Journal of Chromatography, 397 (1987) 141–151
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1164

POLYMER SUPPORT SYNTHESIS

XII*. DERIVATIZATION WITH THE 4-DECYLOXYTRITYL GROUP AS AN AID IN THE AFFINITY CHROMATOGRAPHY OF OLIGO- AND POLYNUCLEOTIDES

HARTMUT SELIGER* and GERALD SCHMIDT
Sektion Polymere, Universität Ulm, Oberer Eselsberg, D-7900 Ulm (F.R.G.)

SUMMARY

In a continuation of previous studies on trityl groups substituted with long alkyl chains as affinity-protecting groups for oligonucleotides, the use of the (4-decyloxyphenyl)diphenylmethyl (DTr) group as an aid in the separation of solid-phase products has been investigated. This substituent, which can be introduced and removed from the 5'-position in a similar manner to the di-*p*-anisylphenylmethyl group, helps in the purification of deoxypolynucleotides with up to 140 bases. Their retention time was shown to depend not only on the length of the nucleotide chain and alkyl substituent, but also on the elution gradient rate. In oligoribonucleotide synthesis, the DTr group allows the purification of partially protected sequences, which are more stable to enzymatic degradation and, therefore, more suitable for handling and storage.

INTRODUCTION

On the basis of earlier work[1], the use of triphenylmethyl protecting groups with long-chain alkyl substituents for the affinity chromatographic purification of oligonucleotides of defined sequence[2], and particularly of solid-phase products[3], has previously been proposed by us. A recent reinvestigation showed that the (4-hexadecyloxyphenyl)diphenylmethyl (HTr) group, which we recommended in the earlier work, causes most oligonucleotides to be too strongly retained on modern C_{18} stationary phases[4]. From our present studies the (4-decyloxyphenyl)diphenylmethyl group now appears more suitable for separations of medium- to large-size oligonucleotides, as they are routinely used for gene synthesis, etc. This paper describes some applications of this affinity-protecting group to current problems of polynucleotide purification and discusses some parameters of the separations.

* For Part XI, see H. Seliger and K. C. Gupta, *Angew. Chem.*, 97 (1985) 711; *Angew. Chem., Int. Ed. Engl.*, 24 (1985) 685.

0021-9673/87/$03.50

Abbreviations

The following abbreviations are used: DMTr = di-*p*-anisylphenylmethyl; DTr = (4-decyloxyphenyl)diphenylmethyl; HTr = (4-hexadecyloxyphenyl)diphenylmethyl; dA^{bz} = 6-N-benzoyl-2'-deoxyadenosine; dC^{bz} = 6-N-benzoyl-2'-deoxycytidine; dG^{ibu} = N-isobutyryl-2'-deoxyguanosine; dT = 2'-deoxythymidine; HPLC = high-performance liquid chromatography; FPLC = fast protein liquid chromatography; RP = reversed phase; TEAA = triethylammonium acetate; PAGE = polyacrylamide gel electrophoresis.

EXPERIMENTAL

Chemicals

The aqueous buffer that was used for high-performance liquid chromatography (HPLC) and fast protein liquid chromatography (FPLC) was 0.1 *M* triethylammonium acetate (TEAA) of pH 7. This was prepared by dilution from a 0.4 *M* stock solution, which, in turn, was obtained by slowly mixing the corresponding amounts of triethylamine (Merck, Darmstadt, F.R.G.) and glacial acetic acid (Merck). The organic eluent component was HPLC-grade acetonitrile (Baker, Phillipsburg, NJ, U.S.A.).

Columns

The columns used for the separations were: μBondapak C_{18} (300 × 7.5 mm I.D.) from Waters Assoc. (Milford, MA, U.S.A.) for HPLC and PepRPC$_{18}$ HR 5/5 (50 × 5 mm I.D.) from Pharmacia (Uppsala, Sweden) for FPLC.

Nucleosides

5'-Decyloxytritylnucleosides were prepared essentially according to the procedure published earlier[2]. The yields of $DTrdA^{bz}$, $DTrdC^{bz}$, $DTrdG^{ibu}$ and DTrdT were between 80 and 93%. The compounds were characterized by their UV spectra ($\lambda_{max} \approx \lambda_{max}$ of parent nucleoside) and by field desorption mass spectra [$(M + H)^+$: m/z = 755, 731, 737 and 642, respectively]. The nucleosides were converted into the corresponding 3'-(β-cyanoethoxy)phosphoamidites according to standard procedures[5,6].

Deoxyoligonucleotides

The shorter deoxyoligonucleotides with chain lengths up to 30 bases were prepared with the SAM I synthesizer (Biosearch, San Rafael, CA, U.S.A.). The 53- and the 61-base fragments were synthesized on a Pharmacia Gene Assembler; the polynucleotides of 95 and 140 bases were obtained with a Biosearch 8600 synthesizer (Biosearch, New Brunswick Scientific, Edison, NJ, U.S.A.).

