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High-performance liquid chromatography of synthetic oligonucleotides

A new affinity protecting group[a]

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ABSTRACT

The use of triphenylmethyl (trityl) protecting groups with long alkyl chain substituents, *viz.*, (4-hexadecyloxyphenyl)diphenylmethyl and (4-decyloxyphenyl)diphenylmethyl, as affinity handles in the purification of oligonucleotides results in significant depurination of oligonucleotides, while removing these protecting groups from the high-performance liquid chromatographically purified oligonucleotides in 80% acetic acid. Excellent resolution of solid-phase synthesized medium- to large-size oligonucleotides using the 4-methoxy-4'-octyloxytrityl (MOTr) group is demonstrated. Moreover, the MOTr group is removed neatly under conditions identical with those used for the deprotection of the dimethoxytrityl group, and hence is less prone to depurination.

INTRODUCTION

Automated solid-phase methods have made the syntheses of oligonucleotides rapid and allowed their widespread employment in molecular biology. The last few years have seen a tremendous improvement in the methods used to prepare modified oligonucleotides [1–4]. Oligonucleotides synthesized by means of these methods have to be purified from by-products after detachment from the solid support.

Many techniques have been used for the purification of chemically synthesized oligonucleotides, including thin-layer (TLC) [5], paper [6], gravity-fed column [7] and high-performance liquid chromatography (HPLC) in ion-exchange [8], normal- [5] and reversed-phase [9] modes. Polyacrylamide gel electrophoresis (PAGE) is also a commonly used technique of purification [10]. However, reversed-phase (C_{18}) column chromatography has become more popular than the others. The reversed-phase

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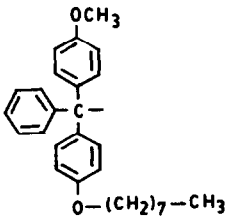
HPLC (RP-HPLC) separation takes advantage of the fact that the conventional 5′-protecting group, *viz.*, dimethoxytrityl (DMTr) (trityl = triphenylmethyl), is a hydrophobic moiety and rather than resolving oligomers differing by a single base, the separation involves resolving 5′-DMTr-DNA from 5′-hydroxy-DNA.

The DMTr group has been found to be suitable for the purification of DNA fragments up to 30 nucleotides in length [11]. However, larger oligonucleotides are difficult to purify using the DMTr group as an affinity handle. In order to overcome the limitations of DMTr group, the use of trityl protecting groups with long alkyl chain substituents, *e.g.*, (4-hexadeacyloxyphenyl)diphenylmethyl (HTr) and (4-decyloxyphenyl)diphenylmethyl (DTr) groups was recommended by Seliger and co-workers [12–14]. Recently,it was observed [15] that the HTr group causes most oligonucleotides to be retained too strongly on C_{18} stationary phase. In general, the main impurities obtained in oligonucleotide synthesis are due to either incomplete capping or 5′-protected failure sequences that are usually shorter than the desired product. These failure sequences seem to be produced by a depurination reaction occurring during the acid treatment used to cleave the DMTr group following each nucleotide addition. The quantitative elution of such small nucleotides with substituents that have too long alkyl chains was either difficult or required a change of solvent. In fact, monomeric HTr-dT was not eluted at all with acetonitrile, but required carbon tetrachloride as eluent.

It has been observed that HPLC-purified oligonucleotides terminated by either an HTr or a DTr group require prolonged exposure to 80% acetic acid in order to remove these protecting groups, thus resulting in significant depurination of the oligonucleotides. It is well documented [16] that the complete removal of the monomethoxytrityl group in 80% acetic acid takes 90 min at 27°C, which is 4.5 times longer than that for the DMTr group (20 min). In view of the above limitations and based on earlier work [17], it was decided to investigate the use of a moderately hydrophobic disubstituted trityl protecting group with 4-methoxy and 4′-octyloxy substituents.



MOTr Group

Model studies demonstrated the utility of the 4-methoxy-4′-octyloxytrityl (MOTr) group as an affinity handle for the purification of medium- to large-size oligonucleotides useful for total gene synthesis and other applications in molecular biology.

