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Note

Separation of the sequence isomers of pyrimidine deoxytetranucleoside triphosphates by high-performance ion-exchange liquid chromatography

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DNA from herring sperm was degraded to pyrimidine oligonucleotides. From this very complex hydrolysate definite mixtures of sequence isomers of pyrimidine nucleotides were isolated[1–4]. These, however, could not be further separated by conventional techniques.

Recently dinucleoside monophosphates and trinucleoside diphosphates were separated by high-performance liquid chromatography (HPLC)[5]. In the present paper we report the separation and identification of the sequence isomers of pyrimidine deoxytetranucleoside triphosphates using the same technique.

EXPERIMENTAL

Apparatus

A high-pressure liquid chromatograph (Hewlett-Packard 1010B, Böblingen, G.F.R.) was equipped with a UV detector (Schoeffel SF 770, Westwood, N. J., U.S.A.) and a high-pressure sampling valve (Valco, Houston, Texas, U.S.A.). The column (stainless steel, 50 cm × 3 mm I.D.) was packed as described[5] and kept at 60° during chromatography. Eluents were degassed under vacuum at 40° and kept at this temperature. The monitoring wavelength was 260 nm. For identification fractions were collected 25 cm behind the detector (outlet tube 0.3 mm I.D.). The collections of one to four runs were sufficient for a subsequent sequence determination.

Materials

Partisil-10 SAX (Chrompack, Berlin, G.F.R.) (particle size 10 μm) was used as supplied. Pyrimidine 5′-deoxynucleotides (Boehringer, Mannheim, G.F.R.), pyrimidine 3′-deoxynucleotides (Sigma, München, G.F.R.), triethylamine (reagent

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grade), tris(hydroxymethyl)aminomethane (p.a.), and glacial acetic acid (p.a.) (Merck, Darmstadt, G.F.R.) were commercially available. Triethylamine was purified by refluxing for 3 h with 2,4-diaminophenol dihydrochloride (reagent grade) (Merck, Darmstadt, G.F.R.) followed by distillation[6]. 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.

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, G.F.R.).

The mixtures* $p(dC_3,dT)p$, $p(dC_2,dT_2)p$, $p(dC,dT_3)p$, and $p(dT_4)p$ were isolated from the partial hydrolysate of herring sperm DNA[7]. $p(dC)_4$ was synthetized[8]. Terminal phosphate groups were removed with alkaline phosphatase. For this, *ca.* 1 mg of each group of sequence isomers was dissolved in 0.5 ml of 0.01 *M* Tris·HCl buffer (pH 7.0) containing 0.01 *M* $MgCl_2$, and incubated with 5 units of alkaline phosphatase at 37° for 4 h. Aliquots (25 μl) were analysed by HPLC.

Characterization of the sequence isomers

The separated compounds (see results and discussion) were characterized by hydrolysis with snake venom phosphodiesterase (PDE I) and spleen phosphodiesterase (PDE II)[9] as follows. The eluted fractions were divided into two parts and freeze dried to remove triethylammonium acetate. One part was dissolved in 0.05 ml of 0.01 *M* Tris·HCl buffer (pH 9.0) containing 0.01 *M* $MgCl_2$ and incubated with 0.005 units of PDE I at 37° for 3 h. The other part was dissolved in 0.05 ml of 0.01 *M* Tris·HCl buffer (pH 7.0) and incubated with 0.005 units of PDE II at 37° for 3 h. For partial enzymatic digestion the incubation times were 15, 30 and 60 min. The conditions for the identification of the deoxynucleotides and deoxydinucleoside monophosphates are given in Table I.

TABLE I

ELUTION TIMES OF PYRIMIDINE DEOXYNUCLEOTIDES AND DEOXYDINUCLEOSIDE MONOPHOSPHATES[5]

Column, Partisil-10 SAX (10 μm), 0.3 × 50 cm, at 60°. Flow-rate, 1.0 ml/min.

	Elution time (min)	*Eluent*
pdC	5.6	0.15 *M* Triethylammonium acetate (pH 3.1)
dCp	8.1	Pressure: *ca.* 60 bar
pdT	11.0	
dTp	13.0	
d(CpC)	4.2	0.01 *M* Triethylammonium acetate (pH 3.1)
d(TpC)	7.0	Pressure: *ca.* 60 bar
d(CpT)	8.0	
d(TpT)	22.0	

* Abbreviations: abbreviations for nucleosides and nucleotides follow CBN Recommendations[10].