Ribooligonucleotide synthesis

The ribooligonucleotide $(rU)_{15}$ was prepared in collaboration with D. Zeh by solid-phase synthesis in the SAM I synthesizer, using 5'-O-trianisylmethyl-2'-O-tetrahydropyranyluridine-3'-O-(β-cyanoethoxy-N,N-diisopropyl)phosphoamidite[7] up to the thirteenth cycle, then the respective 5'-O-(4-decyloxyphenyl)diphenylmethyluridine derivative for the last cycle. The workup was as described previously[8], except that the tetrahydropyranyl group was not deblocked.

Apparatus

HPLC was performed with a DuPont (Wilmington, DE, U.S.A.) Series 8800 system. The FPLC results were obtained with a Pharmacia FPLC-500 system.

RESULTS

Introduction of the (4-decyloxyphenyl)diphenylmethyl (DTr) group

This group was introduced selectively into the 5'-position of base-protected nucleosides by following essentially the procedure described earlier[2] (Fig. 1). The yields of protected nucleosides were 80–93%, as isolated by column chromatography. The correct structure was established by ^{1}H NMR and field desorption (FD) mass spectrometry. The (4-decyloxyphenyl)diphenylmethylnucleosides were converted into the 3'-O-(β-cyanoethoxy-N,N-diisopropyl)phosphoamidites by literature procedures[5,6].

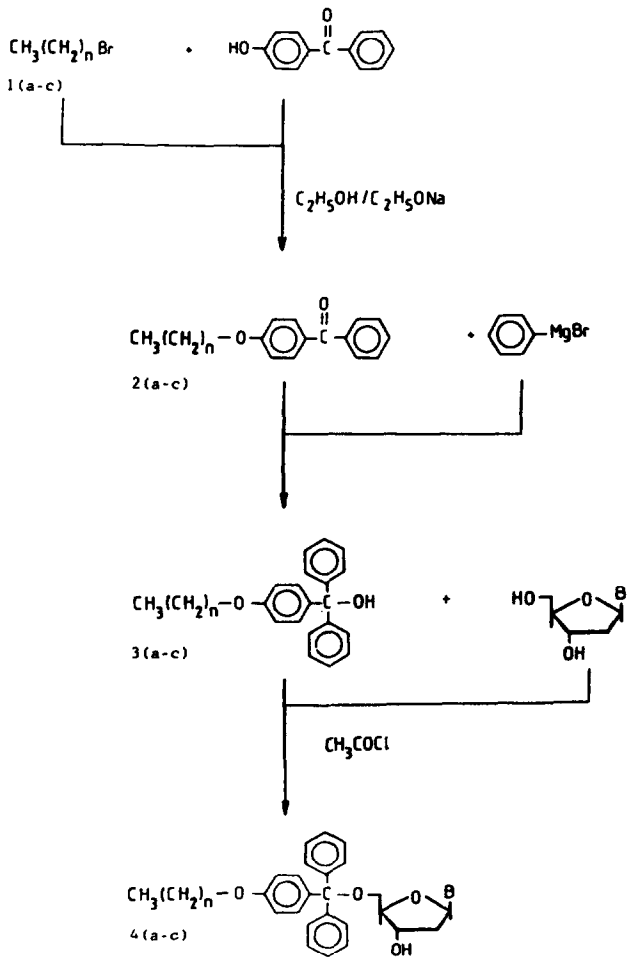

Fig. 1. Preparation of 5'-(4-alkoxyphenyl)diphenylmethyl nucleosides.

Deoxyoligonucleotide synthesis

The oligo- and polydeoxynucleotides were prepared by the solid-phase method in automated synthesis machines by following mainly the standard phosphoamidite protocol[9,10]. For simplicity, we mostly used commercial 5'-O-di-*p*-anisylphenylmethyl nucleoside-3'-O-(β-cyanoethoxy-N,N-diisopropyl)phosphoamidites as reagents for all but the last cycle. In the last chain elongation, the appropriate 5'-O-(4-decyloxyphenyl)diphenylmethyl nucleoside-3'-O-(β-cyanoethoxy-N,N-diisopropyl)phosphoamidite was used. In this instance, no change of the solvents or of the reaction and wash times was necessary. Alternatively, the phosphoamidites of 5'-O-(4-decyloxyphenyl)diphenylmethyl nucleosides can be used throughout all cycles with comparable yields. The only change, in this instance, would be a slightly longer deprotection time (*ca.* 1 min instead of 0.5 min).

Workup of oligodeoxynucleotides

After completion of the elongation, the oligonucleotide products were cleaved from the polymer support with concomitant deprotection of the internucleotidic bonds and the nucleobases, as described in the literature[11]. These "solid-phase products", containing a mixture of oligonucleotides of different lengths and stripped of all protecting groups except the trityl substituents, were generally the substrates for the HPLC studies discussed in the following sections.

