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PREPARATION OF OLIGOADENYLATES WITH 2'–5' LINKAGE USING Pb^{2+} ION CATALYST

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Abstract—2'–5' Linked oligoadenylates up to a pentamer were formed selectively by Pb^{2+} ion-catalyzed polymerization of adenosine-5'-phosphorimidazolide in aqueous solution. Small amounts of oligoadenylate isomers containing both 2'–5' and 3'–5' internucleotide linkage were obtained simultaneously. The structure of the linkage isomers was established by sequential enzyme and alkaline digestion.

Adenosine-5'-phosphorimidazolide (ImpA) is an activated nucleotide and it hydrolyzes to adenylic acid (pA) in aqueous solution. Several years ago, we showed that some divalent metal ions promote the polymerization of ImpA in aqueous solution to yield oligoadenylates.[1,2] Pb^{2+} ion has the highest activity and gives high yield of oligoadenylates.[2] Enzyme digestion of the resulting diadenylates revealed that the 2'–5' linked isomer is predominant. However, no detailed characterization of the reaction and of the products has been carried out from a synthetic point. This reaction may provide a simple preparative method for oligoadenylates with 2'–5' linkage. We have now studied the separation of the resulting isomeric oligoadenylates and determined the structure by sequential enzyme digestions. Here we wish to report the preparation and the structure determination of various 2'–5' linked oligoadenylates from ImpA by Pb^{2+} ion catalyst.

Recently an unusual 2'–5' linked oligonucleotide has been discovered from interferon-treated cell,[3] and attracted widespread interest for its strong inhibitory activity in protein biosynthesis.[3,4] Application of the Pb^{2+} ion-catalyzed oligoadenylate formation for the synthesis of the oligonucleotide inhibitor of protein biosynthesis has been published elsewhere.[5]

RESULTS AND DISCUSSION

ImpA was easily hydrolyzed to pA in aqueous solution. Addition of lead nitrate to a clear solution of ImpA gave white precipitates which indicated the complexation of ImpA with Pb^{2+} ion. The mixture was kept for 5 days at room temperature with stirring for polymerization. The starting ImpA disappeared almost completely in 5 days. Versenol was added to the reaction mixture to break the Pb^{2+}-nucleotide complexes. The reaction mixture turned homogeneous, indicating the formation of Pb^{2+}-Versenol complex. Analysis of the products with HPLC and paper chromatography showed the formation of several isomeric oligoadenylates in addition to the hydrolysis of ImpA to pA. Analysis with HPLC confirmed the complete formation of the Pb^{2+}-Versenol complex. We found that isomeric mixture of short chain oligonucleotides were well separated by QAE Sephadex A-25 column chromatography. Thus, column chromatography of the reaction products on QAE Sephadex A-25 (bicarbonate form) gave good separation of the linkage isomer of the oligoadenylates. Elution pattern is shown in Fig. 1. The Pb^{2+}-Versenol complex was eluted in the fraction number 106–118. The distribution and the yield of the products are listed in Table 1 along with the identified structure.

