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Note

Separation and sequencing of the sequence isomers of pyrimidine deoxypentanucleoside tetraphosphates by high-performance liquid chromatography

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Chemical synthesis of DNA fragments with a defined sequence is extremely long and complex. A simpler alternative is isolation of fragments from partially hydrolysed DNA. Mixtures of sequence isomers of pyrimidine oligonucleotides were recently isolated on a preparative scale from hydrolysates of depurinated herring sperm DNA[1]. These, however, could not be further separated by conventional techniques. Using high-performance liquid chromatography (HPLC), sequence isomers of pyrimidine di-, tri- and tetranucleotides were successfully separated and sequenced[2,3]. In the present work, separation and sequencing of sequence isomers of pyrimidine pentanucleotides by HPLC was achieved, demonstrating the applicability of this particular approach in preparation of desired DNA fragments.

EXPERIMENTAL

Apparatus

Separations were performed using a Varian Model 8500 liquid chromatograph (Varian Assoc., Walnut Creek, Calif., U.S.A.) equipped with a high-pressure sampling valve (Valco, Houston, Texas, U.S.A.). The column effluents were monitored at 260 nm using a variable-wavelength detector (cell volume, 8 μl) (Schoeffel SF 770, Westwood, N.J., U.S.A.). Filtration of the solvents was carried out using a pyrex filter holder (filter pore size, 0.22 μm) (Millipore, Bedford, Mass., U.S.A.). Samples were filtered by means of a Swinney filter (Millipore). A guard column packed with Co:Pell ODS (Whatman, Clifton, N.J., U.S.A.) was used between the sampling valve and the column. For identification, fractions were collected 25 cm behind the detector (outlet tube 0.3 mm I.D.). All separations were carried out on a 30 × 0.4 cm I.D. MicroPak AX-10 column (Varian Assoc.), which is a difunctional weak anion-exchange bonded phase prepared on LiChrosorb Si-60 silica (10 μm)[4]. This column

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provides efficient separations with either aqueous buffer or aqueous buffer–organic solvent mobile phases, and has successfully been used for separation of nucleosides, nucleotides and bases[4].

Materials

Pyrimidine deoxydinucleoside monophosphates were obtained from P-L Biochemicals (Milwaukee, Wisc., U.S.A.). Pyrimidine 5'-deoxy- and 3'-deoxynucleotides, tris(hydroxymethyl)aminomethane, 2'-deoxycytidine and 2'-deoxythymidine were from Sigma (St. Louis, Miss., U.S.A.). Triethylamine (Eastman Kodak, Rochester, N.Y., U.S.A.) was purified by refluxing for 3 h with 2,4-diaminophenol dihydrochloride followed by distillation[5]. A stock solution of 2 *M* triethylammonium acetate (pH 4.5) was prepared by addition of triethylamine to an acetic acid solution. Working solutions were prepared by dilution of the stock solution and titration with glacial acetic acid to the desired pH value. Glass-distilled acetonitrile was purchased from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.) and used as supplied. Buffer solutions were prepared using deionized, glass-distilled and filtered water.

Alkaline phosphatase (EC 3.1.3.1.), snake venom phosphodiesterase (EC 3.1.4.1.) and spleen phosphodiesterase (EC 3.1.4.18) were obtained from Boehringer Mannheim (Indianapolis, Ind., U.S.A.).

The mixtures p(dC,dT_4)p, p(dC_2,dT_3)p and p(dC_3,dT_2)p* were isolated from the partial hydrolysate of herring sperm DNA[1]. Terminal phosphate groups were removed with alkaline phosphatase. For this, *ca.* 1 mg of each group of sequence isomers was dissolved in 0.25 ml of 0.01 *M* Tris·HCl buffer (pH 7.0) containing 0.01 *M* $MgCl_2$, and incubated with 2 units of alkaline phosphatase at 37° for 4 h. Aliquots (25 μl) were analysed by HPLC.