HPLC separation of oligo- and polydeoxynucleotides

Comparison of chromatographic properties of model oligonucleotides with 5'-protecting groups having different alkyl chain lengths. The rationale for the selection of the DTr group for affinity separations was arrived at from a comparison of the chromatographic properties of decathymidylate as oligonucleotide substituted at the 5'-terminus with different (4-alkoxyphenyl)diphenylmethyl groups. Fig. 2 shows the chromatogram of a mixture of crude solid-phase products from the synthesis of di-*p*-anisylphenylmethyl-, (4-*n*-butyloxyphenyl)diphenylmethyl-, (4-decyloxyphenyl)diphenylmethyl- and (4-hexadecyloxyphenyl)diphenylmethyl decathymidylate. This comparison shows that the elution volume of the di-*p*-anisylphenylmethyl derivative is approximately doubled for the DTr and tripled for the HTr derivative. In spite of this enhanced retention of the HTr-oligonucleotide, in this standard acetonitrile gradient system, the DTr group appears to be a more suitable "purification handle". This is based on the consideration that if a support product were to contain a small percentage of trityl-substituted oligonucleotides of very low molecular weight, arising, *e.g.*, from incomplete capping, such small oligonucleotides with substituents that have too long alkyl chains will be eluted by nearly 100% acetonitrile, *i.e.*, their quantitative elution will be either difficult or require a change of solvent. In fact, the monomeric HTrdT was not eluted at all with acetonitrile, but required tetrachloromethane as eluent. This consideration does not completely rule out the use of the HTr group, but limits it to special separation problems where a more lengthy elution process is justified.

Comparison of chromatographic behaviour of model DTr-oligonucleotides of different chain lengths. Our study was then extended to the comparison of model oligonucleotides differing not only in the nature of the 5'-protecting group, but also in the length of the nucleotide chain. Fig. 3 shows the elution profile after simultaneous

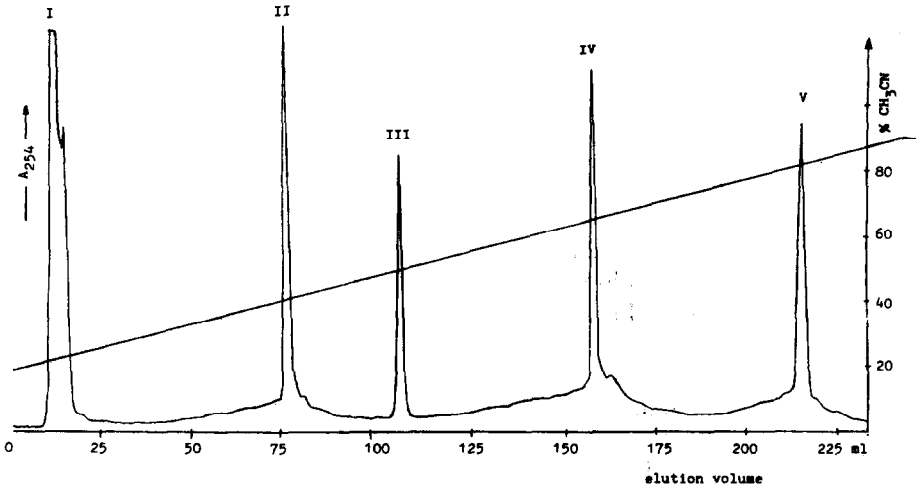

Fig. 2. Comparison of the elution volumes of decathymidylates with 5′-(4-alkoxyphenyl)diphenylmethyl affinity-protecting groups of the type 4-$CH_3(CH_2)_n$–O–C_6H_4–$C(C_6H_5)_2$– of different alkyl chain length, *n*. Peaks: I = bulk of non-tritylated truncated chains; II = di-*p*-anisylphenylmethyl-; III = (*n*-butyloxyphenyl)diphenylmethyl- (*n* = 3); IV = (*n*-decyloxyphenyl)diphenylmethyl- (*n* = 9); V = (*n*-hexadecyloxyphenyl)diphenylmethyl- (*n* = 15). Conditions: Column, μBondapak C_{18} (300 × 7.5 mm I.D.); eluent, 20–90% acetonitrile in 0.1 *M* TEAA (pH 7) in 120 min; flow-rate, 2 ml/min; gradient, 1% acetonitrile per 3.5 ml; detection, 254 nm.