Peaks 1, 2 and 3 were identified as A, $A^{2'}p^{5'}A$ and $A^{3'}p^{5'}A$, respectively. Dephosphorylation of pA, $pA^{2'}p^{5'}A$ and $pA^{3'}p^{5'}A$ gave these compounds. Pb^{2+} ion is known to catalyze the degradation of RNA.[6] Pb^{2+} ion might be a catalyst for dephosphorylation. The longer reaction time and high pH also favored dephosphorylation. Peak 4 was 3',5'-cyclic AMP which was probably formed from ImpA by intramolecular phosphodiester bond formation. Peak 5 contained pA, a simple hydrolyzed product. The compound in peak 6 was AppA which could be obtained from ImpA and pA. The major peaks, 9, 13, 16 and 19, contained a series of fully 2'–5' linked oligoadenylates from a dimer to a pentamer. The yields of the dimer, trimer, tetramer and pentamer were 24.4%, 9.0%, 3.1% and 0.9%, respectively, after allowing for the hypochromicity ratio of each oligoadenylate. The dimer and the trimer were practically pure. Peaks 16 and 19 contained other minor products and needed further purification to get the pure compounds. Internucleotide linkage was checked by sequential enzyme digestions using bacterial alkaline phosphatase, nuclease P_1 and venom phosphodiesterase and by alkaline hydrolysis. The 2'–5' linked oligoadenylates were insensitive to nuclease P_1 and RNase T_2. The compounds in peaks 12 and 16 were 3'–5' linked dimer and trimer, respectively. The structure was confirmed by cochromatography with authentic samples and the enzyme digestion. The yields of 3'–5' linked isomers were less than one tenth of those of the corresponding 2'–5' linked isomers. Peaks 14 and 15 contained the linkage isomers of triadenylate, $pA^{2'}p^{5'}A^{3'}p^{5'}A$ and $pA^{3'}p^{5'}A^{2'}p^{5'}A$, respectively. The yields were lower than that of the 2'–5' isomer and higher than that of the 3'–5' isomer. The structure of the isomers was ascertained by sequential enzyme digestions with bacterial alkaline phosphatase and nuclease P_1. Tetraoligonucleotides with two 2'–5' linkages and one 3'–5' linkage in different positions were present in peaks 17 and 18. The material in peak 10 was $AppA^{2'}p^{5'}A$ which could be formed from either AppA with ImpA, pA with $ImpA^{2'}p^{5'}A$ or ImpA with $pA^{2'}p^{5'}A$. Peaks 7, 8 and 11 were unidentified, though they were confirmed not to be cyclic oligoadenylates.

The concentration of ImpA and Pb^{2+} ion catalyst affects oligoadenylate formation. Lower concentration of ImpA and Pb^{2+} ion facilitated the hydrolysis of ImpA to pA and decreased the yield of oligoadenylates. Higher concentration favored long chain oligoadenylates. However, the total yield of oligoadenylates decreased because of the formation of the unidentified product in large amount.

The major features of this reaction are preferential

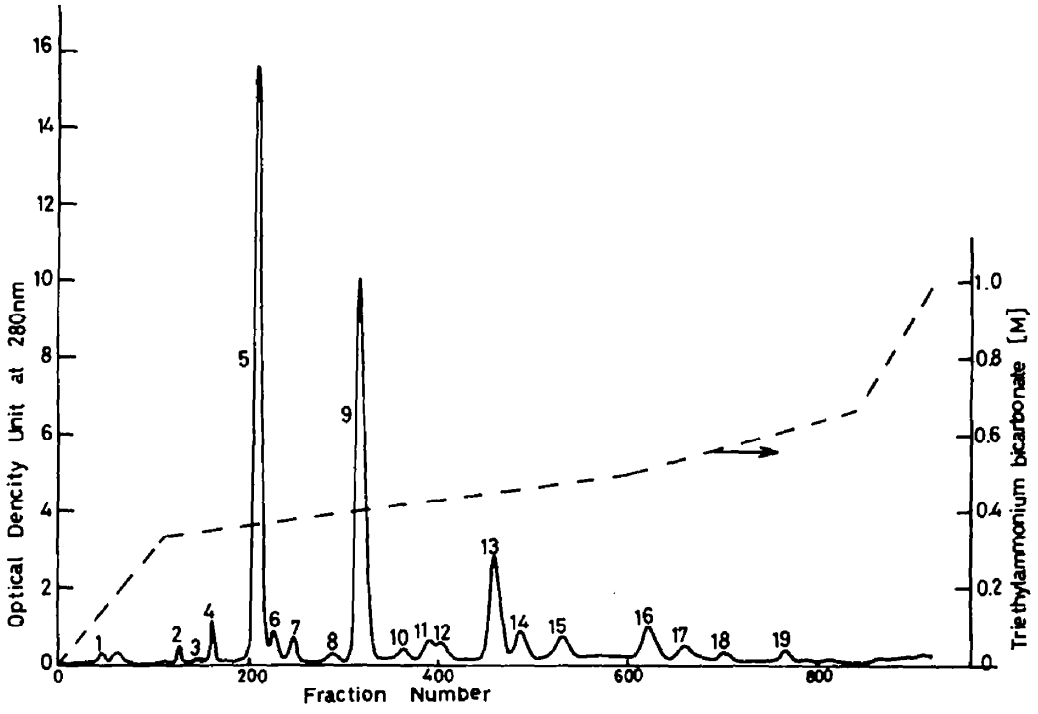


Fig. 1. Elution curve of oligoadenylates obtained from ImpA. Column chromatography was carried out on QAE-Sephadex A-25 (HCO_3^-). Broken line shows triethylammonium bicarbonate gradient.