Sequencing of the separated isomers

The separated isomers were characterized by either complete or partial digestion with snake venom phosphodiesterase (PDE I) and spleen phosphodiesterase (PDE II)[2,3,7–9]. The eluted fractions were freeze-dried to remove triethylammonium acetate. For digestion with PDE I the freeze-dried samples were dissolved in 0.05 ml of 0.01 *M* Tris·HCl buffer (pH 9.0) containing 0.01 *M* $MgCl_2$ and incubated with 0.001 unit of PDE I at 37°. The time for partial digestion as described was found to be 3 min while for complete digestion 30 min. Similarly, for hydrolysis with PDE II the samples were dissolved in 0.05 ml of 0.01 *M* Tris·HCl buffer (pH 7.0) and incubated with 0.002 units of PDE II at 37° for 15 min for partial digestion and 1 h for complete digestion. The released nucleosides, dinucleoside monophosphates and nucleotides were analysed by HPLC as described in Table I. For this, we slightly altered the procedure described by Edelson *et al.*[4] for the simultaneous analysis of nucleosides and nucleotides on the same column. We used triethylammonium acetate buffer instead of KH_2PO_4 buffer as eluent. The elution times of the nucleotides are rather long, but using this procedure nucleosides, dinucleoside monophosphates and nucleotides can simultaneously be analysed in a single analytical run.

* Abbreviations for nucleic acid constituents follow CBN recommendations[6].

TABLE I

ELUTION TIMES OF PYRIMIDINE DEOXYNUCLEOSIDES, DEOXYDINUCLEOSIDE MONOPHOSPHATES AND DEOXYNUCLEOTIDES

Column, MicroPak AX-10 (10 μm), 30 × 0.4 cm I.D. at room temperature.

	Elution time (min)	*Eluent*
dT	2.0	A: 80% acetonitrile–20% 0.01 *M* triethylammonium acetate (pH 3.1); B: 0.5 *M* triethylammonium acetate (pH 3.4); linear gradient of 1% B per minute starting from 5% B; flow-rate: 2.0 ml/min.
dC	3.5	
d(TpT)	11.5	
d(TpC)	15.0	
d(CpT)	17.0	
d(CpC)	20.5	
pdT	36.0	
pdC	41.0	
dTp	39.0	
dCp	48.0	

RESULTS AND DISCUSSION

From the hydrolysates of the chemically depurinated herring sperm DNA only three mixtures of sequence isomers of pyrimidine pentanucleotides were isolated: (dC,dT_4), (dC_2,dT_3) and (dC_3,dT_2). The fourth possible combination (dC_4,dT) has not so far been found in the hydrolysate. The (dC,dT_4) fraction consists of five sequence isomers and all of them were separated and sequenced. Of the ten sequence

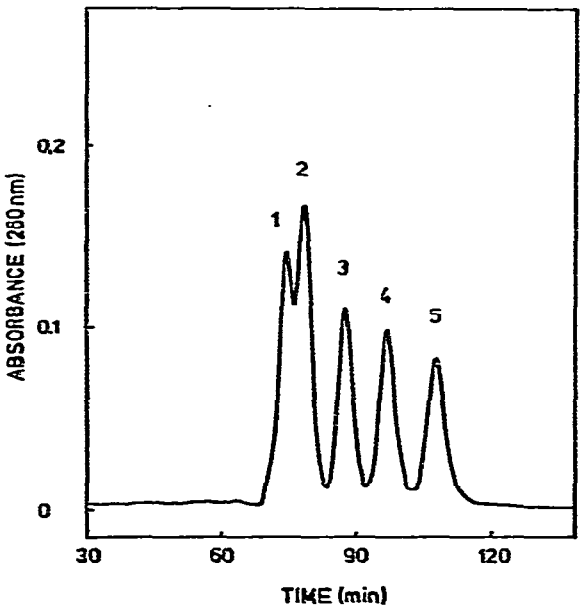

Fig. 1. Separation of the sequence isomers (dC, dT_4). Column, MicroPak AX-10 (10 μm), 30 × 0.4 cm I.D. Temperature, 70°. Eluent, 0.5 *M* triethylammonium acetate (pH 3.4). Flow-rate, 1.5 ml/min. Peaks: 1 = d(TpTpTpTpC); 2 = d(TpTpTpCpT); 3 = d(TpTpCpTpT); 4 = d(TpCpTpTpT); 5 = d(CpTpTpTpT).

isomers in the mixtures (dC_2, dT_3) and (dC_3, dT_2) only six and four isomers, respectively, were separated and sequenced.