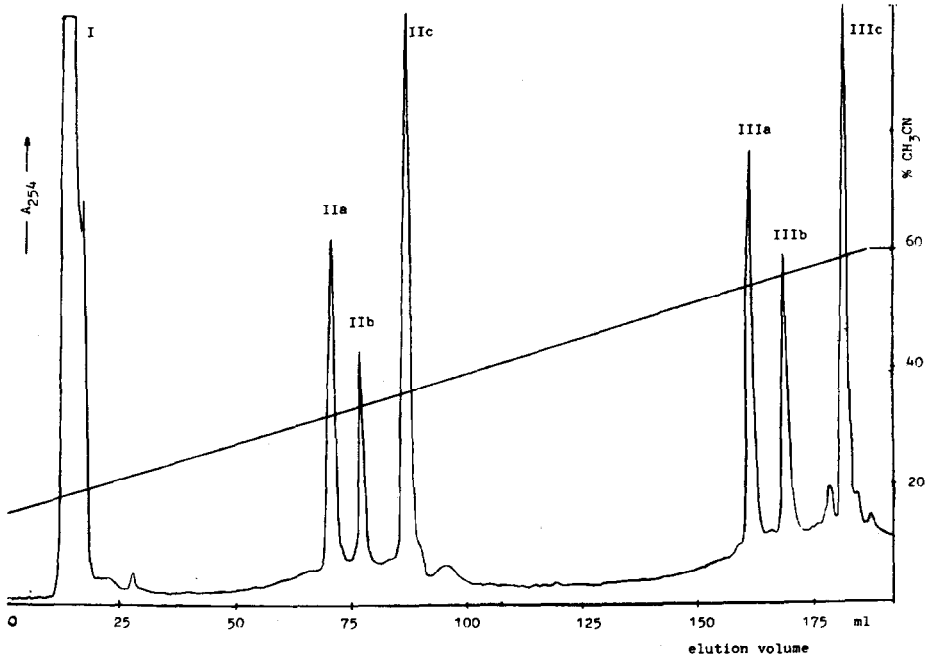

Fig. 3. Comparison of the elution volumes of simultaneously injected deoxythymidylates of chain lengths of 10, 20 and 30 bases, terminated by either 5′-(4,4′-dimethoxy)trityl- or 5′-(4-decyloxyphenyl)diphenylmethyl groups. Peaks: I = bulk of non-tritylated truncated chains; IIa, b, c = $DMTr(dT)_{30}$, $DMTr(dT)_{20}$, $DMTr(dT)_{10}$; IIIa, b, c = $DTr(dT)_{30}$, $DTr(dT)_{20}$, $DTr(dT)_{10}$. Conditions: Column, μBondapak C_{18} (300 × 7.5 mm I.D.); eluent, 20–60% acetonitrile in 0.1 *M* TEAA (pH 7) in 85 min; flow-rate, 2 ml/min; gradient, 1% acetonitrile per 4.74 ml; detection, 254 nm.

injection of 5'-O-di-*p*-anisylphenylmethyl- and 5'-O-(4-decyloxyphenyl)diphenylmethyloligothymidylate having chain lengths of 10, 20 and 30 bases. As can be seen in Fig. 3, the acetonitrile content of the eluent for $DTr(dT)_{30}$ is still 1.5 times that of the buffer eluting $DMTr(dT)_{10}$. This demonstrates that, although an increase in the length of the oligonucleotide reduces the elution volume, in both instances, the overall retention conferred by the longer alkyl substituent far exceeds the influence of the polyanionic chain.

Purification of deoxypolynucleotides. The result shown in Fig. 3 encouraged us to attempt the purification of long polynucleotides, composed of all four bases. That the DTr group is, in fact, valuable for this purpose is demonstrated by the examples shown in Figs. 4 and 5, which include sequences of up to 140 bases. Even at this extreme chain length, the peak corresponding to the target sequence is still separated from the fraction of the total non-tritylated truncated chains, although the resolution is marginal. A definite improvement was found on chromatographing this mixture in the FPLC system described in the legend of Fig. 6. The near-baseline separation and peak symmetry suggested a high purity of the fractions. This was, in fact, confirmed by polyacrylamide gel electrophoresis of the detritylated sequences after 5'-phosphorylation with [γ-^{32}P]ATP and polynucleotide kinase[10], which showed a single radioactive spot on autoradiography. The correct length and sequence of the polynucleotides were further established by the Maxam–Gilbert technique[12]. The cloning and further use of these fragments for gene synthesis projects will be described elsewhere.

Influence of acetonitrile gradient rates. Comparing the elution volumes of the same model compounds obtained under different HPLC conditions, in Figs. 2 and

