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Note

Reversed-phase liquid chromatography of protected oligonucleotide diesters

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High-performance liquid chromatography (HPLC) is playing an increasingly important role in the separation of nucleobases, nucleosides, nucleotides[1,2] and protected[3] and unprotected oligonucleotides[4]. During the synthesis of oligonucleotides, it is not the preparation itself but the purification that is the time consuming step. Therefore, rapid methods with high recoveries are under development, because such methods are necessary especially for the synthesis of larger amounts of material for biophysical investigations. Recently, reversed-phase columns with a styrene–divinylbenzene copolymer as the stationary phase have been introduced and applications in high-[5] and low-pressure chromatography[6,7] have been published.

For studies of the spectroscopic properties of oligonucleotides, larger amounts of material are necessary. As we were still using the diester method for the synthesis, we decided to use reversed-phase liquid chromatography (RP-LC) for the purification and mass spectrometry (field desorption and fast atom bombardment) for the identification of the products. Both methods are intrinsically fast and reliable. It was necessary to find a lipophilic phosphate protecting group to form derivatives suitable for the separation by RP-LC group to form derivatives suitable for the separation by RP-LC and stable enough for mass spectrometric identification. We chose 2-(β-thionaphtyl)ethanol, which can easily be synthesized in high yields[8]. As the protecting group it fulfills our requirements with respect to the condensation and deprotection chemistry and also to the chromatography and mass spectrometry. Here we describe the methods we have used for the separation and purification of protected intermediates.

EXPERIMENTAL

Synthesis of nucleotides

The nucleotides were synthesized via the diester method according to the literature[9] with only minor modifications.

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Synthesis of 2-(β-thionaphthyl)ethanol (TNE)[8]

To a solution of 10 g (62.5 m*M*) of 2-thionaphthene in 80 ml of toluene, 2.5 g (62.5 m*M*) of sodium hydroxide in 6 ml of water were added and the mixture was heated to 80°C. After addition of 5 g (62.5 m*M*) of 2-chloroethanol the mixture was boiled for 12 h. After extraction with two 50-ml volume of 5% sodium hydroxide solution and 50 ml of water, the water layers were re-extracted with toluene and the combined organic layers were dried with sodium sulphate and reduced to *ca.* 50 ml *in vacuo*. The product was isolated by chromatography on a 60 mm I.D. silica gel column (300 g). Elution with chloroform yielded starting material pure enough for the next preparation. The product was recovered by extraction with chloroform–ethyl acetate (20:1, v/v); the yield was typically 11.5 g (*ca.* 90%); m.p. 61°C. Its structure was confirmed by IR, mass and NMR spectroscopy.

Chromatography

HPLC was carried out with a chromatographic system consisting of a Model 6000A pump, a Model 440 UV detector and a U6K injection system (Waters, Milford, MA, U.S.A.). The columns were (a) μBondapak C_{18} (10 μm, 25 cm), (b) PRP-1 (10 μm, 15 cm) (Hamilton, Reno, NE, U.S.A.) and (c) laboratory-made [10 μm silanized LiChrosorb Si 60, (Merck, Darmstadt, F.R.G.)]. The columns were filled using the balanced density technique with a filling system (Shandon, Astmoor, U.K.), the pressure being 550 bar. The mobile phase was 0.1 *M* ammonium acetate (AA) or triethylammonium acetate (pH 7.5) (TEAA) with various amounts of acetonitrile.

Preparative chromatography was carried out with various XAD-4 (Serva, Heidelberg, F.R.G.) columns using a Uvicord 3 (LKB, Bromma, Sweden) detector.

