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Use of the 4-methoxy-4′-octyloxytrityl group as an affinity handle for the purification of synthetic oligonucleotides[a,b]

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ABSTRACT

The use of triphenylmethyl protecting groups with long-chain alkyl substituents, *viz.*, (4-hexadecyloxyphenyl)diphenylmethyl (HTr) and (4-decyloxyphenyl)diphenylmethyl (DTr), as affinity handles in the purification of oligonucleotides results in significant depurination while removing these affinity groups from the high-performance liquid chromatographically purified oligonucleotides in 80% aqueous acetic acid. The separation and purification of solid-phase synthesized medium-to-large sized model oligonucleotides using the 4-methoxy-4′-octyloxytrityl (MOTr) group was investigated. Model studies demonstrated an excellent resolution of synthetic oligonucleotides and the MOTr group was removed under conditions identical with those used for the conventional 4,4′-dimethoxytrityl (DMTr) group, hence being less prone to depurination.

INTRODUCTION

High-performance liquid chromatography (HPLC) has become an important separation technique for the analytical and preparative separation of synthetic oligonucleotides. Different standard columns containing normal-phase silica gel [1], ion-exchange [2–6] and reversed-phase (RP) [7–15] materials have been used extensively for this purpose. During the solid-phase synthesis of oligonucleotides, a "capping" step involving acylation of the 5′-OH group is applied after each coupling then, on completion of the synthesis, if there are no other side-reactions, only the desired oligomer should carry a 5′-terminal 4,4′-dimethoxytrityl (DMTr) protecting group. The other DNA species, the "failure sequences", bear the base labile 5′-O-acetyl cap which is subsequently hydrolysed during work-up to leave a mixture of components that differ significantly in hydrophobicity from the desired product. The RP-HPLC separation takes advantages of this fact that rather than resolving oligomers differing

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in chain length, the separation involves resolving a 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 [16], but oligomers larger than 30 nucleotides in length pose problems if the DMTr group is used as an affinity handle. In order to overcome this problem, the use of a triphenylmethyl protecting group with long-chain alkyl substituents, *e.g.*, the (4-hexadecyloxyphenyl)diphenylmethyl (HTr) group, for the affinity chromatographic purification of oligonucleotides of defined sequence and particularly of solid-phase synthesized products was recommended by Seliger and co-workers [17,18]. Recently, the same group [19] observed that the HTr group causes most oligonucleotides to be retained too strongly on a C_{18} stationary phase. The HTr-bearing oligonucleotides, particularly those of very low molecular weight, arising from incomplete capping will be eluted by nearly 100% acetonitrile, *i.e.*, their quantitative elution will be difficult or require a change of solvent. In fact, the monomeric (4-hexadecyloxyphenyl)diphenylmethyl deoxythymidine (HTrdT) was not eluted at all with acetonitrile, but required carbon tetrachloride as an eluent. These limitations of the HTr group prompted Seliger and Schmidt [20] to suggest an alternative group, (4-decyloxyphenyl)diphenylmethyl (DTr), for the purification of medium-to-large sized synthetic oligonucleotides.

It has been observed that the complete deprotection of HPLC-purified oligonucleotides containing either an HTr or DTr group required a considerably longer time in 80% acetic acid, resulting in significant depurination of the HPLC-purified oligonucleotides. The complete removal of the monomethoxytrityl group [20] takes 90 min at 27°C, which is almost 4.5 times longer than that of the dimethoxytrityl group [21] in 80% acetic acid at room temperature. The use of a triphenylmethyl protecting group with 4-methoxy-4′-alkoxy substituents was investigated in this study. 4-Methoxy-4′-octyloxytrityl, with a moderate-sized alkyl substituent on the trityl group, has been found to be a better choice for the purification of medium-to-large sized oligonucleotides useful for total gene synthesis and other applications in molecular biology.