EXPERIMENTAL

Reagents

Acetic acid, triethylamine and aluminium oxide were purchased from E. Merck

(India), HPLC-grade acetonitrile from Spectrochem (India) and DMTr-dT from the CSIR Centre for Biochemicals (India). A 0.1 *M* triethylammonium acetate (TEAA) buffer was used in all HPLC analyses, and was prepared by dilution of a 1 *M* stock solution, which was prepared in the following manner: 5.0 g of aluminium oxide was added to 75 ml of triethylamine and the suspension was stirred for 15 min. Then 20 ml of acetic acid were added to 69.7 ml of aluminium oxide-treated triethylamine and distilled water was added to 400 ml. The pH was adjusted to 7.0 with acetic acid. The solution was diluted to 500 ml with distilled water and filtered with 0.22-μm Millipore Durapore filter.

Column

HPLC was performed using either a μBondapak C_{18} column (300 × 3.9 mm I.D., 10 μm) from Waters Assoc. (Milford, MA, USA) or a Zorbax ODS column (250 × 4.6 mm I.D., 5 μm) from DuPont (Wilmington, DE, USA). Both columns were equipped with a precolumn (30 × 8 mm I.D.) packed with Nucleosil 7 C_{18}.

Apparatus

A Model LC-4A HPLC system (Shimadzu, Kyoto, Japan) consisting of a dual-plunger reciprocating pump, a helium degassing unit, an SPD-2AS UV spectrophotometric detector and a C-R3A data processor, was used for all purifications.

*Synthesis of 5'-O-(4-methoxy-4'-alkoxytrityl)thymidine (**3a–e**)*

4-Methoxy-4'-alkoxytrityl groups were selectively introduced at the 5'-hydroxyl position of thymidine by a known method [13] with a slight modification (Fig. 1). In each instance a yield of 85–92% was obtained. The intermediates were characterized by their NMR and UV spectra ($\lambda_{max} \approx \lambda_{max}$ of parent nucleoside).

*Synthesis of 5'-O-(4-methoxy-4'-octyloxytrityl)thymidine-3'-O-(methyl-N,N-diisopropylamino)phosphoramidite (**4**)*

The phosphoramidite **4** was synthesized essentially according to the method reported in the literature [18,19]. 5'-O-(4-Methoxy-4'-octyloxytrityl)thymidine (1.0 g, 1.5 mmol) was reacted with chloro-N,N-diisopropylaminomethoxyphosphine (0.45 ml, 2.3 mmol) in dichloromethane (5 ml) containing diisopropylethylamine (1.1 ml, 6.2 mmol) as an acid scavenger. After work-up, **4** was obtained as a white foam in 89% yield; TLC [dichloromethane–ethyl acetate–triethylamine (45:45:10)], R_F = 0.76.

Removal of 4-methoxy-4'-alkoxytrityl group

The kinetics of the hydrolysis of the 4-methoxy-4'-alkoxytrityl group in **3a–e** in 80% acetic acid was studied in the following manner. A solution of **3a** (4.58 mg) in 1 ml of 80% acetic acid was kept at room temperature with occasional swirling. Aliquots were removed at 1-min intervals and applied to a TLC plate (Fluka precoated plates with fluorescent indicator, 254 nm). The TLC plate was developed with chloroform–methanol (9:1) and spots were revealed under UV light or by spraying with perchloric acid solution with subsequent heating of the plate at 100°C. In each instance hydrolysis was essentially complete in 15–20 min.