Silylation of silica gel[10]

A 5-g amount of LiChrosorb Si 60 (*ca.* 18.5 mmol of silanol functions) was boiled under reflux in a mixture of 5 ml of concentrated nitric acid and 45 ml of contentrated hydrochloric acid for 5 h. After cooling, the silica gel was washed thoroughly with doubly distilled water until pH 5 had been reached. The material was dried for 10 h at 100°C under high vacuum, mixed with 15 ml of dry toluene, 7.5 ml of (58 mmol) distilled di-*n*-butylamine and 5.5 g of $CH_3(CH_2)_{17}Si(OCH_3)_3$ (Si 118) (Degussa, Hanau) and refluxed under argon for 19 h. The mixture was filtered through a glass frit and the silica gel was washed with 80 ml each of toluene, dichloromethane and, finally, methanol. The product was suspended in 30 ml of methanol–water (1:1) and boiled for 10 min, filtered again and rinsed with 60 ml of methanol and acetone. After drying at 100°C and 0.01 Torr, elemental analysis gave C 19.35% and H 3.60%.

For end-capping, the material was boiled for 20 h under reflux in a solution of 1.65 ml of hexamethyldisilazane (HMDS) (6.8 mmol) in 16.5 ml of dry toluene. The material was filtered and rinsed with 100 ml each of dry toluene, methanol and acetone. After drying at 100°C and 15 Torr and at 120°C and 0.01 Torr, elemental analysis gave C 19.63% and H 3.68%.

RESULTS AND DISCUSSION

First, the performance or the laboratory, made columns will be discussed brief-

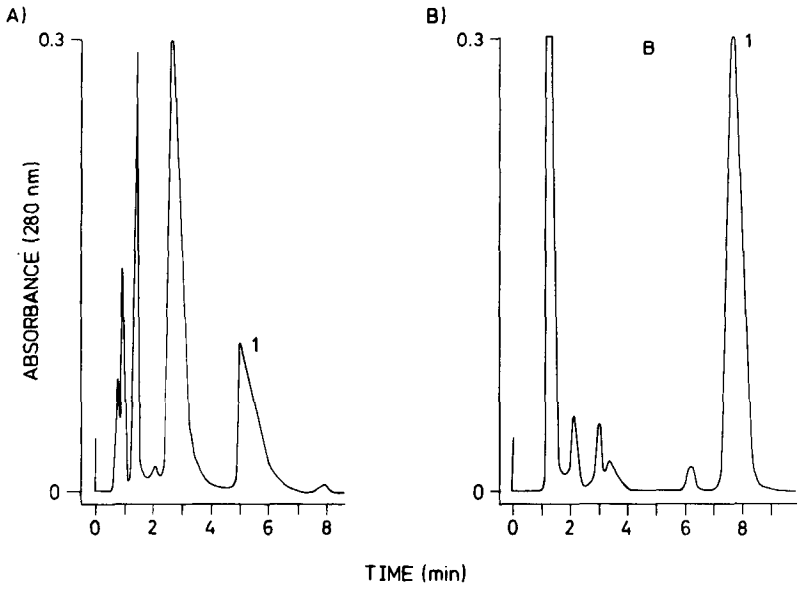

Fig. 1. Chromatography of two preparations of d[(TNE)panC](= 1) on two different laboratory-made columns (A and B). Eluents, (A) 28% and (B) 30% acetonitrile in 0.1 *M* ammonium acetate; flow-rate, 1.5 ml/min; detection, 280 nm. Both columns filled with silica gel, modified with Si 118 (for details, see Experimental); only column B was treated with HMDS. (Abbreviations used: anC = anisoylcytidine; d = desoxy-; p = phosphate.)

