POLYMERIZATION of 5'-DESOXYRIBONUCLEOTIDES WITH β-IMIDAZOLYL-4(5)-PROPANOIC ACID[1]

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Summary: β-imidazolyl-4(5)-propanoic acid was found to act as a specific catalyst in the polymerization of the unprotected 5'-desoxyribonucleotides. Only the 3'-5' internucleotide diester bond is formed and the overall yield of the di- and oligonucleotides is about 40 - 50%.

The synthetic procedure for deoxy-oligonucleotides developed in recent years in several laboratories (1 - 3) involves the activation of the phosphate moiety of the suitably protected nucleotides in a condensation or polymerization process. This process, however, inadvertently yields the undesirable pyrophosphate-linkage besides the 3'-5'-internucleotide diester bond. We wish to report a completely different approach to the polymerization process. Under appropriate conditions, β-imidazolyl-4(5)-propanoic acid (IPA) was found to catalyze the polymerization of unprotected deoxynucleotides giving only 3'-5' linked polynucleotides in 40 - 60% yield.

Experimental

Polymerization: In 100 ml. DMF, 0.50 mMole thymidine-5'-phosphate, disodium salt (Sigma) and 1.0 mMole IPA, (A grade, Calbiochem) were dissolved at 50°C. The reaction mixture was under reflux for 15 minutes, cooled to room temperature, and then the DMF was removed at 44°C in vacuo.

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The residue was dissolved in a small amount of water, adjusted to pH 8 with ammonia and was applied to DEAE-column chromatography.

Separation: Descending paper chromatography was carried out with S. and S. No. 2043B paper in the following 3 solvent systems: A, isopropanol-concd. NH_4OH-water (7 : 1 : 2, V/V); B, isobutyric acid - 1M NH_4OH-Na_2-ETDA (0.1M) (100 : 1.6, V/V); C, n-propanol-concd. NH_4OH-water (55 : 10 : 35, V/V). Paper electrophoresis was carried out on Whatman 3MM strips in 0.05 M triethylammonium bicarbonate buffer (pH 7.5) or in 0.05 M ammonium acetate buffer (pH 4.5). The apparatus is similar to Savant flat plate model. Column chromatography for the separation of the reaction products was first done on DEAE-cellulose (HCO_3^- form), as described by Khorana et al (4) and peak No. 2 to peak No. 10 were rechromatographed on DEAE-cellulose (Cl^- form, 5). The effluent was analyzed with an Isco UA2 u.v.-analyzer.

Acetic anhydride treatment: Portion of the total reaction mixture was converted to pyridinium salt by a column procedure and then was treated by the acetic anhydride method described by Khorana et al (6). The treatment products were analyzed by paper chromatography.

Removal of terminal phosphate: 5µl. of E. coli alkaline phosphatase (Worthington, BAPF) solution (0.1 mg/ml in 0.05 M Tris buffer) was added to the substrate containing 0.1 µmole of terminal phosphate in 0.03-0.04 ml buffered by Tris (0.05 M, pH 8). The mixture was incubated at 37° for 4 hrs. and was then analyzed by paper chromatography developed in solvent C for 2 - 4 days.

Degradation of oligonucleotides by snake venom phosphodiesterase: 40 µl. of enzyme (Worthington, 5 mg/ml water) was added to the oligonucleotide solution containing 0.2 µmole of nucleoside units in 20 µl. of 1 M NH_4CO_3 buffer, pH 9.0. The enzyme preparation has been checked and standardized with a

given amount of TpT. The mixture was incubated at 37° for 8 hours and was then analyzed by paper chromatography in solvent system A. From the quantitative analyses of the chromatograms, thymidine to 5'-thymidine phosphate was obtained for each compound in the series of $T(pT)_{n-1}$ for the chain length determination.

Degradation of the oligonucleotides by spleen diesterase: 20 µl. of the enzyme (Worthington, 16 units/ml water) was added to 20 µl. solution of oligonucleotides containing 0.2 µmole of nucleoside units and 0.25 M Na succinate pH 6.5. The enzyme again was previously checked and standardized with TpT. After incubation at 37° for 8 hours, the mixtures were analyzed by paper chromatography in solvent A. Quantitative analyses of the chromatograms provided the ratio of thymidine to 5'-thymidine phosphate for the chain length determination and showed the absence of pyrophosphate linkage.