RESULTS AND DISCUSSION

The separation of the whole fraction of pyrimidine deoxytetranucleoside triphosphates with a linear gradient of 0.03–0.5 *M* triethylammonium acetate (pH 3.1–3.4) is shown in Fig. 1. dC_4 is eluted first, followed by the series of sequence isomers (dC_3,dT), (dC,dT_2) and (dC,dT_3); dT_4 is eluted last. If this strong gradient's used not all 16 pyrimidine deoxytetranucleoside triphosphates are completely separated. This can be done by chromatographing the various fractions with smoother gradients as shown below.

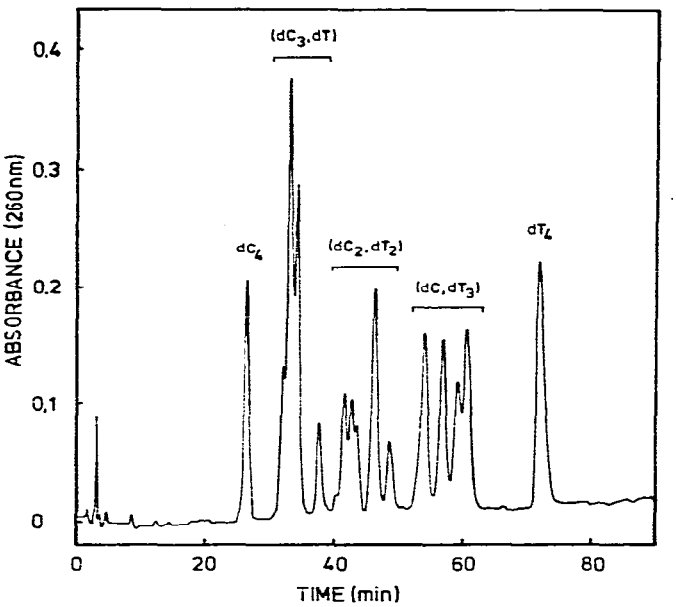

Fig. 1. Separation of pyrimidine deoxytetranucleoside triphosphates. Column, Partisil-10 SAX (10 μm), 0.3 × 50 cm; temperature, 60°; pressure, *ca.* 60 bar; eluents, linear gradient of 0.03 *M* (pH 3.1) to 0.5 *M* (pH 3.4) of triethylammonium acetate; duration of gradient elution, 120 min; flow-rate, 1.0 ml/min.

Fig. 2 shows the separation of all four sequence isomers of general formula (dC_3,dT) with a linear gradient of 0.03–0.35 *M* triethylammonium acetate (pH 3.1–3.4) at 60°.

The sequence analysis of these compounds was achieved by enzymatic digestion as described under experimental. Complete enzymatic digestion led to the following products:

