Journal of Chromatography, 389 (1987) 165–176
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 19 176

COLUMN CHROMATOGRAPHIC PURIFICATION OF GUANYLATE-RICH SYNTHETIC OLIGODEOXYRIBONUCLEOTIDES

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(Received October 20th, 1986)

SUMMARY

The purification of the guanylate-rich DNA fragments d(T_4G_4), d(G_4T_4), d($G_4T_4G_4$), d($T_4G_4T_4$) and d($T_4G_4T_4G_4$) using column chromatography on a preparative scale is described. The crude oligonucleotides were obtained after deprotection of the chemically synthesized compounds. The separation can be performed with commonly used sorbents (DEAE-cellulose, QAE-Sephadex, Nucleosil C_{18}, Partisil 10-SAX), however with high losses during the chromatography. Guanylate-rich oligonucleotides of different chain lengths associate with each other, thus causing identical compounds to be contained within different peaks. At the same time, part of the product remains irreversibly adsorbed on the sorbent. The recoveries could be improved by application of ion-pair reversed-phase high-performance liquid chromatography. The oligonucleotides were fractionated with linear increasing gradients using acetonitrile as the organic modifier and tetrabutylammonium hydrogensulphate as the ion-pair reagent.

INTRODUCTION

The synthesis of guanylate-rich DNA fragments is much more laborious than the synthesis of comparable oligonucleotides, which contain only few or no guanylate monomer units in their sequences. Besides distinctly lower yields obtained in the condensation reaction, there are additional difficulties in separation, isolation and identification, which have not been solved.

We have synthesized the guanylate-rich oligonucleotides d(T_4G_4), d(G_4T_4), d($G_4T_4G_4$), d($T_4G_4T_4$) and d($T_4G_4T_4G_4$) in preparative amounts. These oligonucleotides correspond to fragments of the terminus of the macronuclear DNA of hypotrichous ciliates[1–6]. The syntheses were carried out in solution according to the phosphotriester method and will be published elsewhere[7]. The same oligonucleotides were prepared in three ways, applying differently protected guanylate monomer units. The results of thirty different condensation reactions, several of which have been performed repeatedly, may be summarized as follows: the synthesis and isolation of the protected guanylate-rich oligonucleotides can be achieved in gram amounts. The

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condensation reactions result in good yields, which are essentially independent of the kind of protecting groups and of the choice of the agents used for the condensation. However, serious difficulties arise during the chromatographic purification of the deblocked oligonucleotides as will be reported in this paper.

EXPERIMENTAL

Materials

Sephadex G-15 and QAE-Sephadex A-25 were obtained from Pharmacia (Uppsala, Sweden), DEAE-cellulose from W. R. Balstone (Maidstone, U.K.), Dowex 50W-X8 from Serva (Heidelberg, F.R.G.) and Nucleosil 7 C_{18} from Macherey & Nagel (Düren, F.R.G.). Tetrabutylammonium hydrogensulphate (TBA) for ion-pair chromatography was from Merck (Darmstadt, F.R.G.).

Fractionation

Column chromatography of the deprotected oligonucleotides. The deprotected oligonucleotides were fractionated on DEAE-cellulose or QAE-Sephadex at a flow-rate of 200 ml/h, according to the conditions listed in Table I. Fractions of about 20 ml were collected. The absorbance of every fifth fraction was measured at 250, 260 and 280 nm. The values measured at 260 nm were plotted *versus* the elution volume (Fig. 1). Fractions were collected within the vertical dotted lines of Fig. 1. On repeated addition of pyridine, the volatile triethylammonium hydrogencarbonate (TEAB) was removed *in vacuo*. The pyridine was removed by co-evaporation with 3% aqueous ammonia. Finally the remaining solution was lyophilized. The sodium chloride–Tris–HCl buffer was removed by gel chromatography on a Sephadex G-15 column. In order to remove the urea, the combined peak fractions were diluted to 1:2.5 in water and pumped on a DEAE-Sephadex column (40 cm × 2 cm) previously equilibrated with water. The column was washed with water until free of chloride, and was then eluted with a 1 *M* sodium chloride solution. The oligonucleotides eluted with the salt were desalted by gel chromatography and lyophilized.

High-performance liquid chromatography (HPLC) of the deprotected oligonucleotides. HPLC was performed according to the conditions summarized in Table II on an analytical column (250 mm × 4.6 mmm I.D.) and a preparative column (250 mm × 8 mm I.D.) equipped with a precolumn (30 mm × 8 mm I.D.) packed with Nucleosil 7 C_{18}. Experiments 1–3 of Table II were carried out at room temperature, 4–6 at 50°C. One A_{260} unit of the oligonucleotide was dissolved in 1–10 μl water and applied to the column. The combined fractions were desalted as follows: the TBA solution obtained was added to 50 ml dichloromethane. A saturated aqueous picric acid solution was added dropwise to the stirred mixture until the aqueous layer had become slightly yellow. After separation of the layers, the aqueous phase was treated with Dowex 50W-X8 (H^+) and chromatographed on a Sephadex G-15 column (40 cm × 4 cm). The fractions containing product were combined, evaporated to dryness *in vacuo* and lyophilized.

