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Preparation and Properties of Oligocytidylates with 2′—5′ Internucleotide Linkage

Hiroaki SAWAI* and Masaji OHNO
Faculty of Pharmaceutical Sciences, University of Tokyo Hongo, Bunkyo-ku, Tokyo 113
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Oligocytidylates upto a pentamer were prepared by lead(II) ion catalyzed polymerization of cytidine 5′-phosphorimidazolide in aqueous solution. 2′—5′ internucleotide linkage was preferentially formed. The yields of the 2′—5′ linked dimer, trimer, tetramer and pentamer were 17.0, 5.5, 1.9, and 0.7%, respectively. Some of their properties were studied by NMR, CD, UV, and chromatography and compared with the corresponding 3′—5′ linked oligomers which were prepared enzymatically.

Biological and physicochemical properties of 2′—5′ linked oligonucleotides have attracted widespread interest because unusual 2′—5′ linked oligoadenylates were isolated from interferon-treated cell and shown to be associated with the interferon's action.[1-3] Previously we have reported the preparation of 2′—5′ linked oligoadenylates by lead(II) ion catalyzed polymerization of adenosine 5′-phosphorimidazolide in aqueous solution.[4] The method provided a very simple preparative procedure for the 2′—5′ oligonucleotides. Preparation of other 2′—5′ linked oligonucleotides has been conducted by a similar procedure.[5,6] Oligoribonucleotides with 2′—5′ internucleotide linkage were preferentially formed by the reaction, though the yield and distribution of the resulting oligomers were different depending on the base moiety. In this paper, we report the synthesis of 2′—5′ linked oligocytidylates from cytidine 5′-phosphorimidazolide by lead(II) ion catalysis. Physicochemical properties of the 2′—5′ linked oligocytidylates are described and compared with the corresponding 3′—5′ linked oligocytidylates which were prepared by partial enzyme digestion of polycytidylate.

Results and Discussion

Cytidine 5′-phosphorimidazolide (ImpC) was prepared from cytidine 5′-phospate and imidazole using di-2-pyridyl disulfide and triphenylphosphine as a condensing agent.[5,7] Polymerization of ImpC was carried out in neutral aqueous solution in the presence of lead(II) nitrate as a catalysis. The starting ImpC disappeared almost completely in 3 d at room temperature. The reaction mixture was treated with Versenol solution to break the Pb^{2+}-nucleotide complex which was checked by HPLC. The solution was applied to a QAE-Sepahdex A-25 column and eluted with a linear gradient of triethylammonium hydrogencarbonate buffer. The elution profile is shown in Fig. 1. The separated products were further purified by paper chromatography when necessary. The peak identification and the yields of the products are given in Table 1. Oligocytidylates upto a pentamer were formed in the reaction. The lead(II) ion catalyzed internucleotide-bond formation is shown in Scheme 1. The oligocytidylates thus obtained were characterized by enzyme digestions, TLC and NMR. The 2′—5′ internucleotide linkage is not degraded with nuclease P_1 (N. P_1) and RNAse T_2 which degrades 3′—5′ internucleotide linkage. Both 2′—5′ and 3′—5′ internucleotide linkages are hydrolyzed with snake venom phosphodiesterase (VPDase) and by alkaline digestion. The internucleotide linkage and the chain length of the oligocytidylates were determined by sequential enzyme digestion using bacterial alkaline phosphatase (BAPF), N. P_1, VPDase and alkaline digestion. The yields of 2′—5′ linked dimer, trimer, tetramer and pentamer were 17.0, 5.5, 1.9, and 0.7%, respectively. The 3′—5′ linked dimer and trimer were isolated in 1.6 and 0.3%, respectively. Two linkage isomers, pC2′p5′pC3′p5′C and pC3′p5′pC2′p5′C, which appeared in peaks 10 and 11 were obtained in 2.8 and 1.4% yields, respectively. Three tetramers, containing one 3′—5′ and two 2′—5′ internucleotide linkage in a different position, were present in peak 12 along with 2′—5′ $(pC)_4$. The compound with a pyrophosphate bond, CppC, was obtained as a minor byproduct. Cytidine 3′,5′-cyclic phosphate which was formed by intramolecular phosphodiester bond formation was present in peak 4. Cyclic dicytidylates were obtained in 11.4% yield in peak 7. In contrast to the linear oligomers, 3′—5′ internucleotide linkage was predominant in the cyclic dicytidylates, as 3′—5′ linkage is conformationally preferable for the cyclic nucleotides. The cyclic dicytidylate with two 3′—5′ linkages was degraded with N. P_1 to cytidylic acid and insensitive to BAPF. The 3′—5′, 2′—5′ linked cyclic dicytidylate was hydrolyzed with N. P_1 to pC2′p5′C. The 2′—5′, 2′—5′ linked cyclic dimer was insensitive to N. P_1 but was digested with VPDase. The major feature of the reaction is preferential formation of 2′—5′ linked linear oligocytidylates. The yield and distribution of the products are comparable to those of the lead-ion catalyzed oligouridylates formation.[5] The yields and the selectivity of the 2′—5′ linked oligomers are lower than those of the oligoadenylates