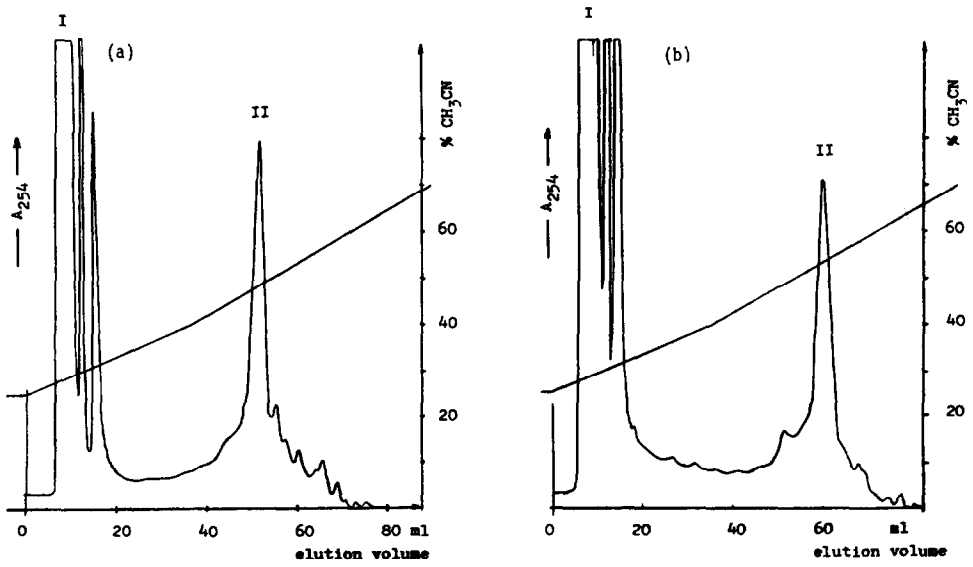

Fig. 4. HPLC elution profiles of deoxyoligonucleotides of (a) 61 bases and (b) 53 bases, containing all four nucleotides and terminated by 5'-DTr groups. Peaks: I = non-tritylated truncated chains; II = tritylated solid-phase products. Conditions: Column, μBondapak C_{18} (300 × 7.5 mm I.D.); eluent, 25–70% acetonitrile in 0.1 *M* TEAA (pH 7) in 45 min; flow-rate, 2 ml/min; gradient, 25–40% with 1% acetonitrile per 2.4 ml, 40–70% with 1.8 ml; detection, 254 nm.

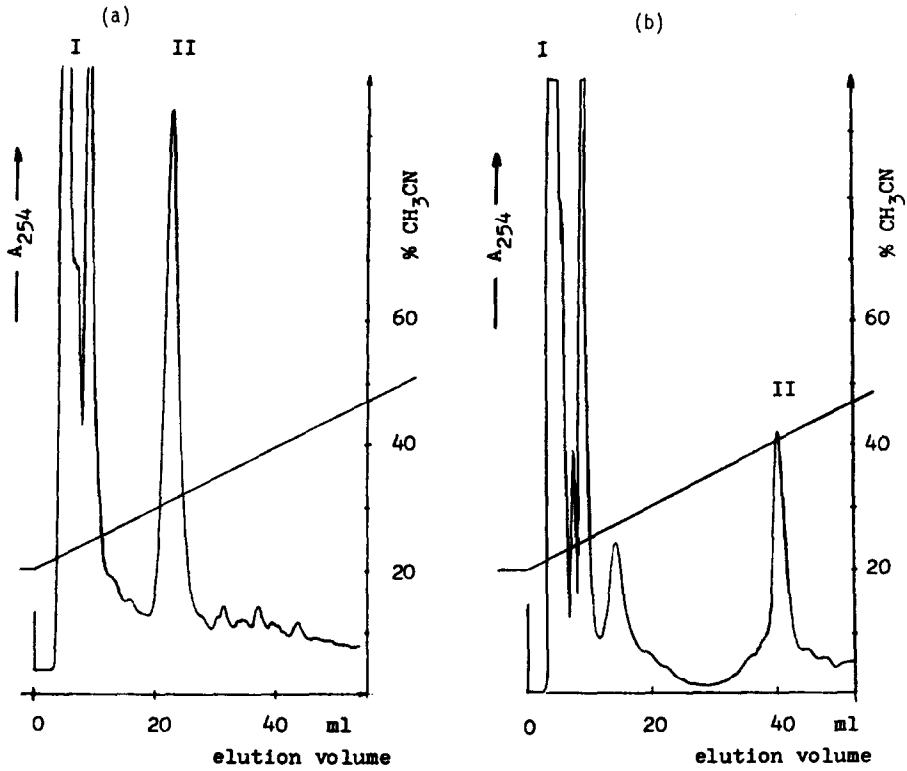

Fig. 5. HPLC elution profiles of deoxyoligonucleotides of defined sequence containing (a) 140 bases and (b) 95 bases and terminated by 5'-DTr groups. Peaks: I = non-tritylated truncated chains; II = tritylated product. Conditions: Column, μBondapak C_{18} (300 × 7.5 mm I.D.); eluent, 20–50% acetonitrile in 0.1 *M* TEAA (pH 7) in 30 min; flow-rate, 2 ml/min; gradient, 1% acetonitrile per 2 ml; detection, 254 nm.