HPLC of $d(T_4G_4T_4G_4)$ after total hydrolysis by formic acid. One A_{260} unit of $d(T_4G_4T_4G_4)$ was treated with 500 μl of 90% formic acid at 170°C during 45 min. The reaction mixture was lyophilized and dissolved in about 200 μl of 50 m*M* aqueous ammonium acetate (pH 6.8). About 0.10 A_{260} units of this solution were

TABLE I

CHROMATOGRAPHIC PURIFICATION OF PROBES (DISSOLVED IN WATER) OBTAINED AFTER THE DEPROTECTION OF THE PROTECTED DODECAMERS AND HEXADECAMERS USING DEAE-CELLULOSE (EXPERIMENTS 1, 2, 3a, 4) OR QAE-SEPHADEX (EXPERIMENT 3)

The columns (diameter 2 cm) were eluted with increasing salt concentration using triethylammonium hydrogencarbonate (pH 7.8) (A) or sodium chloride–0.05 *M* Tris–HCl (pH 7.6) + 7 *M* urea (B).

Experiment No.	*Deprotected oligonucleotide*	*Applied probe (A_{260} units/ml)*	*Column length (cm)*	*Elution conditions*				
				Temperature (°C)	*Step No.*	*Eluent*	*Volume (l) and salt concentration (M)**	
							Mixing vessel	*Reservoir*
1**	$T_4G_4T_4$	10 200/300	50	50	1	B	1.0, 0.05	–
					2	B	2.0, 0.05	2.0, 0.15
					3	B	2.0, 0.15	2.0, 0.30
					4	1 *M* NaCl	1.0	–
2	$G_4T_4G_4$	4500/150	25	25	1	A	1.0, 0.10	–
					2	A	2.0, 0.10	2.0, 0.40
					3	A	0.5, 1.0	–
3	$T_4G_4T_4G_4$	14 500/350	25	25	1	B	1.0, 0.05	–
					2	B	2.0, 0.05	2.0, 0.50
					3	B	1.0, 0.5	–
					4	1 *M* NaCl	0.7	–
3a	$T_4G_4T_4G_4$***	3900/100	25	50	1	B	1.0, 0.05	–
					2	B	2.0, 0.05	2.0, 0.20
					3	B	1.5, 0.20	1.5, 0.35
					4	1 *M* NaCl	1.0	
4	$T_4G_4T_4G_4$	6700/200	25	50	1	B	1.0, 0.05	–
					2	B	2.0, 0.05	2.0, 0.20
					3	B	1.5, 0.20	1.5, 0.35
					4	1 *M* NaCl	1.0	

* When B is used as the eluent *M* refers only to the sodium chloride concentration.
** The total amounts of deprotected oligonucleotides were chromatographed in three experiments.
*** Rechromatography of the mixture of d(T_4G_4) and d($T_4G_4T_4G_4$) isolated from experiment 3.

fractionated on a Nucleosil 7 C_{18} column (250 mm × 4.6 mm I.D.) with 50 m*M* ammonium acetate (pH 6.8) as the eluent (see Fig. 5).

RESULTS AND DISCUSSION

The DNA fragments d($G_4T_4G_4$), d($T_4G_4T_4$) and d($T_4G_4T_4G_4$) were obtained from the corresponding fully protected oligonucleotides after cleavage of the protecting groups and chromatographic separation of the oligonucleotides. The crude product d($T_4G_4T_4$), obtained after deprotection of 1.05 g dodecamer, was fractionated on DEAE-cellulose in three portions, employing a linear increasing gradient of sodium chloride containing 7 *M* urea at 50°C, as indicated in Table I (experiment 1).