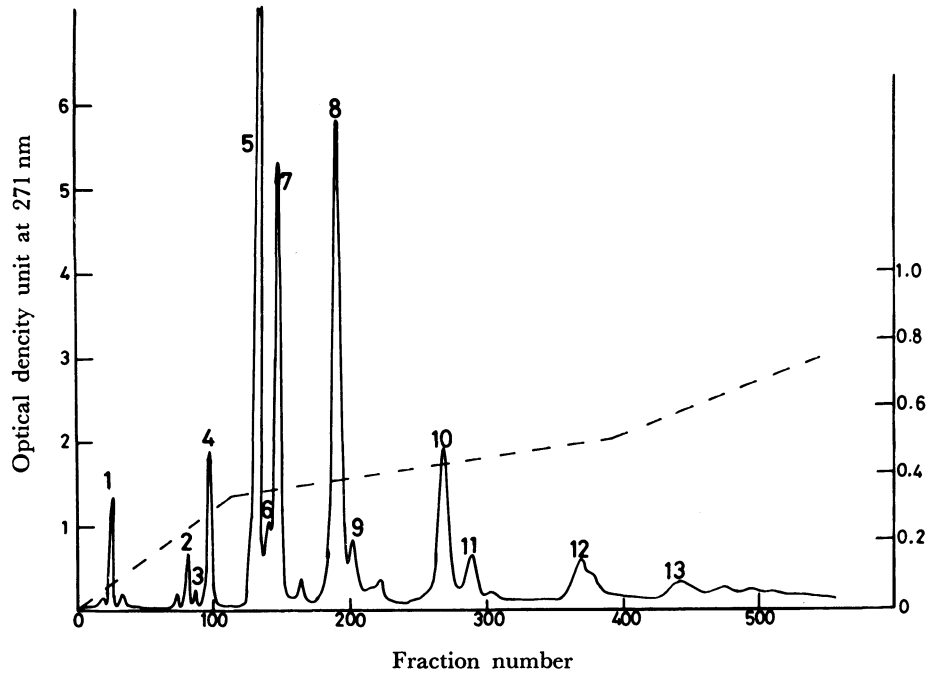
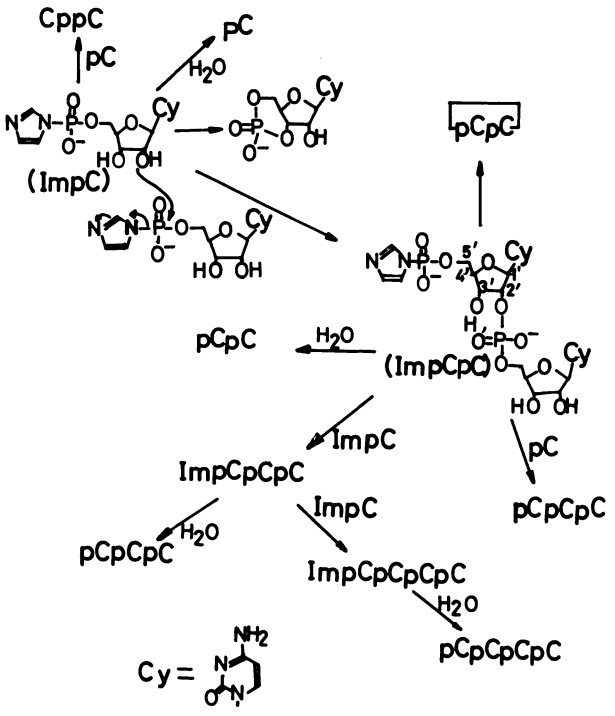


Fig. 1. Chromatography of the Products on QAE-Sephadex A-25 column. A linear gradient of triethylammonium hydrogencarbonate was used for elution (dotted line). The characterization of the peaks is listed in Table 1.