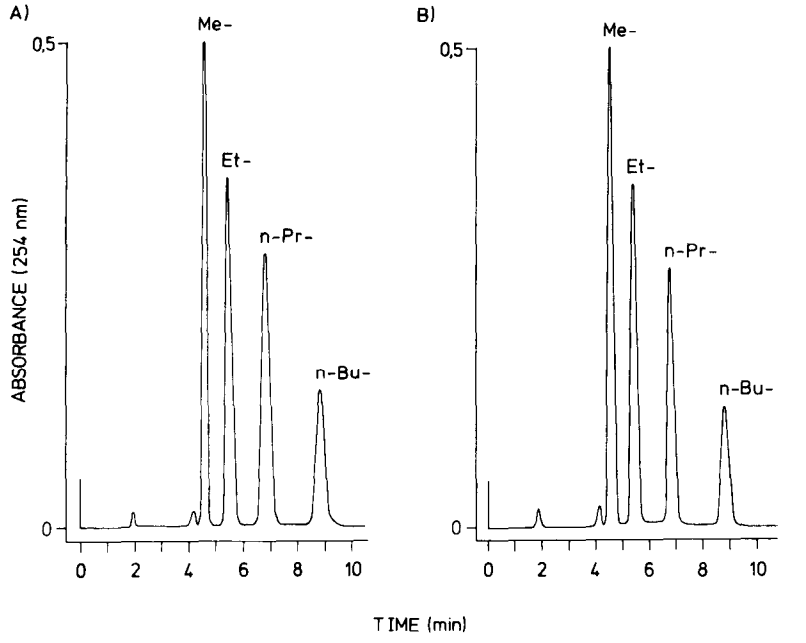

Fig. 2. Separation of a mixture of methyl, ethyl, *n*-propyl and *n*-butyl benzoates on columns A and B. Eluents: methanol–water, (A) 11:2 and (B) 4:1; flow-rate, 2 ml/min; detection, 254 nm.

ly. In Fig. 1 the influence of end-capping is demonstrated for the chromatography of nucleotides: the chromatogram recorded using the HMDS-treated column contains symmetrical peaks with reduced widths, whereas the peaks eluting from the untreated column are broadened and unsymmetric[11]. From Fig. 2, it is evident that this difference cannot be detected by evaluating the columns with a mixture *n*-alkyl benzoates in the usual way, because the peak shapes in the two chromatograms are identical.

For a more detailed picture, some of the properties of a typical column made in our laboratory are summarized in Table I.

From a comparison with commercial columns used in the separations of nucleotides it can be stated that the differences in chromatographic behaviour between conventional ODS materials and polymers of the XAD type (*e.g.*, PRP-1) are comparable to those found from batch to batch of the same material. The main advantage of the polymer is its chemical stability[12]: in our hands, a PRP-1 column survived more than 1000 injections of nucleotide mixtures in buffer solutions prior to determination.

In order to facilitate the identification of products in reaction mixtures and to avoid co-elution, we studied the retention behaviour of nucleotides with respect to the eluent (acetonitrile–0.1 *M* AA). As an example, two chromatograms of product mixtures are shown in Fig. 3.

We used the retention times and capacity factors obtained with a series of

TABLE I

CHARACTERISTICS OF THE LABORATORY-MADE COLUMNS A AND B

Stationary phase, LiChrosorb Si 60 (Merck), modified with Si 118; particle size, 10 μm; column dimensions, 250 × 4.6 mm .I.D.

Parameter	*Column A (20.43% C*)*	*Column B (19.35% C**)*
Retention times and k' values of benzoates ($2/k'$)***:		
Eluent (methanol–water)	11.2	4.1
Flow-rate (ml/min)	1.0	0.8
(1) Methyl	276/1.46	450/1.20
(2) Ethyl	326/1.90	571/1.78
(3) Propyl	408/2.63	774/2.77
(4) Butyl	528/3.70	1101/4.37
Selectivity (a):		
2–1	1.30	1.48
3–1	1.80	2.31
4–1	2.53	3.64
efficiency (theoretical plates/m):		
(1) Methyl-	21 500	19 500
(2) Ethyl	18 000	22 000
(3) Propyl-	17 000	23 000
(4) Butyl	17 000	24 500

* Carbon content measured by elemental analysis.

** Carbon content measured by elemental analysis; value after HMDS treatment, 19.63%.

*** The following equations were used: $k' = (rt - dt)/dt$ where rt = retention time, dt = dead time; $\alpha = k'_2/k'_1$; theoretical plates, $N = 5.54(rt/e_{0.5})^2$, where $w_{0.5}$ = peak width at half-height.