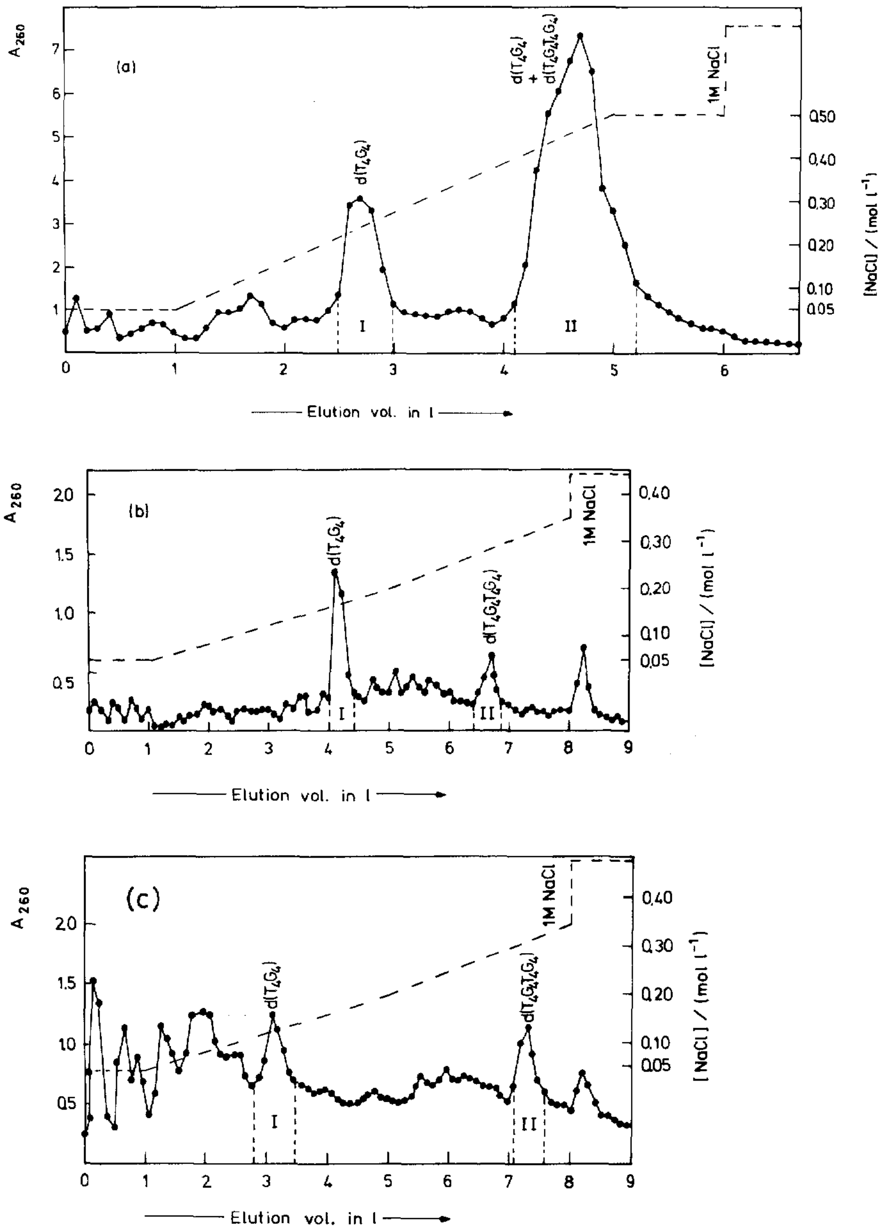

Fig. 1. Chromatographic purification of $d(T_4G_4T_4G_4)$ resulting after deprotection of the protected hexadecanucleotides, which were synthesized using different strategies. (a) Fractionation (experiment 3, Tables I, III) of the first hexadecanucleotide $d(T_4G_4T_4G_4)$ on a QAE-Sephadex column at 25°C with an increasing sodium chloride gradient, buffered to pH 7.6 by 0.05 *M* Tris–HCl. (b) Rechromatography of the mixture corresponding to peak II resulting from (a) on a DEAE-cellulose column (experiment 3a, Tables I, III) at 50°C with an increasing sodium chloride gradient in 7 *M* urea, buffered to pH 7.6 by 0.05 *M* Tris–HCl. (c) Fractionation (experiment 4, Tables I, III) of the second hexadecanucleotide $d(T_4G_4T_4G_4)$ using the same conditions as in (b). Column: 25 cm × 2 cm. Flow-rate: 200 ml/h. Within the dotted lines, fractions of peaks I and II were pooled, desalted and lyophilized.

TABLE II

CONDITIONS AND RESULTS OF ION-PAIR REVERSED-PHASE HPLC OF OLIGONUCLEOTIDES ON A NUCLEOSIL 7 C_{18} COLUMN (LENGTH 250 mm)

Eluents: A = 7.5 m*M* tetrabutylammonium hydrogensulphate (TBA) pH 7.0; B = 7.5 m*M* TBA pH 7.0 in 75% aqueous acetonitrile; C = 5 m*M* TBA pH 6.8; D = 5 m*M* TBA pH 6.8 in 70% aq. acetonitrile.

Experiment No.	*Chromatographed oligonucleotides*			*Column diameter (mm)*	*Elution conditions*	*Retention time (min)*	*Fig.*
	Designation	*Amount (A_{260} units)*	*Yield (%)*				
1	d(G_4T_4)	60.0	91	8.0	70% A, 30% B	9.23	2a
2	d($T_4G_4T_4$)	2.5	98	4.6	50% A, 50% B	12.92	–
3	d($T_4G_4T_4$)	50.0	94	8.0	50% A, 50% B	12.22	2b
4	d(T_4G_4)	0.5	92	4.6	60% C, 40% D changed within 48 min to 20% C, 80% D	26.22	3a
5	d($G_4T_4G_4$)	0.3	60	4.6		31.78	3b
6	d($T_4G_4T_4G_4$)	0.3	91	4.6		36.23	3c

Of the A_{260} units applied to the column, 39% were due to d($T_4G_4T_4$) and 18% to d(G_4T_4) . The remaining 43% consisted of removed protecting groups and of several oligonucleotides of shorter chain length. Fractions which contained d($T_4G_4T_4$) or d(G_4T_4) from three experiments were pooled and worked up, thus giving 351 mg of dodecamers and 150 mg of octamer. On the basis of the fully protected dodecamer, the yield of d($T_4G_4T_4$) was 53%.