$$d(TpCpCpC) \xrightarrow{\text{PDE I}} 3\ pdC + dT$$

$$d(TpCpCpC) \xrightarrow{\text{PDE II}} 2\ dCp + dTp + dC$$

$$d(CpTpCpC) \xrightarrow{\text{PDE I}} 2\ pdC + pdT + dC$$

$$d(CpTpCpC) \xrightarrow{PDE\ II} 2\ dCp + dTp + dC$$

$$d(CpCpTpC) \xrightarrow{PDE\ I} 2\ pdC + pdT + dC$$

$$d(CpCpTpC) \xrightarrow{PDE\ II} 2\ dCp + dTp + dC$$

$$d(CpCpCpT) \xrightarrow{PDE\ I} 2\ pdC + pdT + dC$$

$$d(CpCpCpT) \xrightarrow{PDE\ II} 3\ dCp + dT$$

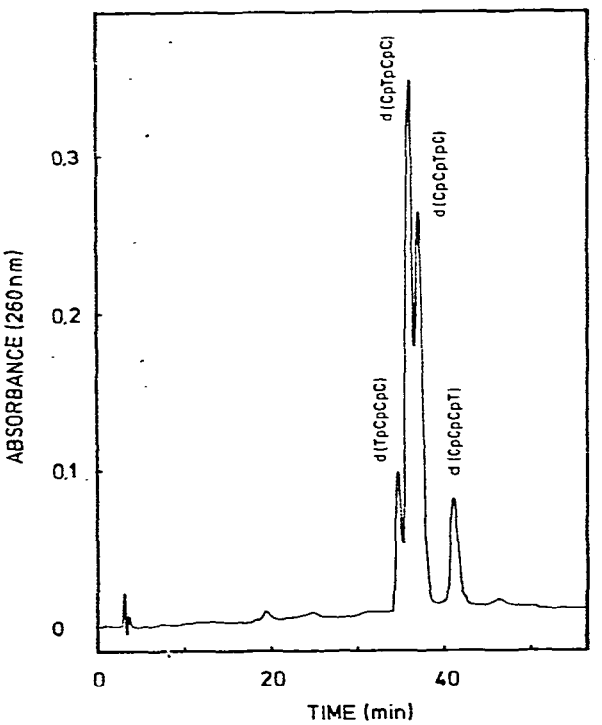

Fig. 2. Separation of the sequence isomers (dC_3, dT). Column, Partisil-10SAX (10 μm), 0.3 × 50 cm; temperature, 60°; pressure, *ca.* 60 bar; eluent, linear gradient of 0.03 *M* (pH 3.1) to 0.35 *M* (pH 3.4) of triethylammonium acetate; duration of gradient elution, 90 min; flow-rate, 1.0 ml/min.

The isomers d(TpCpCpC) and d(CpCpCpT) are immediately characterized by the relative amounts of cytosine and thymine nucleotides obtained after complete digestion. This is not the case with the isomers d(CpTpCpC) and d(CpCpTpC) (see the equations). However, their partial digestion leads to deoxynucleotides and deoxydinucleoside monophosphates.

$$d(CpTpCpC) \xrightarrow[\text{partial digest}]{PDE\ I} d(CpT) + 2\ pdC$$

$$d(CpTpCpC) \xrightarrow[\text{partial digest}]{\text{PDE II}} d(CpC) + dCp + dTp$$

$$d(CpCpTpC) \xrightarrow[\text{partial digest}]{\text{PDE I}} d(CpC) + pdC + pdT$$

$$d(CpCpTpC) \xrightarrow[\text{partial digest}]{\text{PDE II}} d(TpC) + 2\,dCp$$

The separation of deoxydinucleoside monophosphates has been described previously[5] and is summarized in Table I.

Fig. 3 shows the separation of all six sequence isomers of general formula (dC_2,dT_2) with a linear gradient of 0.05–0.5 *M* triethylammonium acetate (pH 3.1–3.4) at 60°. The identification of d(TpCpCpT) and d(CpTpTpC) was possible by complete enzymatic digestion, whereas for the identification of the other isomers, d(TpTpCpC), d(TpCpTpC), d(CpTpCpT), and d(CpCpTpT) the method of partial digestion (see above) had to be applied.

The separation of all four isomers of general formula (dC,dT_3) is shown in Fig. 4. The eluent was a linear gradient of 0.15–0.5 *M* triethylammonium acetate (pH 3.1–3.4) at 60°. The sequence isomers d(TpTpTpC) and d(CpTpTpT) were