TABLE 1. OLIGOCYTIDYLATES OBTAINED FROM ImpC BY Pb^{2+} ION CATALYSIS

Peak No.	ODU_{271}	h(%)[a]	Identified Structure	Yield/%[b]
1	68.9		C	1.7
2	34	5.7	C2′p5′C	0.8
3	10	4.8	C3′p5′C	0.2
4	117		3′,5′-cyclic CMP	2.8
5	1243		pC	29.9
6	30		CppC	0.6
7	445	5.7[c]	-pCpC- (3′—5′, 3′—5′)	8.7
			(3′—5′, 2′—5′)	1.0
			(2′—5′, 2′—5′)	1.7
8	650	8.2	pC2′p5′C	17.0
9	64	5.7	pC3′p5′C	1.6
10	298	14.5[c]	pC2′p5′C2′p5′C	5.5
			pC2′p5′C3′p5′C	2.8
11	86	15.0	pC3′p5′C2′p5′C	1.4
			pC3′p5′C3′p5′C	0.3
12	212	15.2	pC2′p5′C2′p5′C2′p5′C	1.9
			pC2′p5′C3′p5′C2′p5′C	0.9
			pC2′p5′C2′p5′C3′p5′C	1.1
			pC3′p5′C2′p5′C2′p5′C	0.5
			Other oligomers	1.1
13	119	17.4	pC2′p5′C2′p5′C2′-p5′C2′p5′C	0.7
			Other oligomers	2.3

a) Hypochromicity estimated from alkaline hydrolysis. b) Yield obtained from UV_{271} after allowing the hypochromicity of each oligocytidylate. Total ODU_{271} of starting ImpC was 4160 (0.45 mmol). c) Mean hypochromicity of the compounds in the peak.



formation.[4] However, this procedure provides a simple synthetic method to the 2′—5′ linked oligocytidylates.

Table 2 lists R_f values of the 2′—5′ and 3′—5′ linked oligocytidylates. Paper chromatographic mobilities of 2′—5′ linked oligomers are large compared to those of the corresponding 3′—5′ linked

oligomers when buffer of high salt concentration was used as a developing solvent (solvent II). The result suggests that 2′—5′ linked oligomers have hydrophilic character and 3′—5′ linked oligomers have hydrophobic character. The same tendency was also observed in the 2′—5′ and 3′—5′ linked oligoadenylates, where the difference of the mobilities were larger.[4,8]

NMR data of the 2′—5′ and 3′—5′ linked oligocytidylates are listed in Table 3. Cytosine H-5 and H-6 protons of the 2′—5′ linked oligomers shows high-field shift compared to those of the 3′—5′ linked oligomers.

Figure 2 shows CD spectra of the 2′—5′ and 3′—5′ linked oligocytidylates at 27 °C. CD spectra of 2′—5′ and 3′—5′ linked tetracytidylates at 4, 27, and 55 °C are shown in Fig. 3. CD band of 3′—5′ linked oligomers is stronger than that of the 2′—5′ linked oligomers and increases with increasing the chain length. Temperature dependance and chain length dependance of the CD spectra of 3′—5′ linked oligomers are larger than those of 2′—5′ linked oligomers