3, we first observed a distinct influence of the acetonitrile gradient rate on the chromatographic behaviour. This finding was further elaborated on and generalized, as shown in Fig. 7, which correlates oligonucleotide chain length, elution volume and acetonitrile content of the eluents with gradient rate and substituent alkyl chain length of the 5'-trityl protecting groups. For both the 5'-DMTr- and 5'-DTr-oligonucleotides the elution volumes of nucleotide chains of the same length are significantly enhanced by lowered rates of acetonitrile gradient. Also, the acetonitrile content of the fractions, in which the respective oligonucleotides are eluted, differs slightly with changing gradient rates. For a constant gradient rate and a given 5'-protecting group the correlation between oligonucleotide length and elution volume seems to be linear and apparently nearly independent of the sequence. Fig. 7 suggests that the separation in RP-HPLC can be further enhanced by proper selection of the elution gradient rates. Additionally, even if a solid-phase product happens to contain a series of tritylated sequence homologues, products containing low concentrations of contaminating neighbouring sequence homologues may be worked up, if appropriately small fractions are collected. Such products, after detritylation[10], may be used directly for ligation and cloning without further purification.

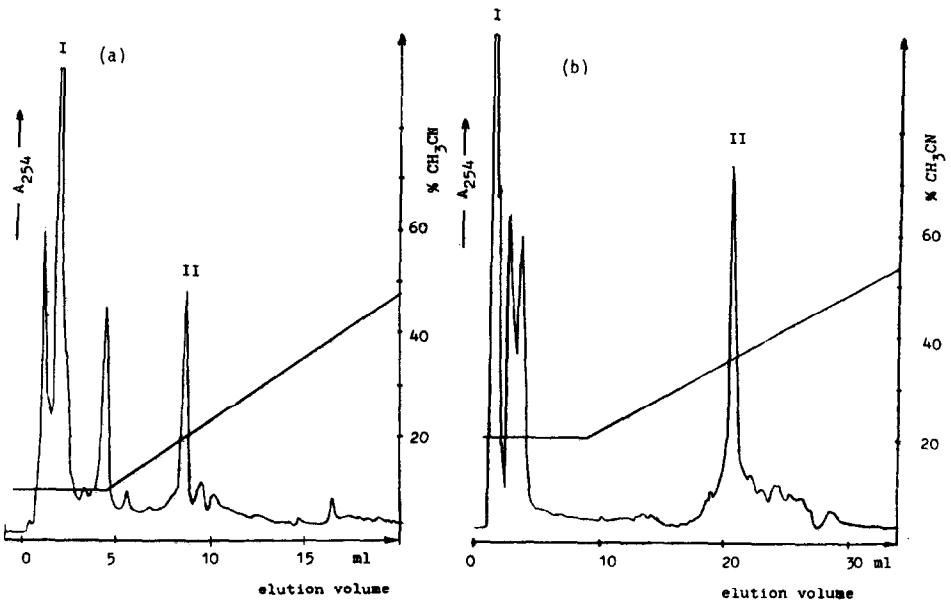

Fig. 6. FPLC elution profiles of deoxyoligonucleotides of defined sequence containing (a) 140 bases and (b) 95 bases and terminated by 5′-DTr groups. Peaks: I = non-tritylated truncated chains; II = tritylated product. Conditions: Column, PepRPC$_{18}$ HR 5/5 (50 × 5 mm I.D.); eluent, (a) 10–50% and (b) 20–50% acetonitrile in 0.1 *M* TEAA (pH 7) in (a) 48 min and (b) 45 min; flow-rate, (a) 0.25 and (b) 0.5 ml/min; gradient, 1% acetonitrile per (a) 0.3 and (b) 0.75 ml; detection, 254 nm.

HPLC separation of partially protected oligoribonucleotides

Oligoribonucleotides, in general, are not only more difficult to synthesize but, as unprotected molecules, they are also more difficult to handle owing to their lability to omnipresent nucleases. This lability is largely reduced as long as the 2′-OH group remains blocked. Unfortunately, solid-phase products containing 2′-tetrahydropyranyl groups in addition to a 5′-DMTr group[8] did not allow a good HPLC separation of the target sequence from the non-tritylated truncated chains in all instances. In this instance the use of a DTr group for enhanced resolution was again obvious. This is demonstrated by the elution profile for the HPLC separations of pentadecauridylate-2′-tetrahydropyranyl ether, protected with a 5′-DTr group (Fig. 8). Whereas the corresponding compound terminating in a 5′-DMTr group could no longer be purified by HPLC, the DTr-protected oligoribonucleotide was fairly well separated from the bulk of truncated chains, allowing a fraction of the main peak to be further deprotected and characterized as the target sequence. By applying the considerations described in the last section, better separations of 2′-protected oligoribonucleotides containing a terminal DTr group can be accomplished, particularly if a lower acetonitrile gradient rate and a RP-HPLC system were used in future experiments.