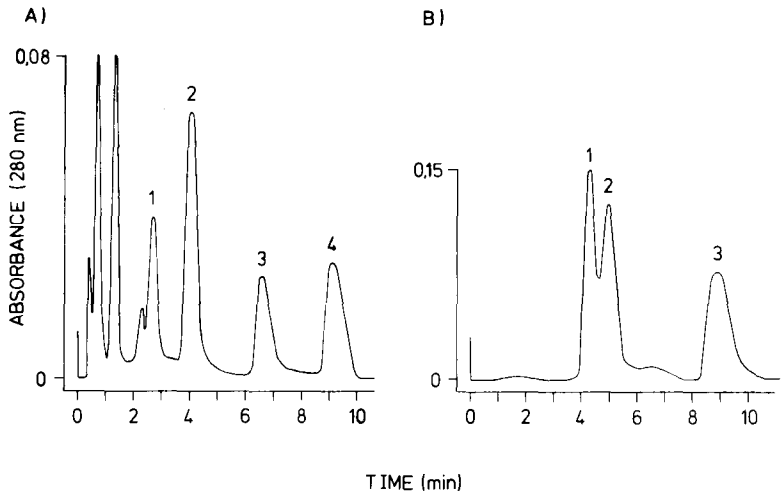

Fig. 3. Typical HPLC trace of the two nucleotide mixtures used to calculate the relationship between the capacity factors and the eluent compositions. Eluents: (A) 24% and (B) 27% acetonitrile in 0.1 *M* ammonium acetate; flow-rate, 1.5 ml/min; detection, 280 nm. (A) 1 = d[(TNE)pT(Ac)]; 2 = d[(TNE) panCpT]; 3 = d[(TNE)panCpT(Ac)]; 4 = d[(TNE)panC]. (B) 1 = d[(TNE)pbzA]; 2 = d[(TNE)pbzA-pibG(Ac)]; 3 = d[(TNE)pbzA(Ac)]. (Abbreviations used: Ac = acetate; bzA = benzoyladenosine; ibG = isobutyrylguanosine; T = thymidine. For other abbreviations see Fig. 1.)

different eluents to demonstrate their dependence on the amount of acetonitrile (Fig. 4). It is evident that there are eluent compositions where the starting materials and products may co-elute or even retention inversion may occur (cross-over in the graphs). Therefore, we used these and similar data to deduce the appropriate eluent composition for our separation problems. This is necessary, because it has been shown with similar systems[3] that the precise prediction of the effects of the various protecting groups is not possible; the effect of the lipophilic anisoyl protecting group may dominate the other effects and the additional introduction of a 3'-acetate group into the molecule causes only minor changes in retention time, whereas in oligonucleotides without the anisoyl group the 3'-protecting group controls the retention behaviour.

For chemically similar compounds, the same adsorption mechanism should prevent retention inversion; this assumption is supported by the data for mixtures of mono- and dinucleotides (Fig. 5), where no retention inversion was found within either group.

As the application of polymeric stationary phases in analytical HPLC was highly successful, we used the same type of copolymer with a larger particle size (XAD-4, 16–50 mesh) for preparative low-pressure chromatography. For the separation of the protected nucleotides it is necessary carefully to equilibrate the resin with 0.1 *M* triethylammonium hydrogen carbonate buffer prior to loading the sample[13]; the separation of the mixtures is acieved with 0.1 *M* triethylammonium hydrogen carbonate–acetonitrile mixtures in ratios variing from 1:5 to 1:3 depending on the base composition and the protecting groups. As a typical example of chromatography on a preparative scale, the separation of the reaction mixture for the synthesis of d[(TNE)panCpT(AC)] is shown in Fig. 6. The complete separation was

achieved by a multi-step gradient, which was found to be superior to a linear gradient. In comparison with the usual RP-HPLC, the same elution sequence was observed and a similar eluent was necessary (28% acetonitrile–0.1 *M* ammonium acetate *vs.* 25–30% acetonitrile–0.1 *M* triethylammonium hydrogen carbonate). In all our experiments the recovery was better than 90% with a sample size up to 1 g.