The crude product of the second dodecamer d($G_4T_4G_4$) was also fractionated by means of DEAE-cellulose. When a small quantity of dodecamer d($G_4T_4G_4$) was chromatographed on a DEAE-cellulose column, no clear peaks could be detected in the region of the dodecamer. This result was quite a surprise, especially since the formation of a fully protected condensation product had been confirmed by thin-layer chromatography. After the deprotection of 600 mg of the corresponding protected d($G_4T_4G_4$), preparative chromatography (experiment 2, Table I) could be performed only with a considerable loss of oligonucleotides. Contrary to the previously described fractionation of d($T_4G_4T_4$), the column was eluted with an increasing concentration of triethylammonium hydrogencarbonate buffer (TEAB) at 25°C. The oligonucleotide leaving the column at a salt concentration of 0.35–0.39 *M* was identified as d(T_4G_4). The required dodecamer d($G_4T_4G_4$) was finally eluted by 1 *M* TEAB (see Table III). 15% of the applied A_{260} units were due to d(T_4G_4) and 20% to d($G_4T_4G_4$). On working up the pooled fractions, 20 mg d(T_4G_4) and 30 mg d($G_4T_4G_4$) were obtained corresponding to a yield of only 8% in relation to the fully protected dodecamer.

This rather low yield might be explained by assuming that part of the dodecamer was degradated during the cleavage of the protecting groups. This explains the elution of numerous short-chain oligonucleotides. Another reason for the low yield lies in the fact that substantial losses of guanylate-rich dodecamers occur during chromatography. Chromatography at elevated temperature (45°C) did not increase

TABLE III

RESULTS OF THE CHROMATOGRAPHIC PURIFICATION (SEE TABLE I) OF THE DEPROTECTED OLIGONUCLEOTIDES

*Experiment No.**	*Oligonucleotide eluted*			*Isolated oligodeoxynucleotide*			
	Salt concentration (M)	*Amount*		*Peak (Fig. 1)*	*Designation*	*Weight (mg)*	*Yield**** *(%)*
		(A_{260} units)	*(%)***				
1	0.13–0.17	1880§	18.4	Not shown	$d(G_4T_4)$	50§	
	0.21–0.25	3980§	39.0	Not shown	$d(T_4G_4T_4)$	117§	52.7§
2	0.35–0.39	670	14.9	Not shown	$d(T_4G_4)$	20	
	1.00	920	20.4	Not shown	$d(G_4T_4G_4)$	30	7.9
3	0.23–0.30	1350	9.3	I(a)	$d(T_4G_4)$	40	
	0.42–0.50	4680	32.2	II(a)	$d(T_4G_4)$ + $d(T_4G_4T_4G_4)$	170	
3a	0.15–0.18	1620	41.5	I(b)	$d(T_4G_4)$	50	
	0.27–0.30	820	21.0	II(b)	$d(T_4G_4T_4G_4)$	30	4.7
4	0.14–0.16	2520	37.6	I(c)	$d(T_4G_4)$	70	
	0.30–0.33	1800	26.9	II(c)	$d(T_4G_4T_4G_4)$	55	22.1

* See Table I.
** Based on the total amount of the probe applied.
*** Based on the protected oligonucleotides.
§ Average of three experiments.

the recovery of guanylate-rich oligonucleotides. In this case, extended elution with strong buffer solution resulted in a broad second peak of nucleotide material. During rechromatography a part of this material was eluted with the normal retention time. Similar problems have been reported[8] when purifying guanylate-rich oligonucleotides on Partisil 10-SAX. Even when chromatographing small amounts, other authors[9] have reported unusually low recoveries (40%) from a PEI column in the case of oligonucleotides containing three or more consecutive deoxyguanosine monomer units.

Chromatography of small quantities of the deprotected hexadecamer $d(T_4G_4T_4G_4)$ on a DEAE-cellulose column yielded no clear peaks in the region where octamers and longer-chain oligonucleotides are eluted. The chromatographic purification of larger amounts of a hexadecamer was performed as follows. A 700-mg amount of the first hexadecamer, which was synthesized using only one nucleobase protecting group, was deprotected and the $d(T_4G_4T_4G_4)$ obtained was fractionated on QAE-Sephadex with an increasing gradient of sodium chloride at 25°C, according to the conditions given in Tables I, III (experiment 3). The elution profile, shown in Fig. 1a, exhibited two main peaks: 9.3% of the applied A_{260} units were contained in peak I and amounted to 40 mg $d(T_4G_4)$ after isolation, peak II contained 32% of the applied A_{260} units and 170 mg of a mixture of $d(T_4G_4)$ and $d(T_4G_4T_4G_4)$ (see Table III). Furthermore, the elution profile indicates two different shorter-chain oligonucleotides eluted previous to the octamer. These oligomeric units might be the result of chain degradation, occurring during cleavage of the protecting groups. For

the isolation of $d(T_4G_4T_4G_4)$, the mixture corresponding to peak II was rechromatographed on a DEAE-cellulose column, see Table III (experiment 3a), with an increasing sodium chloride gradient, containing 7 *M* urea, at 50°C. The elution profile (Fig. 1b) again showed two main peaks, with numerous smaller side peaks forming a high baseline. It is remarkable that oligonucleotides were still eluted from the DEAE-cellulose column at 50°C with 1 *M* sodium chloride, although the same mixture was eluted from the more basic anion exchanger QAE-Sephadex during the separation with 0.42–0.50 *M* sodium chloride at 25°C (see Fig. 1a). From the fractions corresponding to peak I, containing 42% of the applied A_{260} units, 50 mg $d(T_4G_4)$ were isolated. The work-up of peak II, containing 21% of the A_{260} units applied, resulted in 30 mg $d(T_4G_4T_4G_4)$, which is only 4.7% of the fully protected hexadecamer.