The separation of the five sequence isomers of general formula (dC, dT_4) is given in Fig. 1. d(TpTpTpTpC) and d(TpTpTpCpT) (peaks 1 and 2) could not be resolved completely, but the resolution was sufficient for the subsequent sequence analysis. The other isomers were completely separated from these and each other. An improvement of the separation was observed by increasing the temperature to 70°. The sequence analysis of the separated isomers was performed as described in the experimental section. d(TpTpTpTpC) and d(CpTpTpTpT) (peaks 1 and 5) were immediately identified by complete digestion with PDE II and PDE I, respectively:

$$d(TpTpTpTpC) \xrightarrow{\text{PDE II}} 4\,dTp + dC$$

$$d(CpTpTpTpT) \xrightarrow{\text{PDE I}} 4\,pdT + dC$$

The other isomers could be sequenced by partial digestion:

$$d(TpTpTpCpT) \xrightarrow[\text{part. digest}]{\text{PDE II}} d(CpT) + 3\,dTp$$

$$d(TpTpCpTpT) \xrightarrow[\text{part. digest}]{\text{PDE I}} d(TpT) + 2\,pdT + pdC$$

$$d(TpTpCpTpT) \xrightarrow[\text{part. digest}]{\text{PDE II}} d(TpT) + 2\,dTp + dCp$$

$$d(TpCpTpTpT) \xrightarrow[\text{part. digest}]{\text{PDE I}} d(TpC) + 3\,pdT$$

The released nucleosides, dinucleoside monophosphates and nucleotides were analysed by HPLC as described in Table I.

The separation of the sequence isomers of general formula (dC_2, dT_3) at 70° is shown in Fig. 2. The absorbance profile represents a resolution of eight peaks. Two pairs of sequence isomers, d(TpCpTpTpC) and d(TpTpCpCpT) (peak 3), d(TpCpTpCpT) and d(CpTpTpTpC) (peak 4) could not be resolved. A separation at 50° showed a small resolution of these compounds, but the separation of the others were not as good as that at 70°, which was found to be the optimal temperature. d(TpTpTpCpC) (peak 1) and d(CpCpTpTpT) (peak 8) were identified by partial digestion with PDE II and PDE I, respectively, while the other isomers had to be digested partially with both of the enzymes:

$$d(TpTpTpCpC) \xrightarrow[\text{part. digest}]{\text{PDE II}} d(CpC) + 3\,dTp$$

$$d(CpCpTpTpT) \xrightarrow[\text{part. digest}]{\text{PDE I}} d(CpC) + 3\,pdT$$

$$d(TpTpCpTpC) \xrightarrow[\text{part. digest}]{\text{PDE I}} d(TpT) + 2\,pdC + pdT$$

$$d(TpTpCpTpC) \xrightarrow[\text{part. digest}]{\text{PDE II}} d(TpC) + dCp + 2\,dTp$$

$$d(TpCpTpTpC) \xrightarrow[\text{part. digest}]{\text{PDE I}} d(TpC) + pdC + 2\,pdT$$

$$d(TpCpTpTpC) \xrightarrow[\text{part. digest}]{\text{PDE II}} d(TpC) + dCp + 2\,dTp$$

etc.

Fig. 3 shows the separation of the sequence isomers (dC_3, dT_2). A resolution of nine peaks was observed. The optimal temperature of separation was found to be 60°. Only four isomers (peaks 1, 2, 8 and 9) could be characterized by partial digestion (see above) while the resolution of the remaining isomers was not sufficient for the subsequent sequence analysis.

The observed elution orders of the separated sequence isomers of pyrimidine deoxypentanucleoside tetraphosphates (see Figs. 1–3) obey the rules given earlier in our recent papers[2,3] for the sequence isomers of di-, tri- and tetranucleotides.