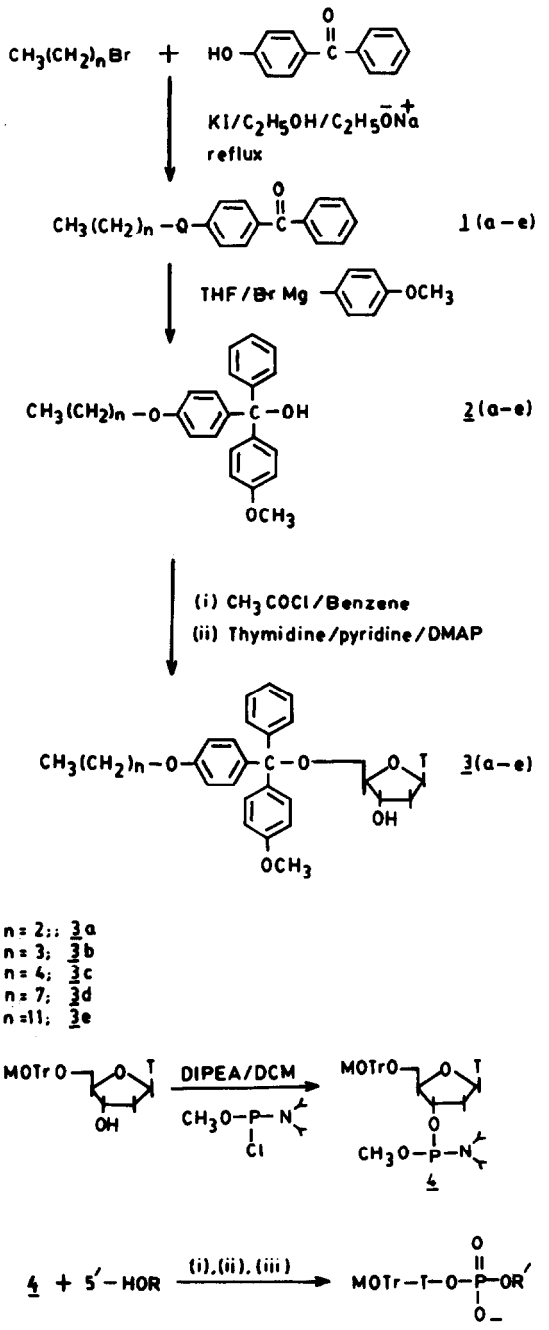


Fig. 1. Scheme for the synthesis of 5'-O-(4-methoxy-4'-alkoxytrityl)thymidines and 5'-O-(4-methoxy-4'-octyloxytrityl)thymidine-3'-O-(methyl-N,N-diisoproylamino)phosphoramidite. In the bottom reaction, (i) = tetrazole; (ii) = aqueous iodine; (iii) = 25% aqueous ammonia, 60°C for 16 h. R = protected oligonucleotide; R' = deprotected oligonucleotide.

Oligonucleotide synthesis and deprotection

Oligonucleotides were synthesized following solid-phase (controlled-pore glass supports) phosphoramidite chemistry [18,19] on a Pharmacia Gene Assembler [20] using methyl or 2-cyanoethyl phosphoramidite derivatives. Oligonucleotides with up to 30 bases were prepared using methyl phosphoramidite and a 60-mer sequence was synthesized with 2-cyanoethyl phosphoramidite. The synthesis was carried out on a 1.3-μmol scale of support-bound first nucleoside. For the synthesis of oligonucleotides with a 5'-MOTr group, in the last coupling step 5'-O-(4-methoxy-4'-octyloxytrityl)thymidine-3'-O-(methyl-N,N-diisopropylamino)phosphoramidite was used. In this final coupling step, no changes to the coupling time or the solvent were made. After the synthesis of the required sequence, the MOTr group was kept intact. The internucleotide methyl phosphate protecting groups were removed with ammonium thiophenoxide [21] treatment. Removal of the exocyclic base protecting groups together with the cleavage of the oligonucleotide from the support was achieved with 25% aqueous ammonia at 60°C for 16 h. With oligo-dT, the support was treated with aqueous ammonia (25%) at room temperature only. The crude oligonucleotides terminated by either DMTr or 4-methoxy-4'-octyloxytrityl groups were then purified by HPLC.

Sample preparation for HPLC purification

After completion of the synthesis, deprotection and cleavage from the support, the ammonia solution containing the crude oligonucleotide was evaporated to dryness in a Savant Speed Vac concentrator. The residue was dissolved in 0.1 *M* TEAA buffer (pH 7.0) and desalted on a Bio-Gel P-2 column using the same buffer as eluent. The desalted product was collected and concentrated and the residue was dissolved in 0.1 *M* TEAA buffer (pH 7.0) (200 μl) and *ca.* 20 μl were applied to the column for HPLC purification.