The greatest portion of the deprotected guanylate-rich oligonucleotides was lost during the preparative fractionation on the ion exchangers QAE-Sephadex and DEAE-cellulose. As clearly demonstrated in Fig. 1a, quite a large part of $d(T_4G_4)$ is associated with $d(T_4G_4T_4G_4)$. Therefore both oligonucleotides are eluted together within peak II, although the octamer differs significantly from the hexadecamer in its negative charge. By rechromatography (experiment 3a, Fig. 1b), using 7 *M* urea and a temperature of 50°C, however, $d(T_4G_4)$ and $d(T_4G_4T_4G_4)$ were separated. Both 7 *M* urea and the increased temperature during the elution counteracted the formation of aggregates. The high baseline in the elution profile (Fig. 1b) also indicated that the mixture corresponding to peak II in Fig. 1a, besides both main products, contained additional oligonucleotides of various chain lengths, which are associated with the main products. On the other hand, it cannot be excluded that octa- and hexadecanucleotides were also eluted in the background. Despite the drastic elution conditions, part of the applied mixtures was retarded to such an extent that it was not eluted without the use of 1 *M* sodium chloride. According to our experience, mixtures of corresponding guanylate-poor oligonucleotides do not exhibit such difficulties. For example, $d(G_4T_4)$ could be separated from $d(T_4G_4T_4)$ (see experiment 1 in Tables I, III), although these oligonucleotides differ less in their negative charges in comparison to $d(T_4G_4)$ and $d(T_4G_4T_4G_4)$.

The purification of a second hexadecamer, which was synthesized using another strategy, resulted in comparable results. After deprotecting 350 mg of fully protected hexadecamer, the solution containing $d(T_4G_4T_4G_4)$ was directly fractionated on a DEAE-cellulose column at 50°C with an increasing sodium chloride gradient, containing 7 *M* urea without any previous separation (see experiment 4, Tables I, III). The elution profile (Fig. 1c) generally corresponds to that in Fig. 1b, except that the bulk of the shorter-chain oligonucleotides was eluted prior to peak I, $d(T_4G_4)$. Although the first hexadecamer, in contrast to the second one, was synthesized using guanylate monomer units with doubly protected guanine residues, both solutions exhibited similar percentages of short-chain oligonucleotides, after the protecting group had been cleaved. Because most of these side products had been removed during the preseparation of the first hexadecamer (experiment 3, Tables I, III), they are lacking in the elution profile (experiment 3a, Fig. 1b) upon rechromatography. The fractions corresponding to peak I (Fig. 1c), which contained 37.6% of the applied A_{260} units, amounted to 70 mg $d(T_4G_4)$. The work-up of peak II, corresponding to 27% of the A_{260} units, resulted in 55 mg $d(T_4G_4T_4G_4)$. On the

basis of the fully protected component, $d(T_4G_4T_4G_4)$ was obtained in 22% yield, whereas the yield of the hexadecamer synthesized according to the other strategy was only 4.1%. It cannot be excluded that the low yields result chiefly from the chromatography and not from insufficient protection of the bases during the synthesis.

The non-specific and irreversible adsorption, which caused the heavy losses of the deprotected oligonucleotides on the ion exchangers, occurred also on Nucleosil C_{18} which is commonly used for reversed-phase HPLC of oligonucleotides. In reversed-phase HPLC on Nucleosil 7 C_{18} recoveries > 90% could be achieved only when analytical amounts (< 3 A_{260} units) were applied. On applying 30 A_{260} units, only 50% were eluted within the expected region. The other part appeared in subsequent peaks or even at the end of the gradient. Especially when fractionating $d(G_4T_4G_4)$, identical compounds had different retention times. Finally, we found that the totally deprotected oligonucleotides could be separated satisfactorily by ion-pair reversed-phase HPLC[10–13], as described below.

In order to remove any contamination, HPLC was performed at room temperature with a preparative Nucleosil 7 C_{18} column (250 mm × 8 mm I.D.) permitting up to 60 A_{260} units to be fractionated. Elution of the columns was achieved by a two-component system (see Table II). The elution was monitored at 260 nm and resulted in the elution profiles shown in Figs. 2 and 3. The oligonucleotides $d(G_4T_4)$ and $d(T_4G_4T_4)$ were eluted under isocratic conditions (experiments 1–3) using 7.5 m*M* tetrabutylammonium hydrogensulphate (TBA) pH 7.0 as eluent A and 7.5 m*M* TBA pH 7.0 in 75% aqueous acetonitrile as eluent B. The oligonucleotides $d(T_4G_4)$, $d(G_4T_4G_4)$ and $d(T_4G_4T_4G_4)$ were fractionated with a linear increasing gradient (experiments 4–6), the concentration of eluent D (5.0 m*M* TBA pH 6.8 in 70% aqueous acetonitrile) increasing from 40 to 80% within 48 min. Eluent C was 5.0 m*M* TBA pH 6.8. The integration of the elution profiles (Figs. 2 and 3) showed that the oligonucleotides, except $d(G_4T_4G_4)$, were contaminated to an extent of less than 10%, demonstrating that the previous column chromatographic separations on DEAE-cellulose or QAE-Sephadex led to oligonucleotides of sufficient purity. The fractions corresponding to the main peaks within the vertical dotted lines were pooled, desalted and lyophilized. As a test of purity, the oligonucleotides were rechromatographed in amounts of 10–15 μg at 50°C on an analytical Nucleosil 7 C_{18} column (250 mm × 4.6 mm I.D.), *e.g.*, experiment 2 (Table II) demonstrates that the purity of the isolated oligonucleotides exceed 98%.