formation of the 2'–5' linked oligoadenylates and no formation of cyclic oligoadenylates. Our procedure provides a very simple method for the preparation of the 2'–5' linked oligonucleotides. The condensation reaction proceeds in aqueous solution and no protecting group is employed.

So far, the mechanistic role of Pb^{2+} ion is unknown, nevertheless we believe that Pb^{2+} ion orients ImpA by coordination in such a way as to promote internucleotide bond formation. Further, Pb^{2+} ion probably facilitates the nucleophilicity of 2'-OH of ImpA. Similar mechanistic roles of Zn^{2+} ion have been proposed in enzymatic and nonenzymatic phosphoryl and nucleotidyl transfer reactions.[7]

EXPERIMENTAL

Materials. Adenosine-5'-monophosphate was purchased from Yamasa. Imidazole was from Tokyo Kasei, and recrystallized from benzene. Triphenylphosphine, Versenol trisodium salt and lead nitrate were obtained commercially. 2,2'-Dipyridyl disulfide was prepared by a modification of the published procedure.[8] ImpA was prepared from pA and imidazole by the method according to Lohrmann and Orgel.[9]

Bacterial alkaline phosphatase and venom phosphodiesterase were supplied by Worthington, nuclease P_1 by Yamasa.

Paper chromatography and HPLC. Paper chromatography was performed on Whatman 3MM paper by the descending technique using the following systems, (1) 1-propanol, concentrated ammonia and water (55:10:35) and (2) saturated ammonium sulfate, 0.1M sodium acetate (pH 6.5) and 2-propanol (79:19:2). The chromatographic mobilities of various compounds are listed in Table 2.

HPLC was taken with a Hitachi 530 apparatus using RPC-5 column (4ϕ × 25 cm). The elution was carried out with a linear gradient of NaCl (0.001M–0.5M) and the compounds were monitored by UV absorption at 254 nm.

Polymerization of ImpA. To the aqueous soln (42.5 ml) of ImpA (1.25 g, 2.6 mmol), 5 ml imidazole buffer (2M, pH 6.5) and then 2.6 ml of 0.25M lead nitrate (0.65 mmol) were added with stirring. White ppts were formed. The mixture was kept at 20° for 5 days with stirring. Versenol buffer (3 ml × 0.25M) was added to the mixture to complex the Pb^{2+} ion. The mixture turned homogeneous by this treatment. Paper chromatography and HPLC of an aliquot showed the formation of oligoadenylates and the disappearance of the starting ImpA. The mixture was diluted with water (200 ml) and subjected to a column chromatography on QAE-Sephadex A-25 bicarbonate form (3.6 cm × 50 cm). The elution was carried out by a stepwise linear gradient of triethylammonium bicarbonate buffer [(1) H_2O–1/3M (2 × 1l); (2) 1/3M–1/2M (2 × 4l); (3) 1/2M–2/3M (2 × 2l); (4) 2/3M–1M (2 × 0.6l)]. 17 ml of each fraction was collected every 12 min. UV absorption of each fraction was measured at 280 nm. The fractions containing the UV absorbing compound were pooled and evaporated *in vacuo*. The carbonate free residue was dissolved in water and lyophilized to give the product as a triethylammonium salt.

Degradation and identification of the products. Identification of the products was carried out by cochromatography with the authentic samples and by sequential enzyme and alkaline digestions.

Digestion with bacterial alkaline phosphatase (BAP) was carried out for 2.5 hr at 37° in a mixture (50 μl) containing the substrate (5 ~ 20 ODU), 0.1M Tris-HCl (pH 8.05), 0.001M $MgCl_2$ and 0.01 units of enzyme.