*HPLC of 5'-O-(4-methoxy-4'-alkoxytrityl)thymidine (**3a–e**)*

The thymidine derivatives **3a–e** were purified on a reversed-phase C_{18} column (μBondapak) using tetrahydrofuran–water–metanol (3:2:2) as the eluent. The pure product peaks were collected and lyophilized. A mixture consisting of HPLC-purified thymidine derivatives, *viz.*, **3a–e** and DMTr-dT, was injected under identical conditions onto the reversed-phase column.

RESULTS AND DISCUSSION

4-Methoxy-4'-alkoxytrityl groups were selectively introduced at the 5'-hydroxyl position of thymidine by a known method [13] with a slight modification (Fig. 1). In each instance, a yield of 85–92% was obtained. The TLC R_F values of 5'-O-(4-methoxy-4'-alkoxytrityl)thymidine derivatives (**3a–e**) obtained on silica gel with chloroform–methanol (9:1) increase with increase in the carbon chain length of the alkoxy function (Fig. 2). This reflects that the increase in the carbon chain length of the alkoxy function increases the hydrophobicity of the 5'-protected thymidine derivatives.

The chromatogram (Fig. 3) obtained by simultaneous injection of **3a–e** and DMTr-dT (used as a reference) onto a C_{18} reversed-phase column shows that the

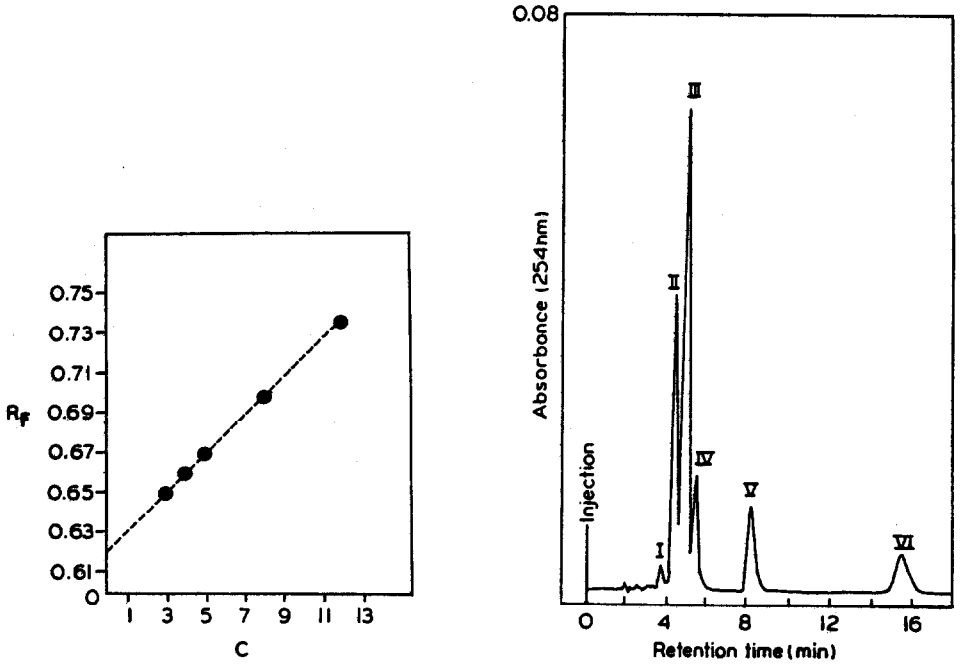


Fig. 2. Dependence of R_F values on the alkyl chain length, C, of compounds **3a–e** on silica gel TLC with chloroform–methanol (9:1).