EXPERIMENTAL

Chemicals and reagents

4-Hydroxybenzophenone, 1-bromopropane, 1-bromobutane, 1-bromopentane, 1-bromooctane, 1-bromododecane, 4-dimethylaminopyridine (DMAP) and diisopropylethylamine (DIPEA) were obtained from Fluka (Buchs, Switzerland), 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite and N,N-diisopropylmethylphosphonamidic chloride were prepared using standard methods [22,23].

Pyridine was purified by distillation from ninhydrin and dried by refluxing over potassium hydroxide followed by distillation and stored over potassium hydroxide pellets under argon. Acetonitrile and dichloromethane were dried by refluxing with calcium hydride (5 g/l) for 16 h followed by distillation and stored over molecular sieves (4 Å). Tetrahydrofuran (THF) was purified by passing it through alumina and dried over lithium aluminium hydride.

Buffer for HPLC

The aqueous buffer for HPLC was 0.10 *M* triethylammonium acetate (TEAA) (pH 7.0). This was prepared by dilution from a 2 *M* stock solution which was ob-

tained in the following manner: 557 ml (4 mol) of triethylamine distilled from ninhydrin were slowly added with stirring to an aqueous solution containing 229 ml (4 mol) of glacial acetic acid at 4°C. When the addition was completed, the solution was diluted to 2 l and the pH adjusted to 7.0 by the addition of acetic acid or triethylamine as needed. The water used was obtained from a Milli-Q water purification system (Millipore). The organic component of the mobile phase was acetonitrile (Spectrochem, Bombay, India).

Apparatus

Thin-layer chromatography (TLC) was performed on silica gel 60 F_{254} plates (Merck, Darmstadt, Germany) with solvents as indicated and compounds were detected under short-wavelength UV light. Proton NMR spectra were recorded on a Perkin-Elmer R-32 spectrometer operating at 90 MHz and a Hitachi FT-60B spectrometer operating at 60 MHz. Melting points were recorded on a Tropical capillary melting-point apparatus and are uncorrected.

HPLC was performed on a Shimadzu LC-4A instrument equiped with a Shimadzu SPD-2AS variable-wavelength UV detector set 254 nm. Analytical HPLC was performed on a Zorbax ODS column (250 × 4.6 mm I.D.), particle size 10 μm (DuPont) and a μBondapack C_{18} column (300 × 3.9 mm I.D.), particle size 5 μm (Waters Assoc.).

Synthesis of 5′-O-(4-methoxy-4′-alkoxytrityl)deoxynucleosides ***3a–e***

4-Methoxy-4′-alkoxytrityl groups were introduced selectively into the 5′-position of base-protected nucleosides by a published procedure [18] with some modifications. The reaction schemes are depicted in Fig. 1. The purity of the intermediates were confirmed by TLC and NMR and IR spectroscopy. 4-Methoxy-4′-octyloxytrityldeoxythymidine was converted into the 4-methoxy-4′-octyloxytrityldeoxythymidine 3′-O-(2-cyanoethyl-N,N-diisopropylamino)phosphoramidites by the method described by Sinha *et al.* [23].

Oligodeoxynucleotide synthesis

Oligonucleotides were synthesized following the standard protocol using solid-phase phosphoramidite chemistry [24] on a Pharmacia–LKB Gene Assembler Plus using methyl or 2-cyanoethyl phosphoramidites. The synthesis was carried out on 1.3 μmol of support-bound first nucleoside. In the last coupling step, a 5′-DMTr or MOTr group containing nucleoside phosphoramidites was used and no change in the coupling time or the solvent was made. After the synthesis of the required sequence, the DMTr or MOTr group was kept intact. The internucleotide phosphate protecting groups were removed following the standard protocol [24]. Removal of the exocyclic base protecting groups and cleavage of the oligonucleotide from the support were achieved with 25% aqueous ammonia at 55°C for 16 h. The crude oligonucleotides with a DMTr or MOTr group at the 5′-terminus were then purified by RP-HPLC.

