Tetrahedron Letters, Vol. 34, No. 44, pp. 7133-7136, 1993
Printed in Great Britain

0040-4039/93 $6.00 + .00
Pergamon Press Ltd

4-(17-Tetrabenzo[*a,c,g,i*]fluorenylmethyl)-4',4''-Dimethoxytrityl Chloride: A Hydrophobic 5'-Protecting Group for the Separation of Synthetic Oligonucleotides.

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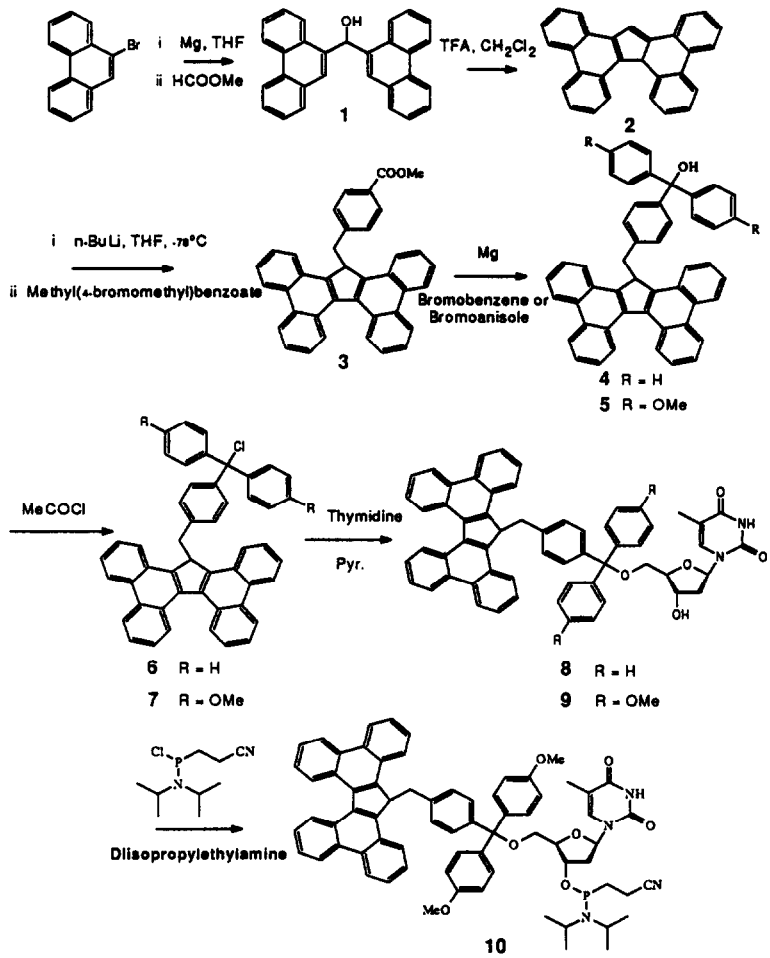
Keywords: RP-HPLC; 4-(17-Tetrabenzo[a,c,g,i]fluorenylmethyl)-4',4''-Dimethoxytrityl Chloride; Tbf-DMTr; DMTr; Protecting group; Oligonucleotide Synthesis.

Abstract: A new highly hydrophobic 5'-hydroxyl protecting group (Tbf-DMTr) has been designed for the purification of synthetic oligonucleotides. Tbf-DMTr-oligonucleotides are strongly retained on RP-HPLC allowing a facile separation from truncated sequences. Subsequently the group can be removed in acidic conditions. The ultra-violet properties of Tbf-DMTr enable easy detection. The synthesis and purification of long oligonucleotides (> 100 mer) is envisaged.

With the development of automated DNA synthesis, oligonucleotides can be synthesised efficiently and in a short period of time. However, syntheses of DNA sequences in excess of 20 nucleotides in length, which involve over 100 chemical steps, produce a large amount of impurities which complicate the purification. Usually the identification of the product is achieved by leaving the 5'-DMTr (di-p-anisylphenylmethyl) group on the final monomer so that the product can be resolved from non 5'-DMTr terminated sequences on RP-HPLC.[1] Unfortunately the change in the overall polarity by the introduction of the hydrophobic DMTr moiety is greatly reduced for longer oligonucleotides. Seliger and Goertz[2] have modified the DMTr group developed by Khorana *et al.*[3] by introducing *para*-alkyloxy groups and thus improving the separation by HPLC. Letsinger and Finan[4] have substituted one phenyl ring by a naphthyl group but this protecting group requires strong acid conditions for its removal. Later, Fourrey *et al.*[5] introduced the fluorescent pyrenyl group. However in both cases only the protected phosphoramidite monomer of thymidine has been successfully prepared. Previous work demonstrated that peptidic derivatives of tetrabenzo[*a,c,g,i*]fluorene (Tbf) are strongly retained on RP-HPLC columns.[6] Therefore we modified the DMTr group by substituting position 4 of one of the phenyl ring with tetrabenzo[*a,c,g,i*]fluorenylmethyl, which still preserves the regioselectivity of the trityl group for primary hydroxyls while forming an extremely hydrophobic 5'-protecting group. Moreover the Tbf moiety shows characteristic UV maxima at 365 and 380 nm which is most useful in monitoring the HPLC purification of oligonucleotides. Our strategy consisted of introducing 4-(17-tetrabenzo[*a,c,g,i*]fluorenylmethyl)-4',4''-dimethoxytrityl chloride (Tbf-DMTr-Cl) as a new hydrophobic protecting group on the 5'-end of long oligonucleotides and exploiting its properties for their purification.