Nuclease P_1 ($N.P_1$) degrades the 3'–5' linked phosphodiester bond and is inactive towards the 2'–5' bond. Digestion with $N.P_1$ was carried out for 2.5 hr at 37° in a mixture (50 μl) containing the substrate (1 ~ 20 ODU), 0.006M Veronal-acetate buffer (pH 5.75) and the enzyme solution (5 μg in 5 μl).

Venom phosphodiesterase (VPD) degrades both 2'–5' and 3'–5' phospbodiester bonds. It also hydrolyzes pyrophosphate bond. Digestion was carried out for 2.5 hr at 37° in a mixture (50 μl) containing the substrate (1 ~ 10 ODU), 0.01M Tris-acetate (pH 8.8), 0.01M $MgCl_2$ and the enzyme soln (0.1 units).

The activities of these enzymes were checked using $A^{2'}p^{5'}A$ and $A^{3'}p^{5'}A$ as model substrates. Incubation times and quantities of the enzymes were confirmed to be enough for complete digestion.

Alkaline hydrolysis was carried out for 1 day at room temp in

Table 1. Oligoadenylates obtained from ImpA catalyzed by Pb^{2+} ion

Peak No.	ODU_{260}	h*1	Identified Structure	Yield (%)*2
1	197		A	0.5
2	138	1.19	$A^{2'}p^{5'}A$	0.4
3	45	1.25	$A^{3'}p^{5'}A$	0.1
4	552		3',5' cyclic AMP	1.4
5	12950		pA	32.4
6	417	1.30	AppA	1.3
7	431		unidentified	1.1*3
8	266		unidentified	0.7*3
9	8138	1.20	$pA^{2'}p^{5'}A$	24.4
10	377	1.22	$AppA^{2'}p^{5'}A$	1.1
11	592		unidentified	1.5*3
12	484	1.16	$pA^{3'}p^{5'}A$	1.4
13	2738	1.31	$pA^{2'}p^{5'}A^{2'}p^{5'}A$	9.0
14	1195	1.22	$pA^{2'}p^{5'}A^{3'}p^{5'}A$	3.6
15	1036	1.26	$pA^{3'}p^{5'}A^{2'}p^{5'}A$	3.1
16	1257	1.27	$pA^{3'}p^{5'}A^{3'}p^{5'}A$ (1) +	0.4
		1.38	$pA^{2'}p^{5'}A^{2'}p^{5'}A^{2'}p^{5'}A$ (9)	3.1
17	651	1.36	$pA^{2'}p^{5'}A^{3'}p^{5'}A^{2'}p^{5'}A$	2.2
18	288	1.34	$pA^{2'}p^{5'}A^{2'}p^{5'}A^{3'}p^{5'}A$	1.0
19	290	1.42	$pA^{2'}p^{5'}A^{2'}p^{5'}A^{2'}p^{5'}A^{2'}p^{5'}A$	0.9

*1 Hyperchromicity calculated from Alkaline hydrolysis.

*2 Yield obtained from UV_{260} after allowing the hyperchromicity of each oligoadenylates. Total ODU_{260} of starting ImpA is 40,000 (2.6 mmol).

*3 Hyperchromicity correction was not made on the value.

a mixture (50 μl) containing the substrate (1 ~ 10 ODU) in 0.5M NaOH soln. Both 2'-5' and 3'-5' phosphodiester bonds were degraded by the alkaline hydrolysis in a different manner from the VPD digestion.

The products, A, $A^{2'}p^{5'}A$, $A^{3'}p^{5'}A$, pA and AppA, were identified by comparing the paper chromatography and HPLC with those of authentic samples. Authentic $A^{2'}p^{5'}A$ and $A^{3'}p^{5'}A$ were prepared by the dephosphorylation of $pA^{2'}p^{5'}A$ and $pA^{3'}p^{5'}A$ with BAP, respectively. AppA was synthesized according to Khorana *et al.*[10] 3',5'-cyclic AMP was identified by cochromatography with an authentic sample which was prepared from pA using dicyclohexylcarbodiimide.[11]

The structure of 2'-5' linked oligoadenylates from the dimer to pentamer was determined by the sequential enzyme and alkaline digestion as follows.