HPLC purification of oligonucleotides using the DMTr or MOTr group as an affinity handle

The crude oligonucleotide obtained as above was dissolved in 0.1 *M* TEAA buffer (pH 7.0) and desalted on a Bio-Gel P2 column using the same buffer as eluent.

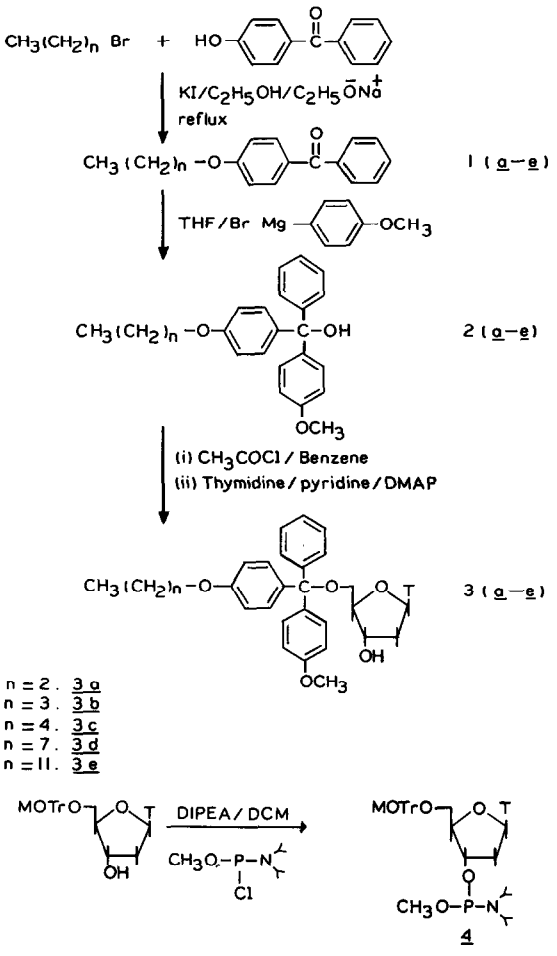

Fig. 1. Scheme for the synthesis of 5′-O-(4-methoxy-4′-alkoxytrityl)thymidine and 5′-O-(4-methoxy-4′-octyloxytrityl)thymidine 3′-O-(methyl N,N-diisopropylamino)phosphoramidite. DCM = dichloromethane.

The desalted product was collected and concentrated under vacuum and the residue was dissolved in 0.1 *M* TEAA buffer (pH 7.0) (500 μl) and then applied to a μBondapak C_{18} or Zorbax C_{18} column. The peak containing the desired material was collected.

Hydrolysis kinetics of 4-methoxy-4′-alkoxytrityl groups from nucleosides ***3a–f***

The kinetics of the hydrolysis of 4-methoxy-4′-alkoxytrityl group in compounds **3a–f** in 80% acetic acid was studied in the following manner: a solution of compounds **3a–f** in 1 ml of 80% acetic acid was kept at room temperature with occasional swirling. Aliquots were removed every 1 min and applied to a TLC plate. The TLC plate was developed in chloroform–methanol (9:1) and the compounds were revealed under UV light or by spraying with perchloric acid solution followed by heating at 100°C in an oven.

RESULTS AND DISCUSSION

We have attempted to investigate the use of the 4-methoxy-4′-alkoxytrityl group as an aid in the separation of solid-phase synthesized oligonucleotides. Two important considerations guided our approach: the first is that the group chosen can be introduced and removed from the 5′-position of nucleosides in a similar manner to the DMTr group, and the second is that the group selected should be moderately hydrophobic and allow the purification of medium-to-large sized oligonucleotides used for gene synthesis.

In order to satisfy these criteria, we selectively introduced 4-methoxy-4′-alkoxytrityl groups at the 5′-position of thymidine by known methods [18] with some modifications in 85–92% yields (Fig. 1).