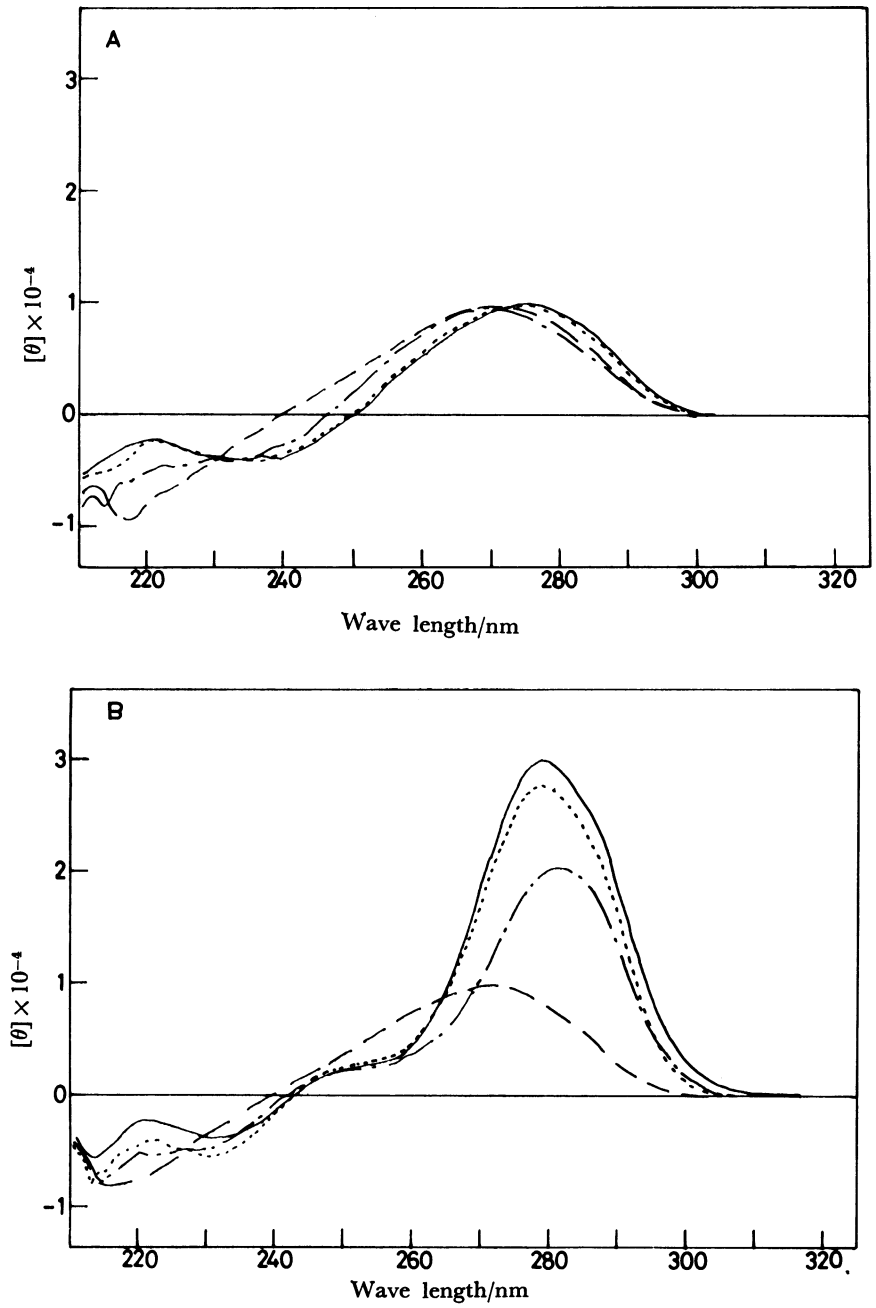


Fig. 2. CD Spectra of Oligocytidylates at 27 °C in 0.1 M Phosphate Buffer (pH 6.7).
A. 2′–5′ Linked Oligocytidylates. B. 3′–5′ Linked Oligocytidylates.
——— pC, — - - — $(pC)_2$, ---- $(pC)_3$, —— $(pC)_4$.