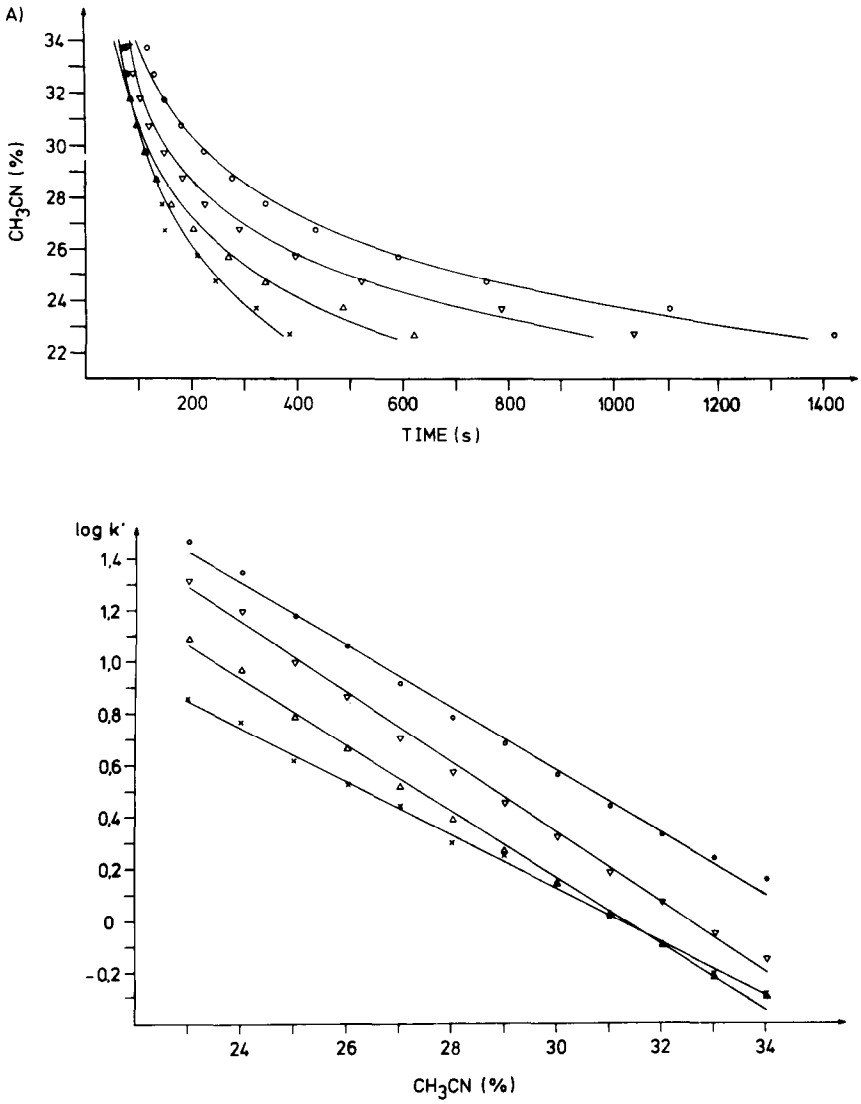

Fig. 4.