The synthesis of Tbf-DMTr-T-phosphoramidite **10** is shown in the Scheme. Tetrabenzo[*a,c,g,i*]fluorene **2** was achieved[7] from 9-bromophenanthrene in a simpler manner than the one described by De Ridder and Martin.[8] An alkylation reaction with methyl-4-bromomethylbenzoate gave the methyl ester **3**. The carbinol compounds **4** and **5** were obtained by a Grignard reaction involving bromobenzene or bromoanisole respectively. The orange coloured **6** and **7** were obtained by treatment of **4** and **5** respectively with acetyl chloride. Tbf-DMTr-Cl **7** and Tbf-Tr-Cl **6** were reacted with thymidine in pyridine to yield the 5'-protected nucleosides **8** and **9** in 40%. A kinetic study of the deprotection of **9** and 5'-DMTr-O-Thymidine was undertaken by monitoring the absorbance of the resulting trityl carbocation at 500 nm against time. The results were treated as a pseudo-first order irreversible reaction and an apparent rate constant of 0.0018 s^{-1} was found for 5'-DMTr-O-Thymidine and 0.0037 s^{-1} for **9**.

Due to the donating inductive effect of tetrabenzo[*a,c,g,i*]fluorenylmethyl, Tbf-DMTr is twice as acid labile as the DMTr. 5'-Tbf-DMTr-thymidine was converted to the cyanoethylphosphoramidite **10** by the method of McBride and Caruthers.[9] The synthesis of oligonucleotide was performed by the phosphite-triester approach using **10** for the last coupling reaction, thus only the desired sequence bore the protecting group having a λ max. at 365 and 380 nm. Due to steric factors, coupling yields between 70 and 88% were obtained compared to >98% obtained for DMTr-protected nucleotides.



Because phosphoramidite monomers have a lower coupling efficiency we synthesised a series of oligonucleotides in the trityl-off mode and protected the terminal 5'-hydroxyl with DMTr-Cl and Tbf-DMTr-Cl while still bound to the solid support. We found that a 0.4 M solution of DMTr-Cl or Tbf-DMTr-Cl in pyridine reacted completely with the 5'-hydroxyl of an oligonucleotide attached to the support. We did not observe any significant change in the HPLC of the product when compared to oligonucleotides synthesised by the trityl-on mode. This method presents the advantage of using Tbf-DMTr-Cl as a single reagent for A, C, T or G-terminated oligonucleotides. It can be injected manually into the column as a pyridine solution or automatically

by the synthesizer without altering the synthesis cycle. Figure 1 shows the RP-HPLC profile of a crude 12-mer oligonucleotide (TTC-GAG-CCA-TAT) at 280 nm bearing DMTr at the 5'-terminus and Figure 2 shows the same crude oligonucleotide bearing a 5'-Tbf-DMTr terminus. The difference in retention time is consistent with the hydrophobicity order of the two protecting groups and the product peak is well separated from the truncated sequences. Using current methodology for the synthesis of short sequences a single peak is usually obtained but for longer sequences a broad product peak or a number of less defined peaks is observed especially if the capping steps in the synthesis cycle have not been efficient or if the product has several stable conformations.