The guanylate-rich $d(G_4T_4G_4)$, however, could not be purified to an extent beyond 60%, by ion exchange or by ion-pair reversed-phase HPLC, as could be concluded from the integration of the elution profile (Fig. 3b). It is possible that the dodecamer was of much higher purity judging from the elution profile. In support of this is the elution of identical guanylate-rich oligonucleotides at different retention times. Furthermore, the dodecamer could be used successfully for enzymatic ligation, as will be described elsewhere[7]. Therefore contaminations up to the presumed amount can certainly be excluded.

Both the purity and sequence of $d(G_4T_4)$, $d(T_4G_4)$ and $d(T_4G_4T_4)$ were confirmed by sequencing the oligonucleotides, carried out according to the well known two-dimensional fingerprint method[14–18]. Contrary to our expectation, the fingerprint method could not be used for the sequencing of $d(G_4T_4G_4)$ and $d(T_4G_4T_4G_4)$. Because of the strong adsorption of the guanylate-rich oligonucleotides on the poly-

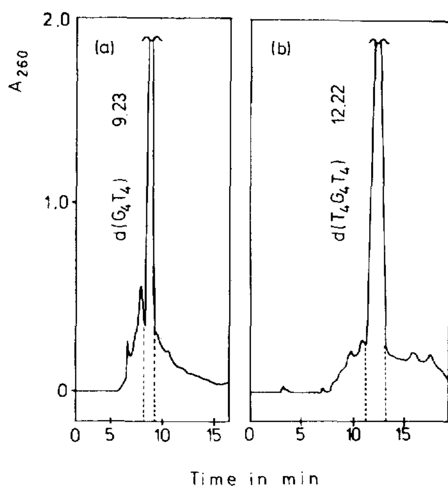

Fig. 2. Ion-pair reversed-phase HPLC of $d(G_4T_4)$ and $d(T_4G_4T_4)$ on a Nucleosil 7 C_{18} column (250 mm × 8 mm I.D.) at room temperature under isocratic conditions (see Table II) with a flow-rate of 2 ml/min. (a) $d(G_4T_4)$ eluted with a mixture of 70% A and 30% B; $d(T_4G_4T_4)$ chromatographed with 50% A and 50% B. A = 7.5 m*M* TBA, pH 7.0; B = 7.5 m*M* TBA, pH 7.0 in 75% aqueous acetonitrile.

saccharide matrix, a significant separation of the partial hydrolysates of these oligonucleotides by means of two-dimensional chromatography failed, thus a fingerprint could not be obtained. Therefore, the partial hydrolysates of the radioactively labelled dodecamer $d([^{32}P]G_4T_4G_4)$ and hexadecamer $d([^{32}P]T_4G_4T_4G_4)$ were separated only one-dimensionally on a polyacrylamide gel under denaturing conditions[19] by means of electrophoresis. The separation of the twelve or sixteen spots confirmed that the oligonucleotides synthesized indeed correspond to dodecamers and hexadecamers, respectively.

$d(T_4G_4T_4G_4)$ was also sequenced according to the method of Maxam and Gilbert[20] (see Fig. 4). This method is based on a specific chemical modification of Cyt, Cyt + Thy, Ade + Gua and Gua in four parallel reactions. During the partial

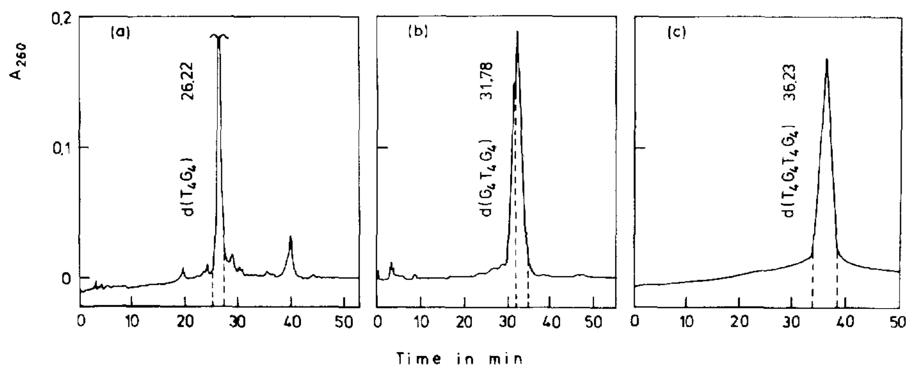

Fig. 3. Ion-pair reversed-phase HPLC of (a) $d(T_4G_4)$, (b) $d(G_4T_4G_4)$ and (c) $d(T_4G_4T_4G_4)$ on a Nucleosil 7 C_{18} column (250 mm × 4.6 mm I.D.) at 50°C with a gradient of 40 to 80% D over 0 to 48 min (see Table II); flow-rate: 1 ml/min. C = 5 m*M* TBA, pH 6.8; D = 5 m*M* TBA in 70% aqueous acetonitrile.