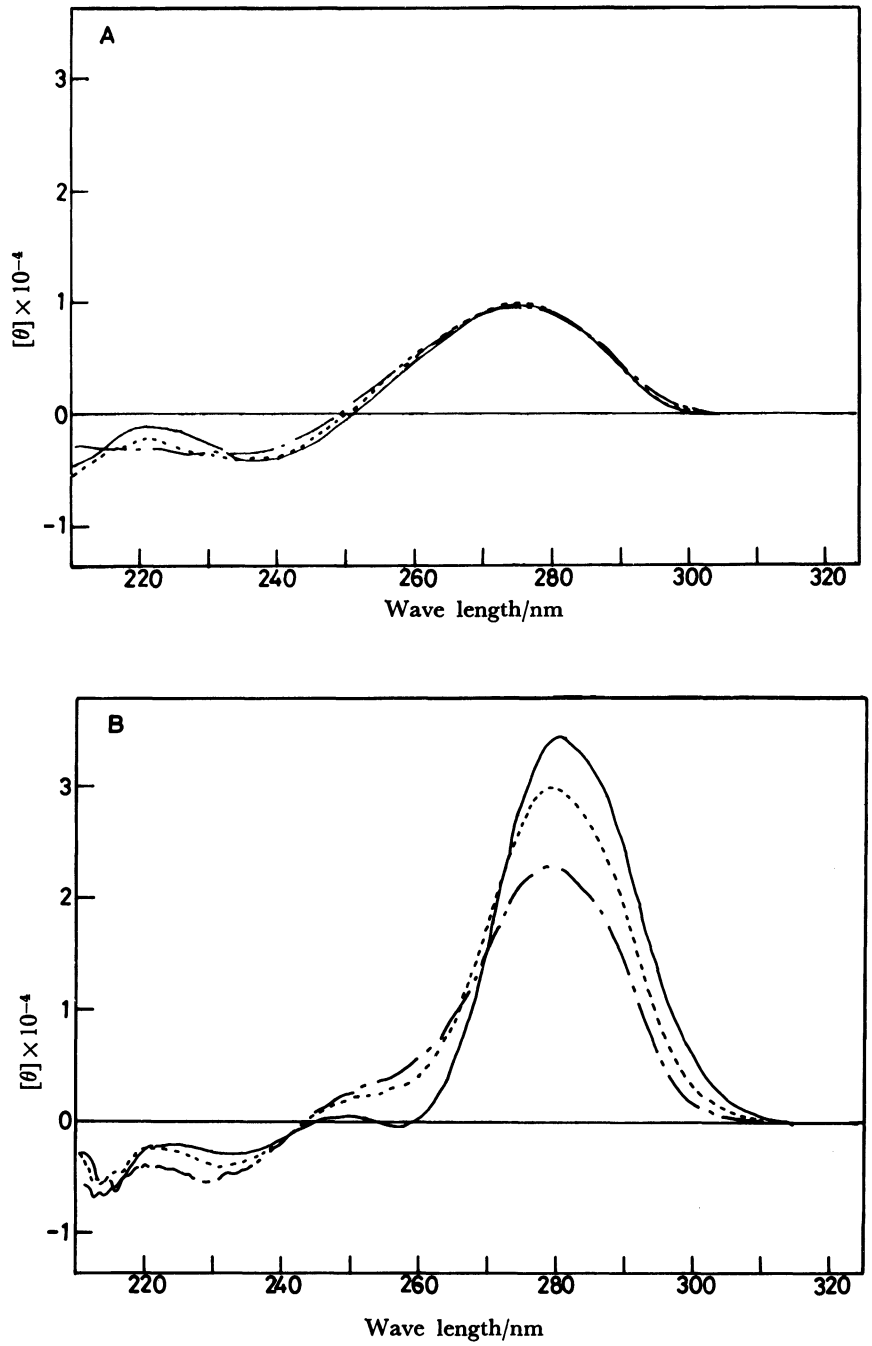


Fig. 3. CD Spectra of Tetracytidylates at 4, 27, and 55 °C in 0.1 M Phosphate Buffer (pH 6.7)
A. 2′—5′ Linked Tetracytidylate. B. 3′—5′ Linked Tetracytidylate.
—— 4 °C, ---- 27 °C, — - — - 55 °C.

which are similar to those of monomeric cytidylic acid. The CD data suggest that 3′—5′ linked oligomers possess higher ordered but more flexible structure than 2′—5′ linked oligomers. The character is also observed in the 2′—5′ and 3′—5′ linked oligoadenylates.[8] We tentatively believe that 3′—5′ linked oligomers can possess various conformations depending on the change of environment such as temperature and salt concentration, which is important for the function of RNA.