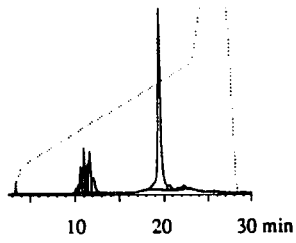


Figure 1

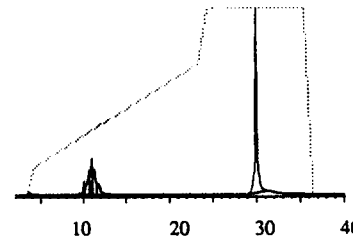


Figure 2

In order to prove the usefulness of this protecting group, we synthesised various DNA sequences of different lengths and terminal nucleotide. We found that the retention of Tbf-DMTr-oligos was so strong that isocratic conditions were sufficient to purify oligonucleotides on RP-C18 SEP-PAK®cartridges. Similar results were obtained with polystyrene cartridges the advantage of which is that the ammonia solution of the crude oligonucleotide can be loaded directly without damaging the stationary phase. Failure sequences are eluted with 10% acetonitrile/90% 0.1 M ammonium acetate, then Tbf-DMTr- is removed with 2% TFA and the product is finally eluted as a single peak with 30% acetonitrile/70% 0.1 M ammonium acetate. This simple procedure can be accomplished within 15 min thus avoiding the time consuming HPLC gradient elution. Further we tested the usefulness of Tbf-DMTr-Cl in the synthesis and purification of longer oligonucleotides. We first synthesised the following 51-mer : 5'-GAT-CTG-TTG-ACA-ATT-AAT-CAT-CGG-CTC-GTA-TAA-TGT-GTG-GAA-TTG-GTC-GAC-3'. Figure 3 shows the HPLC profile of the crude 51-mer at 280 nm (Solvent A: 0.1M ammonium acetate; Solvent B: 50% acetonitrile) and Figure 4 shows the HPLC profile of the appropriate uv fractions (Solvent A: 0.1M ammonium acetate; Solvent B: 20% acetonitrile) after deprotection in 80% acetic acid and desalting over Sephadex®G-25 column. Capillary gel electrophoresis (Figure 5) indicates clearly that the by-product is a much shorter sequence resulting from an incomplete capping step. Figure 6 shows the product as a single peak on capillary gel electrophoresis after preparative HPLC purification.

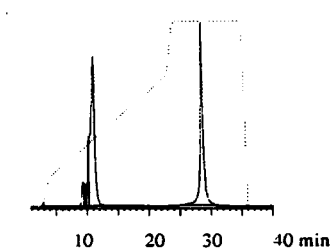


Figure 3

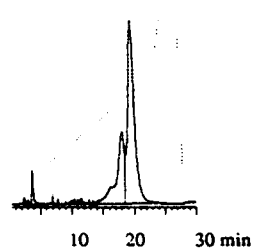


Figure 4

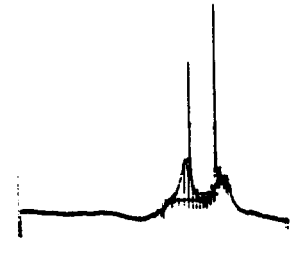


Figure 5

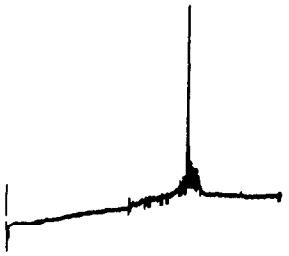


Figure 6

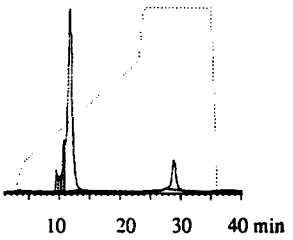


Figure 7

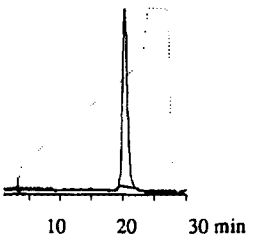


Figure 8

The following 102-mer was synthesised and purified in the same manner: 5'-GAT-CTG-TTG-ACA-ATT-AAT-CAT-CGG-CTC-GTA-TAA-TGT-GTG-GAA-TTG-GTC-GAC-GAT-CTG-TTG-ACA-ATT-AAT-CAT-CGG-CTC-GTA-TAA-TGT-ATG-GAA-TTG-GTC-GAC-3'. Figure 7 shows the RP-HPLC profile of the crude at 280 nm (Solvent A: 0.1M ammonium acetate; Solvent B: 50% acetonitrile). After purification by RP-HPLC (Solvent A: 0.1M ammonium acetate; Solvent B: 20% acetonitrile) according to our methodology, the product was obtained as a single peak; Figure 8.

In summary, we have shown that Tbf-DMTr-Cl can be used to introduce the Tbf-DMTr group directly onto the support-bound oligonucleotide, without lowering the synthesis yield. The removal of this new protecting group in acid is twice as fast as DMTr and its ultra-violet properties enable easy detection at DNA non-damaging wavelengths. Due to the high hydrophobicity of Tbf-DMTr, impurities can be separated by isocratic conditions on small RP-C18 silica gel or polystyrene cartridges. Furthermore, the purification of long oligonucleotides > 100 units is easily achieved by RP-HPLC. The trityl analogue 4 is currently under study for applications in carbohydrate synthesis.

Acknowledgements

We would like to thank Dr. T. Brown and Dr. D. Brown for their helpful advice during this work, and Raylo Chemicals Ltd for their financial support.

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(Received in UK 5 August 1993; accepted 3 September 1993)