hydrolysis only the modified nucleobases are supposed to be eliminated and the polynucleotide chain should be cleaved at the point where these nucleobases are missing. The partial hydrolysate obtained is separated into fragments of different chain lengths by gel electrophoresis, resulting in the autoradiogram of Fig. 4. The sequence of the hexadecamer from the 5'- to the 3'-terminal is obtained by following the most blackened bands in the four lanes from the top (hexadecamer) to the bottom (monomer unit). The interpretation of the autoradiogram is given in the right part of Fig. 4. The degradation pattern confirms the sequence of $d(T_4G_4T_4G_4)$. Possible failure sequences of synthetic oligonucleotides cannot be detected conclusively and can therefore not be excluded. For example, the "C lane", to which the "Cyt degradation" was added for control purposes, contains strong bands in its upper part, which might be correlated with C contaminations. However, this is to be excluded in this case, because only T- and G-monomer units have been employed in the synthesis.

The presence of other nucleobases was independently excluded by totally degrading the hexadecamer chemically. Using formic acid, the oligonucleotide was degraded, according to well known methods[21,22], to its nucleobases. The total hydrol-

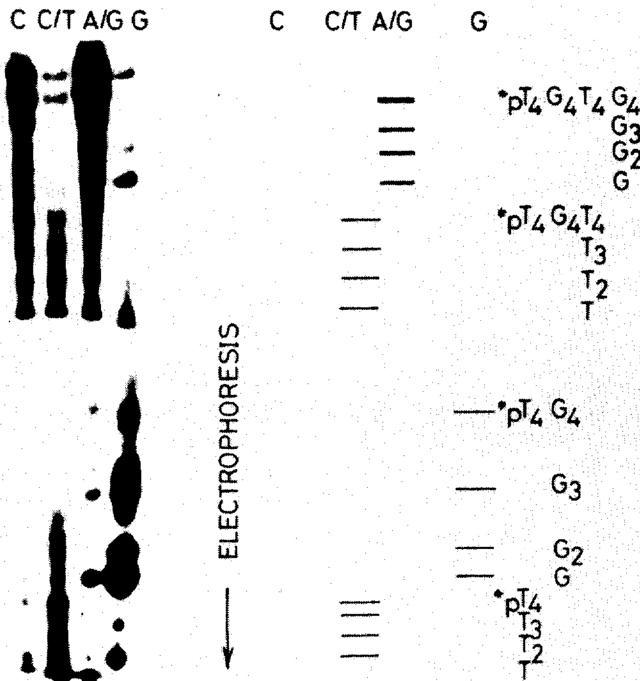

Fig. 4. Left part: autoradiogram after gel electrophoresis of the nucleotide-spectific degraded $d([^{32}P]T_4G_4T_4G_4)$ using the Maxam and Gilbert method. C, C/T, A/G, G denote C-specific, C + T cleavage, A + G cleavage and G-specific cleavage of the oligonucleotide. The chemically degraded oligonucleotide is fractionated on a "20% polyacrylamide gel" (0.025 cm × 20 cm × 40 cm) with 50 m*M* Tris–borate–1 m*M* EDTA buffer. Electrophoresis proceeded at 2.5 kV/6 mA for 2 h. Right part: interpretation of the sequence patterns. *p denotes $[^{32}P]$.

ysate was fractionated on a Nucleosil 7 C_{18} column by means of reversed-phase HPLC under isocratic conditions. Only two peaks were obtained (see Fig. 5b). As is seen from the elution profile (Fig. 5a) obtained by chromatography of the four nucleobases under identical conditions, the retention times (9.76 and 11.69 min) match those of Gua (9.77 min) and Thy (11.67 min), respectively. Having found only the two nucleobases expected in the total hydrolysate, the presence of other nucleobases within the hexadecamer synthesized can be excluded. From the integration of the peak areas of Fig. 5b and in view of the molar absorption coefficients at 260 nm (Thy, 9600 l mol^{-1} cm^{-1}; Gua, 13 700 l mol^{-1} cm^{-1}), a molar ratio of Thy: Gua of 1.02:1 was calculated. The nucleobase composition of $d(T_4G_4T_4G_4)$ determined was very close to that expected (1.00:1).

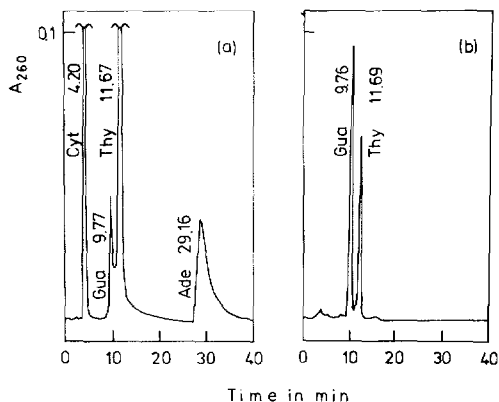

Fig. 5. Reversed-phase HPLC on a Nucleosil 7 C_{18} column (250 mm × 4.6 mm I.D.) at room temperature. Eluent: 50 m*M* ammonium acetate, pH 6.8; flow-rate, 1 ml/min. Applied probe: (a) the test mixture of the four nucleobases Cyt, Gua, Thy and Ade; (b) about 0.1 A_{260} units of the totally hydrolysed $d(T_4G_4T_4G_4)$.