Fig. 3. HPLC profile of a mixture of **3a–e** and DMTr-dT. Column, μBondapak C_{18} (300 mm × 3.9 mm I.D.); tetrahydrofuran–water–methanol (3:2:2); flow-rate, 1 ml/min. Peaks: I = DMTr-dT; II = **3a**; III = **3b**; IV = **3c**; V = **3d**; VI = **3e**.

retention times of these derivatives increase with increase in the carbon chain length of the alkoxy function. This, however, is contrary to their behaviour on a silica gel TLC plate. This can be explained in terms of hydrophobic interactions between the substituted trityl groups with similar alkyl chains bound to the reversed phase of the column.

The rationale of using a disubstituted trityl group (MOTr) for the affinity purification of oligononucleotides was that a very long alkyl chain substitution in the trityl group would make it much too hydrophobic and hence short nucleotides, *i.e.*, monomers or dimers resulting from incomplete capping or depurination, would be retained on the C_{18} column for a longer time or might not be eluted at all from the column. This has already been reported with the HTr group [15]. Further, with a monosubstituted trityl group (HTr or DTr), the time required for the removal of these protecting groups from the HPLC-purified oligonucleotide was found to be very long, which can cause significant depurination of the purified oligonucleotides. The 4-methoxy-4′-octyloxytrityl group is moderately hydrophobic in comparison with the HTr and DTr groups and, being disubstituted, it can be removed from the HPLC-purified oligonucleotides under conditions identical with those used for the deprotection of the DMTr group.

Fig. 4 shows the elution profile of a mixture consisting of DMTr-d(T_9C),

DMTr-d($T_{19}C$) and DMTr-d($T_{29}C$) on a C_{18} reversed-phase column. The difference in the retention times of the truncated sequences and the desired oligonucleotide decreases with increase in the oligonucleotide length, because as the length of the oligonucleotide increases the net hydrophobic contribution of the DMTr group to the oligonucleotide is counterbalanced by polar nature of the polyanionic chain. Hence, in oder to obtain a better resolution between the truncated sequence and the desired oligonucleotide, a reasonably hydrophobic group was required.

The limitations of the DMTr group were further demonstrated by injecting a mixture consisting of MOTr-d(T_9C), MOTr-d($T_{19}C$) and MOTr-d($T_{29}C$) (Fig. 5) under conditions identical with those used for the analysis of a mixture of DMTr-d (T_9C), DMTr-d-($T_{19}C$) and DMTr-d($T_{29}C$) (Fig. 4). As can be seen from the retention times, the proportion of acetonitrile in the mobile phase required to elute MOTr-d($T_{29}C$) is still greater than that required for DMTr-d(T_9C). Hence, under identical conditions, the retention time of an oligomer bearing a 5'-MOTr group is much higher than that for the corresponding oligomer bearing a 5'-DMTr group (see further Figs. 4 and 5). This demonstrates that, although an increase in the length of the oligonucleotide reduces the retention time, the overall retention time conferred by the longer alkyl substituent far exceeds the influence of the polyanionic chain.

The utility of the MOTr group for the purification of polynucleotides was further demonstrated by synthesizing a longer sequence, *viz.*, MOTr-d($T)_{60}$. Fig. 6

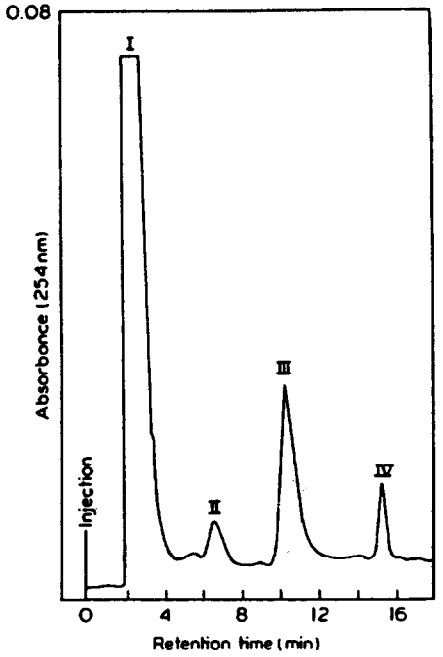


Fig. 4. HPLC profile of a mixture of DMTr-d(T_9C), DMTr-d($T_{19}C$) and DMTr-d($T_{29}C$). Column, Zorbax ODS (25 mm × 4.6 mm I.D.); flow-rate 1 ml/min; gradient from 20 to 40% B in 20 min, with solvent A = 0.1 *M* TEAA (pH 7.0) and solvent B = acetonitrile. Peaks: I = truncated sequences; II = DMTr-d ($T_{29}C$); III = DMTr-d($T_{19}C$); IV = DMTr-d(T_9C).