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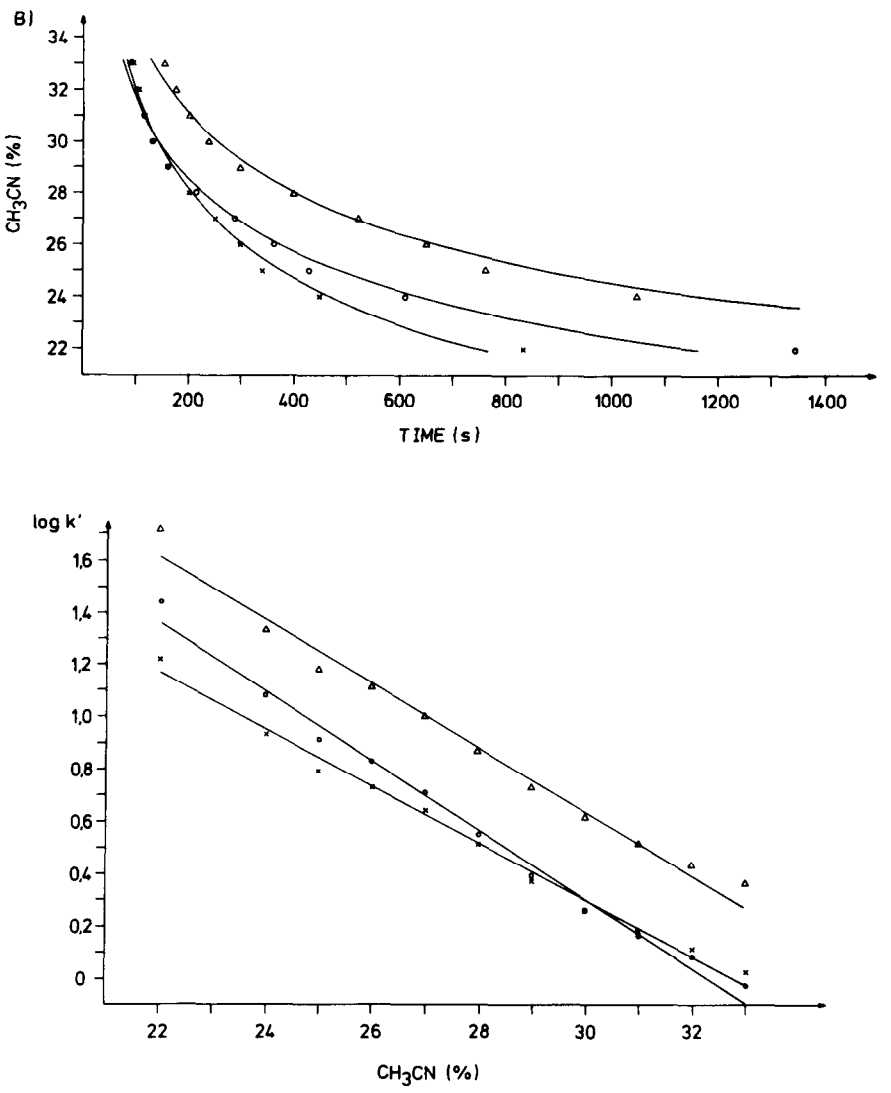

Fig. 4. Retention times and log k' values *versus* eluent compositions (acetonitrile in 0.1 *M* ammonium acetate) of two mixtures of products from the synthesis of (A) d[(TNE)panCpT(Ac)] and (B) d[(TNE)pbzApibG(Ac)]. Chromatography on an analytical PRP-1 column; in both instances inversion of retention was observed. (A) ×, △, ▽, ○ as 1,2,3,4, respectively, in Fig. 3A. (B) ×, ○, △ as 1,2,3, respectively, in Fig. 3B.

CONCLUSION

Reversed-phase liquid chromatography, in particular on copolymers of the XAD type, allows the separation of protected nucleotides diesters not only for analytical purposes but, more important, on a preparative scale. Although the time for