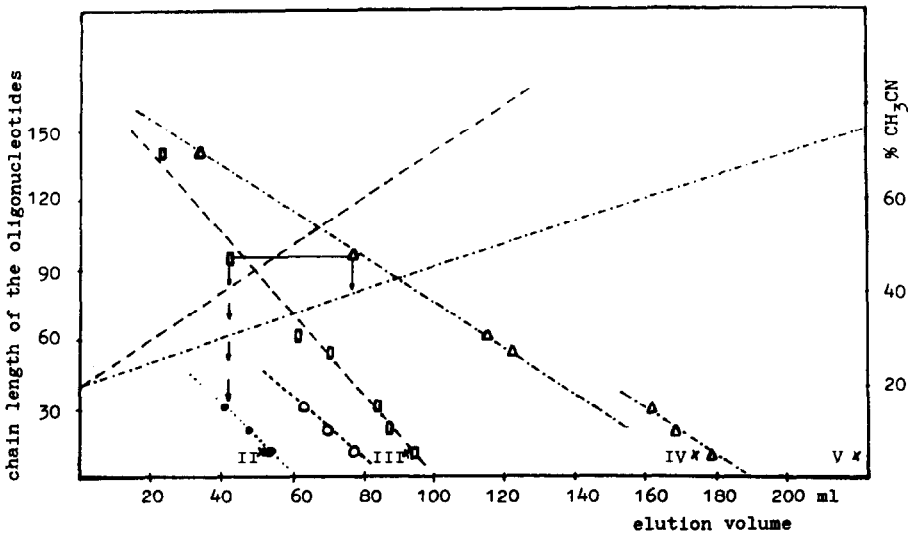

Fig. 7. Correlation between elution volume, acetonitrile content of eluent and elution gradient rate for deoxyoligonucleotides of different chain lengths and 5'-affinity-protecting groups. ●...●, DMTr-oligonucleotides; gradient rate, 1% acetonitrile per 3.2 ml. ○...○, DMTr-oligonucleotides; gradient rate, 1% acetonitrile per 4.75 ml. □—□, DTr-oligonucleotides; gradient rate, 1% acetonitrile per 2 ml. △-.-.-△, DTr-oligonucleotides; gradient rate, 1% acetonitrile per 4 ml, or per 4.75 ml for chains of < 30 bases. ×, Decathymidylates with 5'-(4-alkoxyphenyl)diphenylmethyl groups of different alkyl chain length, *n* (see Fig. 1). II = Di-*p*-anisylphenylmethyl-; III = (*n*-butyloxyphenyl)diphenylmethyl-; IV = (*n*-decyloxyphenyl)diphenylmethyl -; V = (*n*-hexadecyloxyphenyl)diphenylmethyl-. The ascending lines represent the acetonitrile content of eluents used in experiments with gradient rates of 1% acetonitrile per 2 ml (——) and 4 ml (-.-.-.-). Example: to read the elution volumes obtained for a DTr-oligonucleotide of 95 bases when using gradient rates of 1% acetonitrile per 2 ml or 4 ml, follow the horizontal line projecting from the ordinate to the two values marked by □ and △. To read the acetonitrile content of the respective eluents, follow the projection of these values (marked by arrows) to the ascending lines —— and -.-.-.-.

DISCUSSION

In our previous studies we first demonstrated the potential of affinity chromatography in separations brought about by hydrophobic and Van der Waals interactions between long alkyl chains attached to trityl groups and similar chains bonded to reversed stationary phases for oligonucleotides synthesized in solution[2] and short oligonucleotides prepared on a solid support[3]. This paper, focusing on the (4-decyloxyphenyl)diphenylmethyl group as an affinity protecting group, extends the range of HPLC purification to all the oligo- and polynucleotides that are currently targets of automated solid-phase preparations. Clearly, with the separation of a 140-base fragment, shown in Figs. 5a and 6a, we have approached the limit that would be reached by marginal resolution of the product peak not from the peak of the combined truncated sequences, but from that arising from base debenzoylation. This limit can eventually be extended to even longer chains by a change of the separation technique (compare HPLC and FPLC in Figs. 5 and 6) and the derivatization (different base-protecting groups). Of course, such affinity HPLC separations are not