Results and Discussion

The elution pattern and the quantitative distribution of the products of the polymerization reaction of the 5'-thymidylic acid are shown in Fig. 1. About 45% of the original u.v. absorbing (280 mµ) materials is now in di- or oligonucleotides. If hypochromicity is taken into account (about 10%), then the yield of 3'-5' diester bond from the monomeric 5'-nucleotide is about 50%. A portion of each peak collected from the elution shown in Fig. 1 was rechromatographed on DEAE column (Cl^- form, pH 5.5). The results described in Table I provide additional information about the distribution of 5'-phosphate terminated oligonucleotides $(pT)_n$ and the dephosphorylated oligonucleotides $T(pT)_{n-1}$. The dephosphorylation (30 - 40%) was presumably not related to the polymerization reaction catalyzed by IPA, since the same extent of dephosphorylation of 5'-thymidine phosphate was observed when this compound

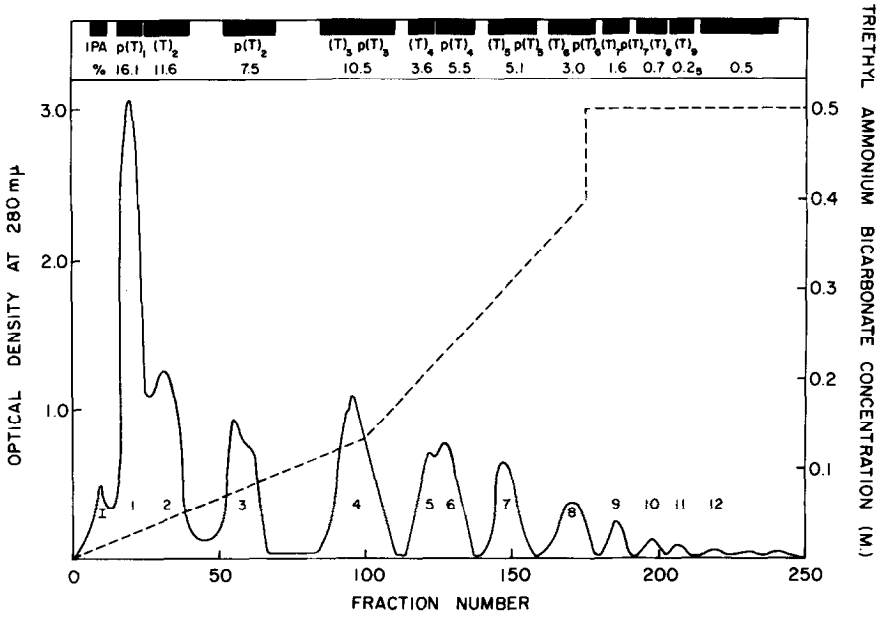

Fig. 1. The elution pattern of the oligonucleotide products obtained by the polymerization of 5'-TMP catalyzed by IPA. Chromatography was carried out on DEAE cellulose (HCO_3^-) column (1.50x30 cm) with a triethylammonium bicarbonate gradient (-----). The eluted fractions were pooled as indicated by the blocked areas. The nature of reaction product in each pooled fraction is shown and the absorbance (280 mμ) is expressed in percentage of the total absorbance of the starting TMP.

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was refluxed for 15 min. in DMF (which has not been vigorously dried) without the catalyst IPA.

The reaction products have been further characterized by three procedures: 1) All the products from each peak in the elution profile were analyzed by paper chromatography in 3 solvent systems and by paper electrophoresis. The Rf values and the relative mobilities observed are in agreement with those reported for these compounds (4). TpT synthesized by this procedure has the same paper chromatographic and electrophoretic properties as the authentic TpT synthesized by the conventional procedure. 2) A part of the reaction mixture was treated with a pyridine/acetic anhydride mixture which

Table I.

Second Chromatographic Separation of Individual Peaks Shown in Fig. 1[a].

