A_{304}) were dissolved in 50% aqueous pyridine (100 ml) and passed through a column (50 ml) of Dowex 50×2 (pyridinium form). The column was washed with 50% aqueous pyridine (300 ml) and the nucleotides were precipitated from combined eluate and washings. These starting materials and Dowex 50×2 (pyridinium form) (2 ml) were dried by coevaporation with pyridine (5 times) then treated with DCC (10 mmol, 2.06 g) in pyridine (8 ml). After shaking for 15 min, DMF (1.0 ml) was added to the homogeneous solution which was immediately evaporated to an immobile gum and stored in a desiccator at 26°. An aliquot deprotected after 3 days reaction contained oligomers up to the pentamer as determined by PPC, and no 5′-C-pyridinium nucleotide could be detected by PPC or PEP. After 7 days, excess DCC, DCU and Dowex resin were removed as in section (i) then the nucleotidic material was dried by coevaporation with pyridine (5 times) and treated with monomethoxytrityl chloride (1.5 mmol, 0.4632 g) in pyridine (20 ml). After stirring for 24 hr at room temperature, PEP showed no detectable untritylated linear nucleotides. Water (20 ml) was added to the reaction mixture (30 ml, 4 times). The aqueous phase was extracted with butan-1-ol until no nucleotidic material was detectable by PEP then the organic phase was back-washed repeatedly with 2% aqueous pyridine to remove pyridinium hydrochloride. The resulting solution was evaporated with repeated addition of 50% aqueous pyridine, then dried by coevaporation with pyridine (5 times). The residue was dissolved in pyridine (20 ml) and acetic anhydride (10 ml) was added. After 48 hr in the dark at room temperature volatile materials were evaporated and the residue was dissolved in 50% aqueous pyridine (60 ml) at 0°. After 24 hr at room temperature, a part (27300 A_{260}, 0.94 mmol with respect to (MeOTr)bzC(Bz)p, by PEP) of this solution was diluted with 95% ethanol (200 ml) and applied to a column (3.4×90 cm) of TEAE-cellulose (acetate form). The column was washed with 95% ethanol (2 l) then 0.05 M TEAA in 95% ethanol (2 l). Elution was performed with a linear gradient of 0.05—0.25 M TEAA in 95% ethanol (total volume, 7 l) followed by a linear gradient of 0.25—1.0 M TEAA in 95% ethanol (total volume 7 l). Fractions of *ca.* 21.5 ml were collected every 5 min. After pooling of material, small aliquots from each peak gave positive tests for the monomethoxytrityl group when applied to chromatography paper and sprayed with 30% H_2SO_4. The material in the peaks was recovered as the powdered pyridinium salts as described above. Identification involved complete deprotection of an aliquot from each fraction PPC in solvent A, then PEP. Samples were also treated with methanolic ammonia alone to establish that no loss of 5′-protection had occurred. The properties of oligocytidylates with 5′-O-monomethoxy-

trityl groups are listed in Table I.

Samples of $(Cp)_2$ $(Cp)_3$ and $(Cp)_4$ obtained by combining material purified by PPC followed by PEP from polymerizations (i) and (ii) were treated with *E. coli* alkaline phosphatase to yield CpC, $(Cp)_2$ and $(Cp)_3C$. These compounds were fully digested with ribonuclease A to give cytidine to cytidine 3′-phosphate ratios of 1 : 1.08, 1 : 2.01 and 1 : 3.45, respectively.

Polymerization of bzA(Bz)p——bzA(Bz)p (pyridinium salt) (0.6 mmol, 2220 A_{305}) plus (MeOTr)bzA(Bz)p (pyridinium salt) (0.4 mmol, 1480 A_{305}) were dissolved in 50% aqueous pyridine (50 ml), passed through a column of Dowex 50×2 (pyridinium form, 40 ml) and treated as above (ii). The dried residue and resin were dissolved in pyridine (4 ml) and DCC (1.03 g, 5 mmol) was added. The resulting solution was shaken at room temperature for 10 min then anhydrous DMF (0.5 ml) was added. The solution was immediately evaporated to an immobile syrup, which was kept in a desiccator at 26°.