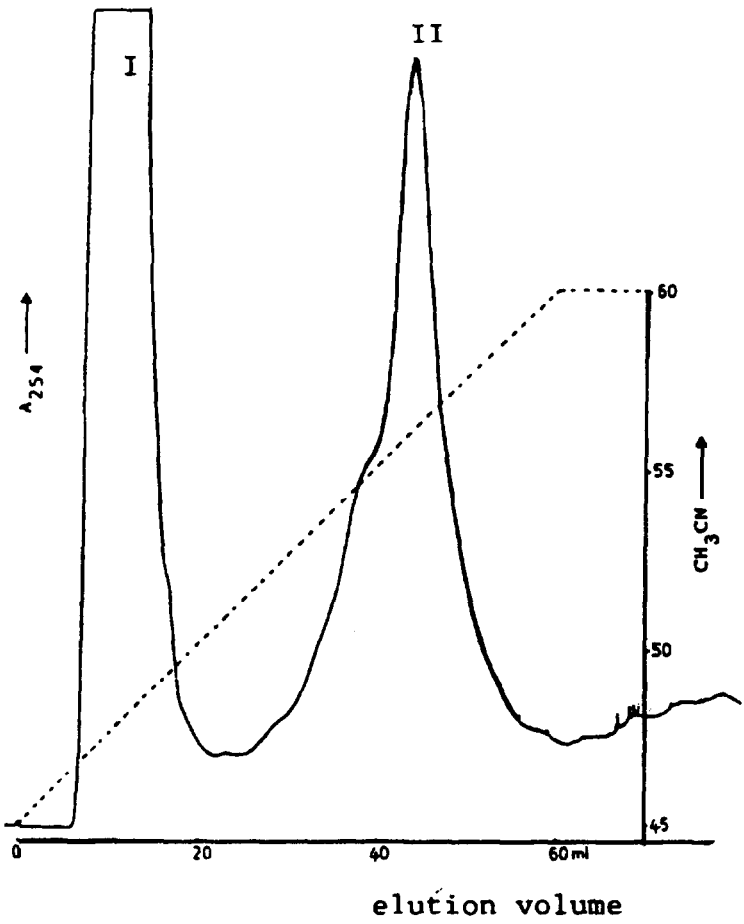

Fig. 8. Separation of 5'-DTr-pentadecauridylate protected at all 2'-positions as a tetrahydropyranyl ether. Peaks: I = non-tritylated truncated chains; II = fraction containing oligoribonucleotide product. Conditions: Column, μBondapak C_{18} (300 × 7.5 mm I.D.); eluent, 45–60% acetonitrile in 0.1 *M* TEAA (pH 7) in 30 min; flow-rate, 2 ml/min; gradient, 1% acetonitrile per 0.25 ml; detection, 254 nm.

designed *a priori* to resolve individual sequence lengths. If the products nevertheless appear as single spots on subsequent polyacrylamide gel electrophoresis[13], this is mainly due to the completeness of capping in the reaction cycles[1]. Even so, we recommend, and routinely carry out, a purity test of the deprotected product by analytical PAGE. Generally, our HPLC method is meant to complement rather than to substitute for electrophoresis, the advantage being, particularly in preparative separations, an increased reproducibility and the possibility of handling larger amounts of solid-phase products. Another advantage is the possibility of purifying partially protected or otherwise modified oligonucleotides. Such sequences, owing to their increased hydrophobicity, may be only marginally amenable, if at all, to purification by PAGE. A first example of this kind is given with the HPLC separation of a 2'-protected oligoribonucleotide. In view of the increasing interest in chemically modi-

fied oligonucleotide structures, this potential should be further exploited, and studies along these lines are under way.

ACKNOWLEDGEMENTS

Expert technical assistance by Mrs. S. Grözinger is acknowledged. The authors thank Pharmacia and Biosearch/New Brunswick Scientific for permission to test syntheses of polynucleotides on automated machines and for generous gifts of materials. This work was financially supported by the Deutsche Forschungsgemeinschaft.

REFERENCES

1 H. Seliger, M. Holupirek and H.-H. Görtz, *Tetrahedron Lett.*, (1978) 2115.
2 H.-H. Görtz and H. Seliger, *Angew. Chem.*, 93 (1981) 708; *Angew. Chem., Int. Ed. Engl.*, 20 (1981) 681.
3 H. Seliger and H.-H. Görtz, *Angew. Chem.*, 93 (1981) 709; *Angew. Chem., Int. Ed. Engl.*, 20 (1981) 683.
4 H. Seliger, A. Herold, U. Kotschi, J. Lyons and G. Schmidt, in K. S. Bruzik and W. J. Stec (Editors), *Biophosphates and Their Analogues, Synthesis, Structure, Metabolism and Activity*, Elsevier, Amsterdam, 1987, p. 43.
5 S. P. Adams, K. S. Kavka, E. J. Wykes, S. B. Holder and G. R. Galuppi, *J. Am. Chem. Soc.*, 105 (1983) 661.
6 L. J. McBride and M. H. Caruthers, *Tetrahedron Lett.*, 24 (1983) 245.
7 H. Seliger, K. C. Gupta, U. Kotschi, T. Spaney and D. Zeh, *Chem. Scr.*, 26 (1986) 56.
8 H. Seliger, D. Zeh, G. Azuru and J. B. Chattopadhyaya, *Chem. Scr.*, 22 (1983) 95.
9 M. D. Matteucci and M. H. Caruthers, *J. Am. Chem. Soc.*, 103 (1981) 3185.
10 H. Seliger, S. Klein, C. K. Narang, B. Seemann-Preising, J. Eiband and N. Hauel, in H. G. Gassen and A. Lang (Editors), *Chemical and Enzymatic Synthesis of Gene Fragments*, Verlag Chemie, Weinheim, 1982, pp. 81*ff*.
11 N. D. Sinha, J. Biernat, J. McManus and H. Köster, *Nucl. Acids Res.*, 12 (1984) 4539.
12 A. Maxam and W. Gilbert, *Methods Enzymol.*, 65 (1980) 499.
13 H. Seliger, C. Scalfi and F. Eisenbeiss, *Tetrahedron Lett.*, 24 (1983) 4963.