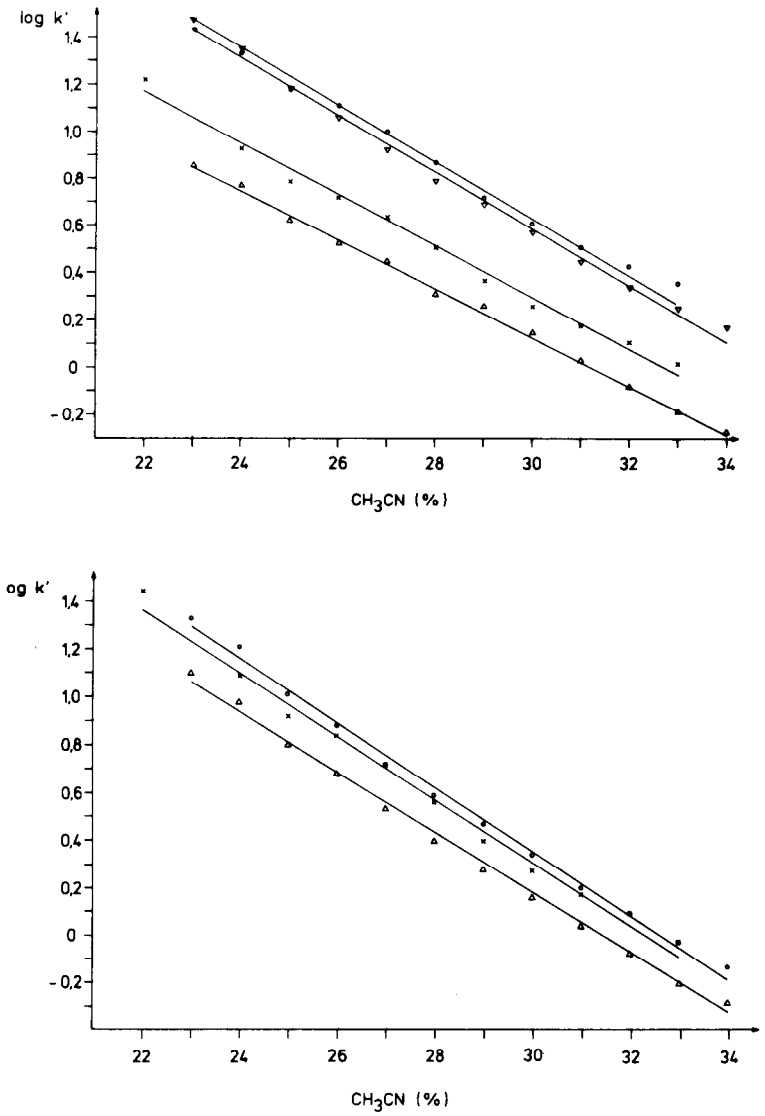

Fig. 5. Log k' values *vs.* eluent composition of mixtures of mononucleotides (above) and dinucleotides (below). Inversion of retention does not occur. Above: ○, d[(TNE)pbz(Ac)]; ▽, d](TNE)panC]; ×, d[(TNE)pbZA]; △, d[(TNE)pT(Ac)]. Below: ×, d[(TNE)pbzApibG(Ac)]; ○, d[(TNE)panCpT(Ac)]; △, d[(TNE)panCpT].

chromatography is longer, the use of XAD-4 resin in low-pressure systems has the advantage of high chemical and therefore long-term stability, high capacity and nearly complete recovery. In the future the use of polymers with smaller particles as stationary phases in preparative LC may increase the capacity and improve the chromatographic results even further. The possibility of co-elution whould be minimized by the use of two or three different eluent compositions in order to find the most suitable system.