Experimental

Materials. Cytidine 5′-phosphoric acid was obtained from Yamasa. Cytidine 5′-phosphorimidazolide was prepared from pC and imidazole using di-2-pyridyl disulfide and triphenylphospine as a condensing agent by the similar method as published procedure.[5,7] Bacterial alkaline phosphatase (BAPF) and snake venom phosphodiestearase (VPDase) were from Worthington. Polycytidylic acid (sodium salt), nuclease p_1 (N. P_1) and nuclease SW were

Table 2. Chromatographic mobilities of the compounds

Compound	R_f value relative to pC	
	Solvent (I)	Solvent (II)
C	1.69	0.87
ImpC	1.67	
3′,5′-cyclic CMP	1.50	0.73
C2′p5′C	1.22	0.84
C3′p5′C	1.24	0.73
pC	1.00	1.00
CppC	1.00	0.88
-pCpC- (mixture)	1.20—1.25	0.61—0.77
pC2′p5′C	0.87	0.93
pC3′p5′C	0.85	0.91
pC2′p5′C2′p5′C	0.73	0.84
pC3′p5′C2′p5′C	0.72	0.81
pC3′p5′C3′p5′C	0.72	0.78
pC2′p5′C2′p5′C2′p5′C	0.58	0.75
2′-5′$(pC)_5$	0.38	0.70

Table 3. ^1H NMR of cytosine and H-1′ proton of oligocytidylates in D_2O at 25 °C (pD 6.7)

Compound	Chemical shift/δ (Coupling constant/Hz)		
	H-6($J_{5,6}$)	H-5($J_{5,6}$)	H-1′($J_{1',2'}$)
pC	8.07 (7.6)	6.14 (7.6)	6.00 (3.7)
C3′p	7.85 (7.6)	6.07 (7.6)	6.00 (5.2)
C2′p	7.79 (7.6)	6.07 (7.6)	5.94 (4.0)
3′—5′$(pC)_2$	8.14 (7.6)	6.07 (7.6)	5.95 (4.0)
	7.93 (7.6)	6.07 (7.6)	5.91 (3.4)
2′—5′$(pC)_2$	7.87 (7.6)	6.08 (7.6)	6.18 (6.4)
	7.81 (7.6)	5.92 (7.6)	5.87 (3.4)
3′—5′$(pC)_3$	8.12 (7.3)	6.03 (7.6)	5.91 (3.1)
	7.93 (7.6)	6.00 (7.3)	5.80 (1.5)
	7.90 (7.6)	6.00 (7.3)	5.79 (2.1)
2′—5′$(pC)_3$	7.80 (7.3)	6.05 (7.7)	6.12 (5.5)
	7.79 (7.6)	5.91 (7.3)	6.10 (5.8)
	7.61 (7.3)	5.91 (7.3)	5.88 (3.7)

purchased from Seikagaku Kogyo.

Paper Chromatography and HPLC. Paper chromagtography was carried out by a descending technique on Whatman 3MM paper using solvent system (I), 1-propanol–aqueous ammonia (55:45), and (II), saturated ammonium sulfate–0.1 M sodium (1 M=1 mol dm^{-3}) acetate (pH 6.5)–2-propanol (79:19:2).

HPLC was performed with a Hitachi 638 using a RPC-5 column (4 mm×25 cm). RPC-5 was prepared from fine grannular poly(chlorotrifluoroethylene) and Adogen 464 by the modification of the method according to Pearson *et al.*[9] Elution was carried out with a linear gradient of $NaClO_4$ solution (0—0.01 M) buffered with 2.5 mM Tris acetate (pH 7.5).

Spectrometric Method. UV absorption spectra were recorded by a Shimazu MPS-5000 instrument in 0.1 M phosphate buffer (pH 6.7). NMR spectra were taken by a JEOL-GX-400 instrument using TSP-d_4 as an internal standard. The sample was passed through a short Dowex 50WX-8 (Na^+ form) column, evaporated under vacuum and repeated coevaporation with D_2O (1 ml×3). Concentration of the sample was about 5 mM per cytidine residue in D_2O (0.1 M phosphate buffer, pH 6.7). CD spectra were measured by a JASCO J-40 spectrometer using a 2 mm cell in 0.1 M phosphate buffer (pH 6.7).