$$pA^{2'}pA \xrightarrow{N.P_1} X$$

$$pA^{2'}pA \xrightarrow{VPD} pA$$

$$pA^{2'}pA \xrightarrow{NaOH} pAp + A(1:1)$$

$$pA^{2'}pA \xrightarrow{BAP} A^{2'}pA \xrightarrow{N.P_1} X$$

$$A^{2'}pA \xrightarrow{VPD} A + pA(1:1)$$

$$A^{2'}pA \xrightarrow{NaOH} Ap + A(1:1)$$

$$pA^{2'}pA^{2'}pA \xrightarrow{N.P_1} X$$

$$pA^{2'}pA^{2'}pA \xrightarrow{VPD} pA$$

$$pA^{2'}pA^{2'}pA \xrightarrow{NaOH} pAp + Ap + A(1:1:1)$$

$$pA^{2'}pA^{2'}pA \xrightarrow{BAP} A^{2'}pA^{2'}pA \xrightarrow{N.P_1} X$$

$$A^{2'}pA^{2'}pA \xrightarrow{VPD} A + pA(1:2)$$

$$A^{2'}pA^{2'}pA \xrightarrow{NaOH} Ap + A(2:1)$$

The tetramer and pentamer were identified in the same procedure.

Oligoadenylates with 3'-5' linkage were identified by cochromatography with authentic samples which were prepared by partial hydrolysis of poly A with pork liver nuclease.[12] The 3'-5' phosphodiester bond was further confirmed by the enzyme digestion.

$$pA^{3'}pA \xrightarrow{N.P_1} pA$$

$$pA^{3'}pA \xrightarrow{NaOH} pAp + A(1:1)$$

$$pA^{3'}pA \xrightarrow{BAP} A^{3'}pA$$

Table 2. Chromatographic mobilities of the compounds

Compounds	Rf value relative to pA	
	Solvent (1)	Solvent (2)
A	1.72	0.60
$A^{2'}p^{5'}A$	1.27	0.41
$A^{3'}p^{5'}A$	1.37	0.22
3',5'p<A	1.69	0.47
ImpA	1.62	-
pA	1.00	1.00
$Ap^{2'}$	1.13	0.80
$Ap^{3'}$	1.11	0.62
AppA	1.09	0.68
$AppA^{2'}pA$	0.78	0.48
$pA^{2'}pA$	0.80	0.81
$pA^{3'}pA$	0.79	0.57
$pA^{2'}p$	0.67	1.30
$pA^{3'}p$	0.67	1.09
$pA^{2'}pA^{2'}pA$	0.52	0.65
$pA^{2'}pA^{3'}pA$	0.51	0.59
$pA^{3'}pA^{2'}pA$	0.50	0.49
$pA^{3'}pA^{3'}pA$	0.52	0.41
$pA^{2'}pA^{2'}pA^{2'}pA$	0.40	0.56
$pA^{2'}pA^{3'}pA^{2'}pA$	0.38	0.42
$pA^{2'}pA^{2'}pA^{3'}pA$	0.38	0.37
$pA^{2'}pA^{2'}pA^{2'}pA^{2'}pA$	0.30	0.23

$PA^{3'}pA^{3'}pA \xrightarrow{N.P_1} pA$

$PA^{3'}pA^{3'}pA \xrightarrow{NaOH} pAp + Ap + A(1:1:1)$

$PA^{3'}pA^{3'}pA \xrightarrow{BAP} A^{3'}pA^{3'}pA \xrightarrow{N.P_1} A + pA(1:2)$

Two linkage isomers of triadenylates which possess one 3'–5' and 2'–5' linkage (14 and 15) were identified as follows.

$pA^{2'}pA^{3'}p \xrightarrow{N.P_1} pA^{2'}pA + pA(1:1)$

$pA^{2'}pA^{3'}p \xrightarrow{NaOH} pAp + Ap + A(1:1:1)$

$pA^{2'}pA^{3'}p \xrightarrow{VPD} pA$

$pA^{2'}pA^{3'}p \xrightarrow{BAP} A^{2'}pA^{3'}pA \xrightarrow{N\,P_1} A^{2'}pA + pA(1:1)$