In summary, 37 pyrimidine oligonucleotides with a defined sequence were

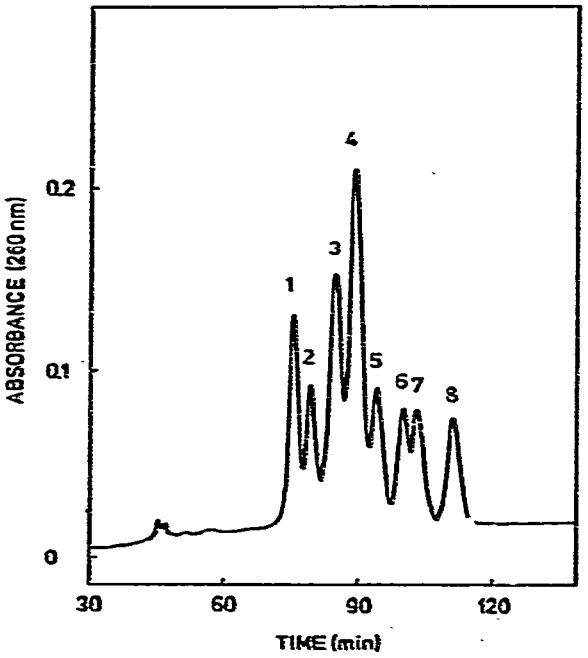

Fig. 2. Separation of the sequence isomers (dC_2, dT_3). Column as in Fig. 1. Temperature, 70°. Eluent: buffer A, 0.01 *M* triethylammonium acetate (pH 3.1); buffer B, 0.5 *M* triethylammonium acetate (pH 3.4); elution was carried out with a linear gradient of 0.5% B per minute starting from 30% A and 70% B. Flow-rate, 1.0 ml/min. Peaks: 1 = d(TpTpTpCpC); 2 = d(TpTpCpTpC); 3 = d(TpCpTpTpC) and d(TpTpCpCpT); 4 = d(TpCpTpCpT) and d(CpTpTpTpC); 5 = d(CpTpTpCpT); 6 = d(TpCpCpTpT); 7 = d(CpTpCpTpT); 8 = d(CpCpTpTpT).

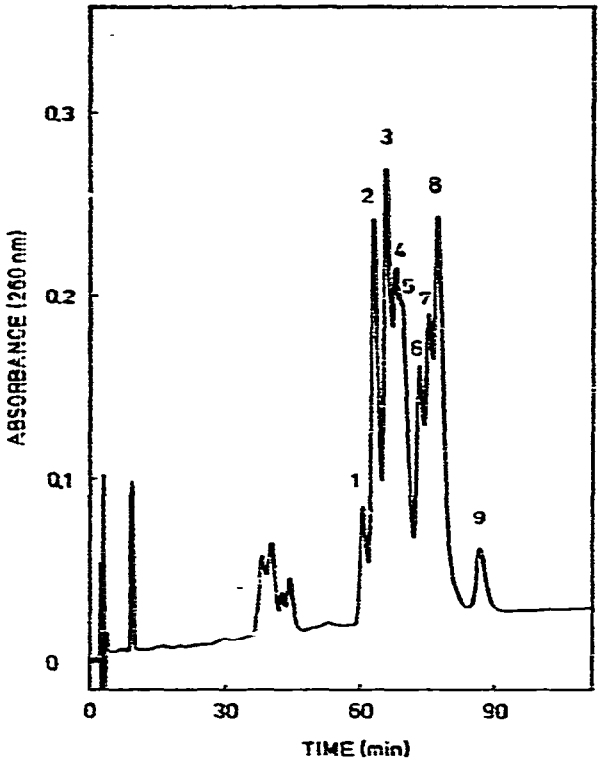

Fig. 3. Separation of the sequence isomers (dC_3, dT_2). Column as in Fig. 1. Temperature, 60°. Eluent as in Fig. 2, elution was carried out with a linear gradient of 0.5% B per minute starting from 50% A and 50% B. Flow-rate, 1.0 ml/min. Peaks: 1 = d(TpTpCpCpC); 2 = d(TpCpTpCpC); 8 = d(CpCpTpCpT); 9 = d(CpCpCpTpT). Pears 3–7 conld not be unequivocally assigned (see text).

TABLE II

PYRIMIDINE OLIGONUCLEOTIDES WITH A DEFINED SEQUENCE ISOLATED FROM HYDROLYSATES OF DEPURINATED HERRING SPERM DNA ON A PREPARATIVE SCALE

Dinucleoside monophosphates[2]
d(TpC), d(CpT)

Trinucleoside diphosphates[2]
d(CpCpT), d(CpTpC), d(TpCpC)
d(CpTpT), d(TpTpC), d(TpCpT)

Tetranucleoside triphosphates[3]
d(CpCpCpT), d(CpCpTpC), d(CpTpCpC), d(TpCpCpC)
d(TpTpCpC), d(TpCpTpC), d(TpCpCpT), d(CpTpCpT), d(CpCpTpT), d(CpTpTpC)
d(TpTpTpC), d(TpTpCpT), d(TpCpTpT), d(CpTpTpT)

Pentanucleoside tetraphosphates (present paper)
d(TpTpTpTpC), d(TpTpTpCpT), d(TpTpCpTpT), d(TpCpTpTpT), d(CpTpTpTpT)
d(TpTpTpCpC), d(TpTpCpTpC), d(CpTpTpCpT), d(TpCpCpTpT), d(CpTpCpTpT)
d(CpCpTpTpT)
d(TpTpCpCpC), d(TpCpTpCpC), d(CpCpTpCpT), d(CpCpCpTpT)

isolated from the hydrolysates of depurinated herring sperm DNA using the HPLC (Table II). The amounts were completely sufficient for enzymatic reactions which are common in molecular biology. The whole procedure takes only a few days rather than a few months as required for a total chemical synthesis.

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