Polymerization of ImpC. To an aqueous solution (6.8 ml) of ImpC (189 mg, 0.4 mmol, 4160 ODU_{270}), 0.8 ml of imidazole buffer (2 M, pH 6.5) and 0.4 ml of 0.25 M lead nitrate were added with stirring. The reaction mixture was kept at 20 °C for 5 d with stirring. Versenol buffer (0.6 ml×0.25 M, pH 7.0) was added to the reaction mixture to remove Pb(II) ion by Versenol-Pb^{2+} complex formation. HPLC of an aliquot showed the formation of oligocytidylates and the disapperance of ImpC. The solution was applied to a QAE-Sephadex A-25 column (30 mmϕ×45 cm). The column was eluted with a stepwise linear gradient of triethylammonium hydrogencarbonate buffer (1) H_2O-0.33 M (2×0.9 l), (2) 0.33 M—0.5 M (2×2 l), (3) 0.5 M—0.75 M (2×1 l). 15 ml of each fraction was collected every 12 min. UV absorption of each fraction was measured at 271 nm. The UV absorbing fractions were pooled and evaporated under vacuum below 30 °C. The excess triethylammonium hydrogencarbonate was removed by repeated evaporation after addition of water. The products were further purified by paper chromatography using solvent system (I) when necessary. The yield was calculated from ODU_{271} after allowing for the hypochromicity of each oligocytidylate. The hypochromicity was estimated by the ratio of UV absorption at 270 nm after and before alkaline hydrolysis of the oligocytidylate.

Enzymatic Synthesis of 3′—5′ Linked Oligocytidylates. Polycytidylic acid sodium salt (25 mg, 0.055 mmol) was digested with nuclease SW (400 units) in the solution (6 ml) containing sodium carbonate buffer (0.05 M, pH 10.3), NaCl (0.1 M), $MgCl_2$ (1 mM) and sodium acetate (2 mM) at 37 °C for 20 h. The reaction mixture was applied to a DEAE-Sephadex A-25 column (25 mmϕ×35 cm) and eluted with a linear gradient of triethylammonium hydrogencarbonate buffer (0.25 M—0.5 M, 2×1 l). 10 ml of each fraction was collected every 10 min. The UV absorbing fractions were pooled and evaporated under vacuum below 30 °C. The 3′—5′ linked di-, tri-, and tetra-cytidylates with 5′-terminal phosphate were obtained in 29% (1350 ODU_{270}, 0.016 mmol), 42% (1180 ODU_{270}, 0.023 mmol) and 18% (780 ODU_{270}, 0.01 mmol) yields, respectively.

Chracterization of the Oligocytidylates. Characterization of the products was carried out by means of sequential enzyme and alkaline hydrolysis. Each oligocytidylate was subjected to paper chromatography in solvent system (I) and (II). The UV absorbing spot on the paper developed with solvent system (I) was eluted with water and incubated with BAPF to remove 5′-terminal phosphate. The mixture was chromatographed on the paper with solvent system (I). The UV absorbing spot on the paper was eluted with water and incubated with N. P_1 to degrade 3′—5′ internucleotide linkage. The mixture was analyzed by HPLC and paper chromatography in solvent system (I). The UV absorbing spot on the paper was eluted with water and incubated with VPDase and NaOH solution to degrade 2′—5′ internucleo-

tide linkage. The degraded products were analyzed by HPLC.

Digestion with BAPF was carried out by incubating a mixture of 0.5—1 μmol of nucleotide material in a 50 μl solution containing 0.1 M Tris-HCl (pH 8.05), 0.001 M $MgCl_2$ and 0.1 unit of the enzyme, at 37 °C for 2.5 h.

Digestion with N. P_1 was performed at 37 °C for 2.5 h in a mixture (50 μl) containing the nucleotide material (0.4—0.8 μmol), 0.006 M veronal acetate buffer (pH 5.75) and an enzyme (5 μg).

Degradation with VPDase was carried out at 37 °C for 2.5 h in a mixture (50 μl) containing the substrate (0.1—0.2 μmol), 0.01 M Tris-acetate (pH 8.0), 0.01 M $MgCl_2$ and enzyme solution (0.01 unit).

Alkaline hydrolysis was carried out at room temperature for 1 d in a mixture (50 μl) containing the substrate (0.1—0.2 μmol) in a 0.5 M NaOH solution.

The structure of the oligocytidylates was further confirmed by NMR, and by comparing the paper chromatography and HPLC with that of the authentic sample when available.

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