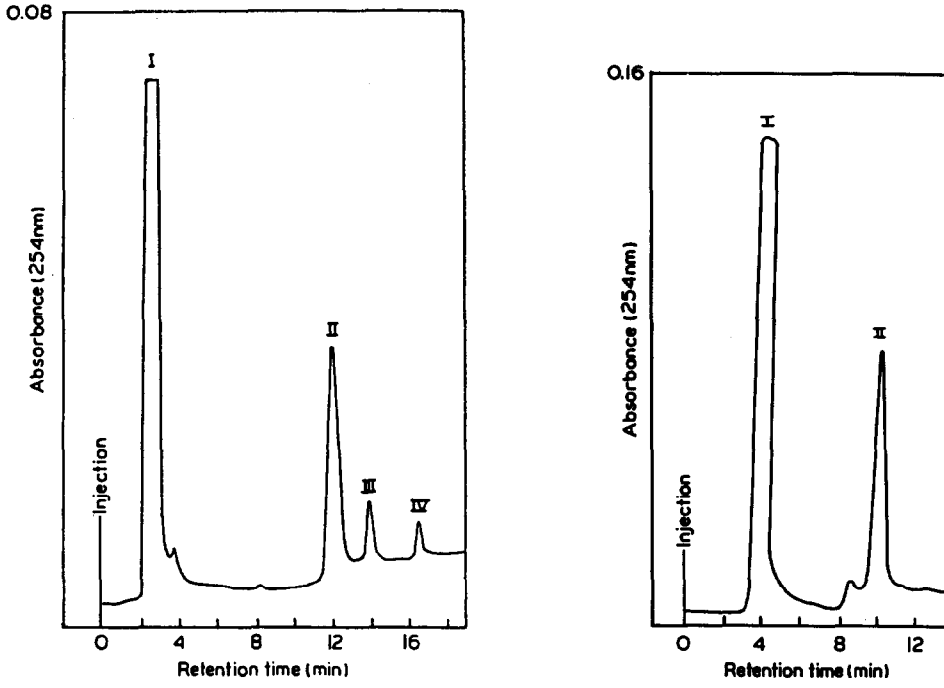


Fig. 5. HPLC profile of a mixture of MOTr-d(T_9C), MOTr-d($T_{19}C$) and MOTr-d($T_{29}C$). Conditions as in Fig. 4. Peaks: I = truncated sequences; II = MOTr-d($T_{29}C$); III = MOTr-d($T_{19}C$); IV = MOTr-d(T_9C).

Fig. 6. HPLC purification of MOTr-d$(T)_{60}$. Column, Zorbax ODS (25 mm × 4.6 mm I.D.); flow-rate, 1 ml/min; gradient from 10 to 70% B in 40 min, with solvent A = 0.1 *M* TEAA (pH 7.0) and solvent B = acetonitrile. Peaks: I = truncated sequences; II = MOTr-d$(T)_{60}$.

shows the HPLC purification profile of MOTr-d$(T)_{60}$. Even with this chain length the desired peak is well resolved from the truncated sequences. The near baseline separation and symmetry of the peak indicated the purity of the product. The product peak was collected, concentrated and reinjected under identical conditions. Again a single peak was obtained, showing the purity of the product.

These results demonstrate that the DMTr group is suitable as an affinity handle for the purification of oligonucleotides. However, the resolution of large oligonucleotides is poor. The MOTr group has been used successfully for the purification of a model polynucleotide, d$(T)_{60}$. The MOTr group is easy to introduce and, being disubstituted, can be removed under conditions identical with those used for the removal of the conventionally used DMTr group, thereby, minimizing the depurination of the HPLC-purified product.

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