$A^{2'}pA^{3'}pA \xrightarrow{NaOH} Ap + A(2:1)$

$pA^{3'}pA^{2'}pA \xrightarrow{N.P_1} pA^{2'}pA + pA(1:1)$

$pA^{3'}pA^{2'}pA \xrightarrow{NaOH} pAp + Ap + A(1:1:1)$

$pA^{3'}pA^{2'}pA \xrightarrow{VPD} pA$

$pA^{3'}pA^{2'}pA \xrightarrow{BAP} A^{3'}pA^{2'}pA \xrightarrow{N.P_1} A + pA^{2'}pA(1:1)$

$A^{3'}pA^{2'}pA \xrightarrow{NaOH} Ap + A(2:1)$

The structure of tetraadenylates with one 3'–5' linkage and two 2'–5' linkages (17 and 18) were confirmed by the following enzyme digestions.

$pA^{2'}pA^{3'}pA^{2'}pA \xrightarrow{N.P_1} pA^{2'}pA^{2'}pA + pA(1:1)$

$pA^{2'}pA^{3'}pA^{2'}pA \xrightarrow{NaOH} pAp + Ap + A(1:2:1)$

$pA^{2'}pA^{3'}pA^{2'}pA \xrightarrow{BAP} A^{2'}pA^{3'}pA^{2'}pA \xrightarrow{N.P_1} A^{2'}pA + pA^{2'}pA$

$A^{2'}pA^{3'}pA^{2'}pA \xrightarrow{NaOH} Ap + A\ (1:3)$

$A^{2'}pA^{3'}pA^{2'}pA \xrightarrow{VPD} A + pA\ (1:3)$

$pA^{2'}pA^{2'}pA^{3'}pA \xrightarrow{N.P_1} pA^{2'}pA^{2'}pA + pA(1:1)$

$pA^{2'}pA^{2'}pA^{3'}pA \xrightarrow{NaOH} pAp + Ap + A\ (1:2:1)$

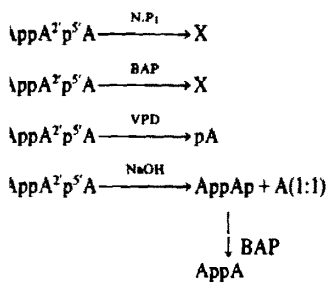
$pA^{2'}pA^{2'}pA^{3'}pA \xrightarrow{BAP} A^{2'}pA^{2'}pA^{3'}pA \xrightarrow{N.P_1} A^{2'}pA^{2'}pA + pA(1:1)$

$A^{2'}pA^{2'}pA^{3'}pA \xrightarrow{NaOH} Ap + A\ (1:3)$

$A^{2'}pA^{2'}pA^{3'}pA \xrightarrow{VPD} A + pA\ (1:3)$

The compound in peak 10 was identified as $AppA^{2'}pA$ by the

ollowing enzyme digestions.



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[12]L. A. Heppel, *Procedures in Nucleic Acid Research*, (Edited by G. L. Cantoni and D. R. Davies) p. 33. Harper & Row, New York (1966).

[13]*Abbreviations.* A, adenosine; pA, adenosine 5'-phosphate; Ap, adenosine 2'(3')-phosphate; ImpA, adenosine-5'-phosphorimidazolide; $A^{2'}p^{5'}A$, adenylyl-[2'–5']-adenosine; $A^{3'}p^{5'}A$, adenylyl-[3'–5']-adenosine; $pA^{2'}p^{5'}A$, 5'-phosphoadenylyl-[2'–5']-adenosine; $pA^{3'}p^{5'}A$, 5'-phosphoadenylyl-[3'–5']-adenosine; AppA, 5,5'-diadenosine pyrophosphate; $pA^{2'}p^{5'}A^{3'}p^{5'}A$, 5'-phosphoadenylyl - [2'–5'] - adenylyl - [3'–5'] - adenoisine; $pA^{3'}p^{5'}A^{2'}p^{5'}A$, 5'-phosphoadenylyl - [3'–5'] - adenylyl - [2'–5'] - adenoisine; Poly A, polyadenylic acid; Versenol, N - hydroxyethylethylendiaminetriacetic acid; $N.P_1$, Nuclease P_1; BAP, Bacterial alkaline Phosphatase; VPD, Venom phosphodiesterase.