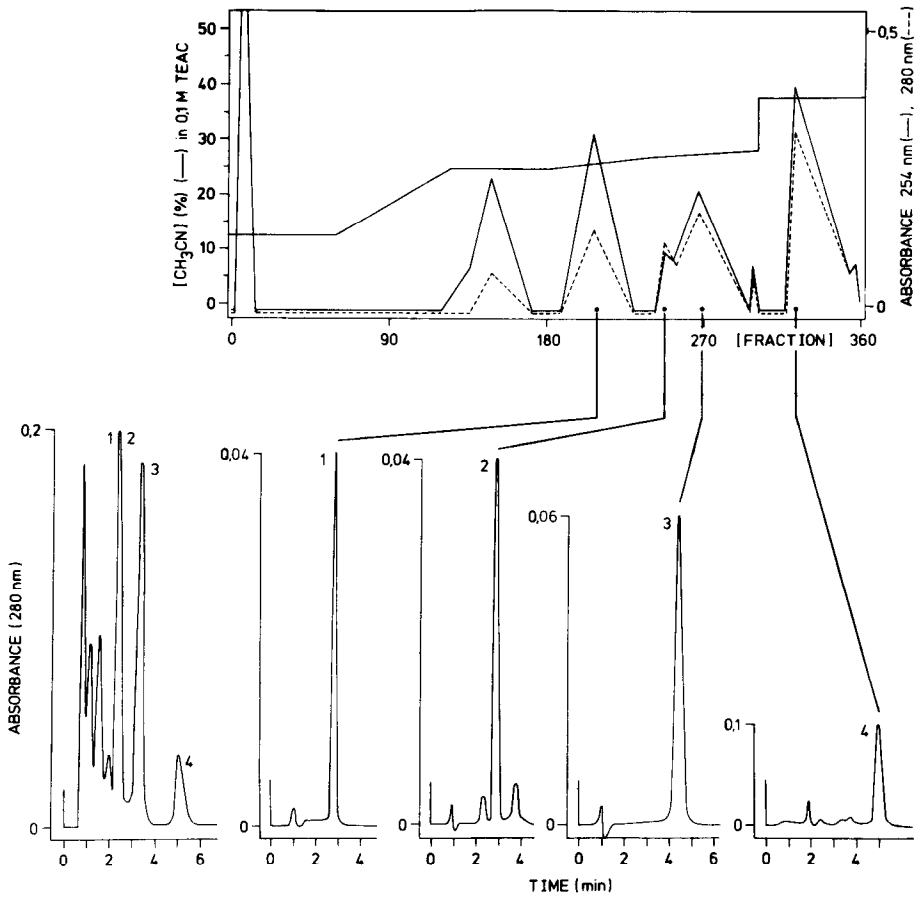

Fig. 6. Preparative separation of the mixture of products of the synthesis of 2 mmol of d[(TNE)panCpT-(Ac)] on an XAD-4 column (schematic). Column, 30 × 4 cm I.D. XAD-4 (16–50 mesh); eluent, acetonitrile in 0.1 *M* triethylammonium hydrogen carborate TEAC, flow-rate 4 ml/min; detection, 254 and 280 nm. The separation was monitored by analytical HPLC. Column, B; eluent, 28% acetonitrile in 0.1 *M* AA; flow-rate, 1.5 ml/min; detection, 280 nm. Not only the product (compound 3) but also the starting materials (compounds 1 and 4) and the byproduct 2 were isolated. 1–4 as in Fig. 3A.

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REFERENCES

1 Ph. R. Brown, A. M. Krztulovic and R. A. Hartwick, *Adv. Chromatogr.*, 18 (1980) 101.
2 A. N. Vul'fson and S. A. Yakimov, *Bioorg. Khim.*, 9 (1983) 365.

3 H.-J. Fritz, R. Belagaje, E. L. Brown, R. H. Fritz, R. A. Jones, G. Lees and H. G. Khorana, *Biochemistry*, 17 (1978) 1257.
4 W. Haupt and A. Pingoud, *J. Chromatogr.*, 260 (1983) 419.
5 D. P. Lee and J. H. Kindsvater, *Anal. Chem.*, 52 (1980) 2425.
6 G. C. Mills, *J. Chromatogr.*, 242 (1982) 103.
7 D. J. Pietrzyk and C. H. Chu, *Anal. Chem.*, 49 (1977) 757.
8 A. M. Kuliev, A. S. Arabova and Z. A. Mamedova, *Izv. Akad. Nauk. Az. SSR*, cited from *C.A.*, (1970) 3613h.
9 H. G. Khorana, K. L. Agarwal, H. Büchi, M. H. Caruthers, N. K. Gupta, H. Kleppe, A. Kumar, E. Ohtsuka, U. L. Rajbhandary, J. H. Van de Sande, V. Sgamarella, T. Terao, H. Weber and T. Jamada, *J. Mol. Biol.*, 72 (1972) 209.
10 V. Rehak and E. Smolkova, *J. Chromatogr.*, 191 (1980) 71.
11 H. Engelhardt, B. Dreyer and H. Schmidt, *Chromatographia*, 16 (1983) 11.
12 D. P. Lee, *J. Chromatogr. Sci.*, 20 (1982) 203.
13 T. Uematsu and R. J. Suhadolnik, *J. Chromatogr.*, 123 (1976) 347.