CONCLUSIONS

The preparative chromatography of guanylate-rich oligonucleotides, employing different separation materials (DEAE-cellulose, QAE-Sephadex, Partisil 10-SAX and Nucleosil C_{18}), can be performed only with considerable loss of oligonucleotides. Therefore, in the oligonucleotide synthesis there is only a limited possibility of separating impurities using chromatography. Guanylate-rich oligonucleotides of different chain lengths associate with each other, thus causing identical compounds to be contained within different peaks and be eluted from the column at different times. At the same time, part of the product remains irreversibly adsorbed on the ion-exchanger matrix. The formation of aggregates between both the oligonucleotides and/or their derivatives and between the oligonucleotides and the polymer matrix is the reason why the desired oligonucleotide cannot be obtained when small quantities of condensation product are worked up by column chromatography.

Remarkably, the dodecamer $d(G_4T_4G_4)$, which could be purified only partially and characterized not unequivocally, however, resulted in the 36mer and other po-

lynucleotides upon enzymatic ligation with the dodecamer $d(T_4G_4T_4)$[7]. This result demonstrates that the oligonucleotides can be used in enzymatic reactions directly after their synthesis, without tedious final purification. Also that the common enzymatic reactions employing oligonucleotides do not require an high standard of purity, because the enzymes are able to select the "fitting compound" from the multitude offered.

The increasing demand for oligonucleotides necessitates their preparation in large amounts. Therefore, preparative synthesis on the largest scale possible is an urgent objective. In our opinion there is no need for considerable improvements in the strategy of the oligonucleotide synthesis, but there is a great demand for more efficient separation methods for purifying the oligonucleotides after the deprotection without major losses.

ACKNOWLEDGEMENT

This work was suported by the Deutsche Forschungsgemeinschaft.

REFERENCES

1 H. J. Lipps and P. Erhardt, *FEBS Lett.*, 126 (1981) 219.
2 H. J. Lipps, W. Gruissem and D. M. Prescott, *Proc. Natl. Acad. Sci. U.S.A.*, 79 (1982) 2495.
3 R. F. Boswell, L. A. Klobutcher and D. M. Prescott, *Proc. Natl. Acad. Sci. U.S.A.*, 79 (1982) 3255.
4 D. Dawson and G. Herrick, *Cell*, 36 (1984) 171.
5 D. Dawson and G. Herrick, *Mol. Cell. Biol.*, 4 (1984) 2661.
6 E. Helftenbein, *Nucleic Acids Res.*, 13 (1985) 415.
7 H. Schott, R. Semmler, K. Closs and H. Eckstein, *Makromol. Chem.*, in press.
8 M. D. Edge, A. R. Greene, G. R. Heathcliffe, P.A. Meacock, W. Schuch, D. B. Scanlon, T.C. Atkinson, C.R. Newton and A. F. Markham, *Nature (London)*, 292 (1981) 756.
9 T. G. Lawson, F. E. Regnier and H. L. Weith, *Anal. Biochem.*, 133 (1983) 85.
10 I. M. Johansson, K.-G. Wahlund and G. Schill, *J. Chromatogr.*, 149 (1978) 281.
11 J. H. Knox and J. Jurand, *J. Chromatogr.*, 149 (1978) 297.
12 A. Tilly Melin, M. Ljungcrantz and G.Schill, *J. Chromatogr.*, 185 (1979) 225.
13 M.Kwiatkowski, A. Sandström, N. Balgobin and J. Chattopadhyaya, *Nucleic Acids Res. Symp. Ser.*, No. 14 (1984).
14 E. Jay, R. Bambara, R.Padmanabhan and R. Wu, *Nucleic Acids Res.*, 1 (1974) 331.
15 C.D.Tu, E. Jay, C.P.Bahl and R.Wu, *Anal. Biochem.*, 74 (1976) 73.
16 R. Frank and H. Blöcker, in H.G. Gassen and A. Lang (Editors), *Chemical and Enzymatic Synthesis of Gene Fragments*, Verlag Chemie, Weinheim, 1982, p. 225.
17 H. Schott and H. Schrade, *J.Chromatogr.*, 284 (1984) 381.
18 H. Schott, H. Schrade and H.Watzlawick, *J. Chromatogr.*, 285 (1984) 343.
19 T. Maniatis, A. Jeffrey and H. van de Sande, *Biochemistry*, 14 (1975) 3787.
20 A. M. Maxam and W. Gilbert, *Methods Enzymol.*, 65 (1980) 499.
21 C. Y. Ko, J. L. Johnson, L. B. Barnett, H. M. McNair and J. R. Vercelotti, *Anal. Biochem.*, 80 (1977) 183.
22 H. J. Fritz, D. Eick and W. Werr, in H. G. Gassen and A. Lang (Editors), *Chemical and Enzymatic Synthesis of Gene Fragments*, Verlag Chemie, Weinheim, 1982, p. 199.