After 5 days an aliquot was deprotected and subjected to PPC and PEP. *Rf* and *Rm* values are shown in Table III. No compound corresponding to 5′-C-pyridinium Ap could be detected. Comparison of the relative proportions of the oligoadenylates and their cyclic counterparts showed that the ratios were essentially the same as those after the final isolation procedure (Table IV). The reaction mixture was dissolved in pyridine (10 ml) added to the homogeneous solution at 0°. The solution was worked up as above (ii). After monomethoxytritylation and acetolysis the mixture was diluted with 95% ethanol (300 ml) and applied to a column (3.4×57 cm) of TEAE-cellulose (acetate column). After an initial wash with 95% ethanol (2 l) elution was performed with a linear gradient of 0.03—0.4 M TEAA in 95% ethanol (7 l total volume) then 1.0 M TEAA (1.0 l) in 95% ethanol. Fractions of 20.5 ml were collected. Figure 2 shows the elution profile and the amounts of nucleotidic material in the various peaks, and their compositions are collected in Table IV. Peaks were pooled and the protected nucleotides were precipitated as their pyridinium salts. Samples of the homologous series of adenylates with 5′-O-monomethoxytrityl groups were prepared by treatment of the precipitated materials with 15 N methanolic ammonia alone. Treatment of this series of compounds with *E. coli* alkaline phosphatase generated the $(Ap)_nA$ series (Table III). Ribonuclease M digestion of these compounds generated Ap plus adenosine. The molar ratios of Ap/A for ApA, ApApA and ApApApA were 1.15, 2.14 ans 3.03 respectively. No nuclease-resistant material could be detected.

Polymerization of U(Bz)p——Pyridinium U(Bz)p (10900 A_{260}, 1 mmol) was polymerized as described above. The results are summarized in Tables V and VI.

Polymerization of ibG(Bz)p——The pyridinium salts of ibG(Bz)p (0.76 mmol) and (MeOTr)ibG(Bz)p (0.26 mmol) were treated with DCC (1.03 g, 5 mmol) in pyridine (10 ml) at 21° for 7 days. Aqueous pyridine (75%, 10 ml) and hexane (5 ml) were added. DCC was removed by extraction with hexane (5 ml, 2 portions) from the filtered aqueous layer. The mixture was rendered anhydrous by evaporation with pyridine and treated with monomethoxytrityl chloride (0.47 g, 1.5 mmol) in pyridine (20 ml) overnight. Aqueous pyridine (50%, 10 ml) was added, then the mixture was concentrated to *ca.* 5 ml and dissolved in butan-1-ol (30 ml). The organic layer was washed with small volumes of water 5 times to remove pyridinium hydrochloride, then concentrated. The nucleotides were precipitated then treated with acetic anhydride (5 ml) in pyridine (10 ml) for 24 hr. Aqueous pyridine (50%, 20 ml) was added and evaporated 4 times to remove acetate ions. The residue was dissolved in 80% ethanol (500 ml) and applied to a column (3×51.5 cm) of TEAE-cellulose preequilibrated with 80% ethanol. Elution was performed with a linear gradient of TEAA (0 to 0.4 M, 8 l). Protected cGpG (632 A_{260} units was eluted at a salt concentration of 0.2 M (Table VII) and was contaminated with linear dinucleotide (GpGp). The deprotected cGpGp was isolated by PPC (Table VIII) in solvent B after methanolic ammonia treatment. The compound was treated with HCl (pH 1) for 1 hr and subjected to PEP to eliminate contaminating 2′,3′-cyclic phosphates. A single spot was obtained and the acid-stable compound was eluted. An aliquot (2.1 A_{260}) was hydrolyzed with ribonuclease T1 (50 μg) in 0.1 M triethylammonium bicarbonate (0.1 ml) for 6 hr at 37°. Under these conditions 28% of the cyclic dimer was hydrolyzed to the mononucleotide, as estimated by PEP. GpG was degraded completely under the same conditions. Ribonuclease M hydrolysis was performed using 20 μg of the enzyme for cGpGp (0.53 A_{260}) in 0.05 M ammonium acetate (pH 5.0, 100 μl) at 37° for 6 hr. No starting material was detected by PEP. Hypochromicity of cGpGp was estimated by measuring the UV spectra before and after hydrolysis of cGpGp as above 6, and was found to be 13.9%. ε_{277} was assumed to be 7.5×10^3, taking $\varepsilon_{277}=8.7\times10^3$ for Gp.

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