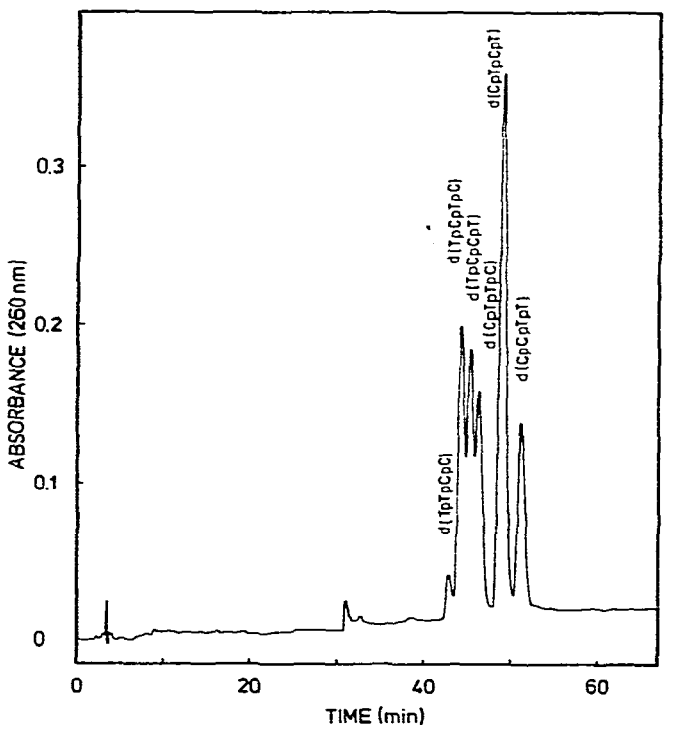

Fig. 3. Separation of the sequence isomers (dC_2, dT_2). Column, Partisil-10 SAX (10 μm), 0.3 × 50 cm; temperature, 60°; pressure, *ca.* 60 bar; eluent, linear gradient of 0.05 *M* (pH 3.1) to 0.5 *M* (pH 3.4) of triethylammonium acetate; duration of gradient elution, 70 min; flow-rate, 1.0 ml/min.

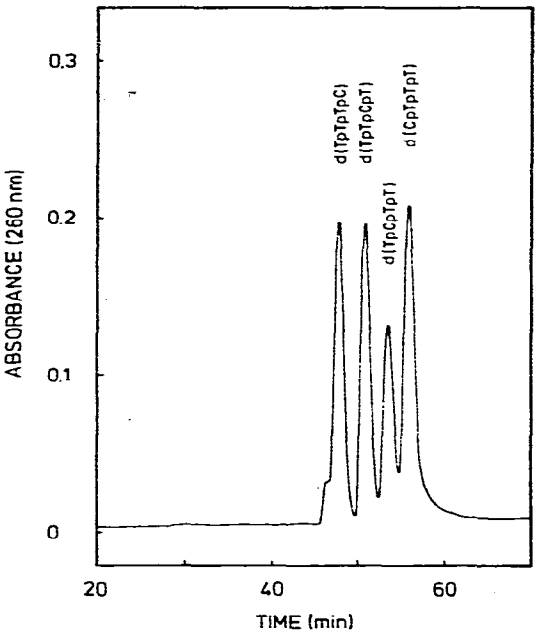

Fig. 4. Separation of the sequence isomers (dC, dT_3). Column, Partisil-10 SAX (10 μm), 0.3 × 50 cm: temperature, 60°; pressure, *ca.* 60 bar; eluent, linear gradient of 0.15 *M* (pH 3.1) to 0.5 *M* (pH 3.4) of triethylammonium acetate; duration of gradient elution, 70 min; flow-rate, 1.0 ml/min.

dentified by complete and d(TpTpCpT) as well as d(TpCpTpT) by partial enzymatic digestion (see above).

The following elution orders of the sequence isomers were observed:
d(TpCpCpC), d(CpTpCpC), d(CpCpTpC), d(CpCpCpT) (Fig. 2);
d(TpTpCpC), d(TpCpTpC), d(TpCpCpT), d(CpTpTpC), d(CpTpCpT), d(CpCpTpT) (Fig. 3);
d(TpTpTpC), d(TpTpCpT), d(TpCpTpT), d(CpTpTpT) (Fig. 4)

This order was as expected because, by extending the rule given earlier for deoxydinucleoside monophosphates and deoxytrinucleoside diphosphates[5], it can been shown that the deoxycytidine component speeds up the elution more strongly the nearer it is to the 3′-terminal of a given oligonucleotide, *i.e.* d(TpC) is eluted before d(CpT). Similarly, the elution order of the sequence isomers (dC,dT_2) is: d(TpTpC), d(TpCpT), d(CpTpT).

In the series of pyrimidine deoxytetranucleoside triphosphates, the elution order of two sequence isomers obeys the above rule if these isomers have a subunit in common. For example, d(CpTpCpT) and d(CpCpTpT) both have the deoxycytidine subunit at the 5′-terminal; this will contribute to the elution to a certain, more or less constant, degree. The elution order is then given by other subunits, *i.e.* d(TpCpT) and d(CpTpT). Their elution order has already been described[5] (see above). One can proceed similarly with other sequence isomers having the same subunit at the 3′-terminal. This procedure can be applied to all sequence isomers of pyrimidine deoxytetranucleoside triphosphates, except for d(TpCpCpT) and d(CpTpTpC) which do not have a subunit in common.

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