In order to understand the effect of alkyl-chain substituents in the trityl function, compounds **3a–f** were simultaneously injected onto a C_{18} column. The elution profile is shown in Fig. 2. It is evident that the retention times of these thymidine derivatives increase with increase in the carbon chain length of the alkyl function. Hence the increase in carbon chain length of the alkyl substituents increases the overall hydrophobicity of the substituted trityl groups. On the basis of these results, it was concluded that the MOTr group could be a useful choice as an affinity handle for the purification of oligonucleotides. A very short alkyl substituent, *viz.*, butyl or hexyl, in the trityl function may not impart a sufficient hydrophobicity to this group to make it effective for the separation of medium-sized oligonucleotides and a very long-chain alkyl substituent in the trityl function would make it too hydrophobic and hence short nucleotides, *viz.*, the monomer or dimer resulting from the incomplete

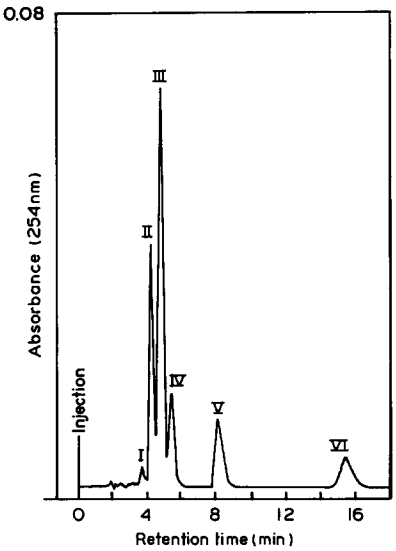

Fig. 2. HPLC profile of a mixture of compounds **3a–f** on a μBondapak C_{18} column. Eluent, THF–water–methanol (3:2:2); flow-rate, 1 ml/min; detection, 254 nm 0.08 a.u.f.s. Peaks: I = **3f**; II = **3a**; III = **3b**; IV = **3c**; V = **3d**; VI = **3e**.

capping, would be retained by the C_{18} column for a longer time or may not be eluted at all from the column completely. This has already been experienced with the HTr group [19].

The hydrolysis of 4-methoxy-4′-alkoxytrityl groups in 80% acetic acid acid was found to be complete in 15 min. Hence the MOTr group can be removed from the purified oligonucleotides under conditions identical with those used for the DMTr group. The reason for using a disubstituted trityl function (MOTr) was mainly its lability in 80% acetic acid. With a monosubstituted trityl function (DTr or HTr) the time required for the removal of these protecting groups from the HPLC-purified oligonucleotides was found to be considerable, which may cause significant depurination of the purified oligonucleotides. The MOTr group was therefore selected for further study as an affinity handle for the purification of solid-phase synthesized oligonucleotides.

Comparison of chromatographic behaviour of model DMTr-oligonucleotides of different chain length with model MOTr-oligonucleotides under identical conditions

We further extended our study 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 injection of 5′-DMTr- and 5′-MOTr-oligonucleotides having chain lengths of 10, 20 and 30 bases. It can be seen that the gradient time, the concentration of the acetonitrile required to elute MOTr $d(T_{29}\ C)$ is almost double that required for DMTr $d(T_{29}\ C)$. It is clear that the difference in retention time of an oligomer bearing a 5′-MOTr is much larger than that of a similar oligomer bearing a 5′-DMTr group. This demonstrates that, although an increase in the length of the oligonucleotide reduces the retention time, the overall retention conferred by the longer alkyl substituent far exceeds the influence of the polyanionic chain.

Encouraged by the results shown in the Fig. 3, we synthesized a longer sequence, *viz.*, MOTr $d(T)_{60}$, to demonstrate the utility of the MOTr group for the purification of long-chain polynucleotides. Fig. 4 shows the HPLC purification profile of MOTr $d(T)_{60}$. Even at this chain length, the peak corresponding to the desired material is well resolved from the truncated sequences. The desired material at 10.2 min was collected, concentrated and re-injected under identical conditions. Again a single peak with the same retention time was obtained.