Peak No.	Product	%[b]	Condition for Chromatography Reservoir[c] LiAc M	Reservoir[c] LiCl M	Mixing Vessel[c] LiAc M	Concn. of Cl^- at the peak (mid-point) M
2	pT	35	0.05	0.10	0.05	0.01
	TpT	65				0.013
3	$(pT)_2$	100	0.05	0.12	0.05	0.061
4	$T(pT)_2$	19	0.01	0.20	0.01	0.058
	$(pT)_3$	81				0.096
5	$T(pT)_3$	33	0.01	0.20	0.01	0.09
	$(pT)_4$	67				0.125
6	$T(pT)_3$	9	0.01	0.20	0.01	0.09
	$(pT)_4$	91				0.125
7	$T(pT)_4$	36	0.01	0.20	0.01	0.13
	$(pT)_5$	64				0.164
8	$T(pT)_5$	46	0.01	0.30	0.01	0.17
	$(pT)_6$	54				0.21
9	$T(pT)_6$	37	0.01	0.30	0.01	0.20
	$(pT)_7$	63				0.23
10	$T(pT)_7$	100	0.01	0.30	0.01	0.23

a. On DEAE-cellulose (Cl^-) column, pH 5.5.
b. % distribution of the u.v. absorbing material in that peak.
c. The volume for both vessels is 2 liters. The system is similar to that in ref. (5).

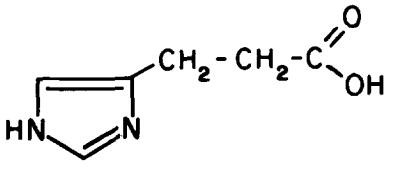
cleaves pyrophosphate linkage. Identical chromatographic patterns were obtained before and after the treatment, indicating the absence of pyrophosphate. 3) The 5'-phospho-terminated oligonucleotides were converted to the corresponding dephosphorylated oligonucleotide series by the action of alkaline phosphatase. These compounds, $T(pT)_{n-1}$, were then degraded completely by spleen diesterase and snake venom diesterase to thymidine and 5'-thymidylic acid. The $^T/_{pT}$ ratio of each compound is in agreement with the assign-

Table II.

Relative R_f Values and Mobilities of Oligothymidylic Acids Synthesized by the Present Procedure.

Compound	R_f Values Relative to pT[a]			Mobility Relative to pT[b]
	A	B	C	
TpT	2.7		1.35	
$(pT)_2$	0.54[c]	0.68[c]	0.86	1.29
$T(pT)_2$	1.28	0.52	1.12	
$(pT)_3$	0.12[c]	0.49[c]	0.70	1.60
$T(pT)_3$		0.33	0.85	
$(pT)_4$		0.29[c]	0.62	1.95
$T(pT)_4$		0.25	0.70	
$(pT)_5$		0.24	0.51	2.10
$T(pT)_5$		0.16	0.57	
$(pT)_6$		0.15	0.41	
$T(pT)_6$		0.12	0.47	
$(pT)_7$		0.11	0.32[c]	
$T(pT)_7$		0.09	0.34	
$T(pT)_8$		0.07	0.29	

a. Solvent systems A, B, and C are described in experimental methods and are the same as system A, B, and E in ref. (4). Chromatograms have been developed for 3 - 5 days.
b. In pH 4.5 buffer described in experimental method, the relative mobilities are slightly higher than those in pH 3.5 buffer reported in ref. (4).
c. Values identical to those published in ref. (4).



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ment of each oligonucleotide from the chromatographic and electrophoretic studies. These results also indicate the presence of 3'-5' phosphodiesters in the oligonucleotides. After the reaction about 70% of IPA was recovered from peak I (Fig. 1) and was identified by its u. v. -spectrum and melting point. The

remaining 30% of IPA decomposes under the reaction conditions, same in the absence and presence of 5'-thymidylic acid. Therefore, this decomposition is presumably due to the instability of IPA under the reaction conditions and not due to the polymerization process. Same yield in the polymerization process was obtained by a 1 : 10 ratio between IPA and 5'-thymidylic acid. These results indicate that IPA functions as a catalyst in the polymerization. The mechanism of the catalysis is currently under investigation. Preliminary studies show that this method is also applicable in the polymerization of all the other unprotected 5'-deoxyribonucleotides.

Besides the merit of this novel process as a synthetic procedure for deoxyoligonucleotides, the above observations may have interesting implications to the action of esterases (7) and to the prebiotic synthesis of nucleic acids.

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