CONCLUSION

RP-HPLC has become an important technique for the purification of solid-phase synthesized oligonucleotides. In this work, a novel affinity handle, 4-methoxy-4′-octyloxytrityl (MOTr) was applied to the separation and purification of medium-to-large sized oligonucleotides useful for routine molecular biology and total gene synthesis. The main advantage of this group is that it can be removed from the HPLC-purified oligonucleotides under conditions identical with those used for the DMTr group, hence minimizing depurination. The utility of this group was demonstrated by purifying a reasonable-sized oligonucleotide, MOTr $d(T)_{60}$.

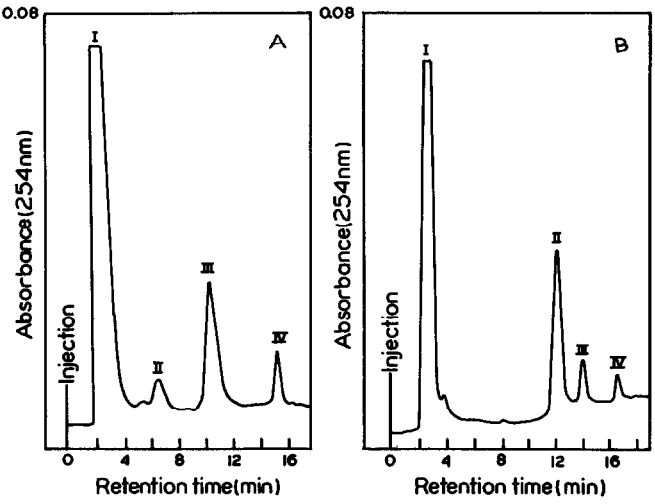

Fig. 3. (a) HPLC profile model DMTr-oligonucleotides with different chain lengths on a Zorbax ODS column (250 × 4.6 mm I.D.); Solvent A, 0.1 *M* TEAA buffer (pH 7.0); solvent B, 100% acetonitrile; gradient from 20 to 40% B in 20 min; flow-rate, 1 ml/min; detection, 254 nm (0.08 a.u.f.s.). Peaks: I = bulk of the non-tritylated truncated sequences; II = DMTr d(T_{29} C); III = DMTr d(T_{19} C); IV = DMTr d(T_9 C). (b) HPLC profile of model MOTr-oligonucleotides with different chain lengths on a Zorbax ODS column (250 × 4.6 mm I.D.). Conditions as in (a). Peaks: I = bulk of the non-tritylated truncated sequences; II = MOTr d(T_{29} C); III = MOTr d(T_{19} C); IV = MOTr d(T_9 C).

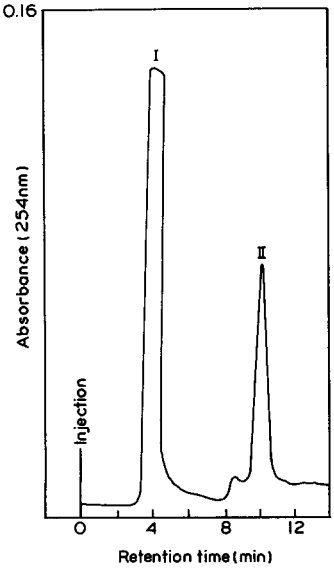

Fig. 4. HPLC purification of MOTr d$(T)_{60}$ on a Zorbax ODS column (250 × 4.6 mm I.D.). Solvent A, 0.1 *M* TEAA buffer (pH 7.0); solvent B, 100% acetonitrile; gradient from 10 to 70% B in 40 min; flow-rate, 1 ml/min; detection, 254 nm 0.16 a.u.f.s. Peaks: I = bulk of the non-tritylated sequences; II = MOTr d$(T)_{60}$.

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