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1,1-BIS-(4-METHOXYPHENYL)-1'-PYRENYL METHYL (bmpm): A NEW FLUORESCENT 5' PROTECTING GROUP FOR THE PURIFICATION OF UNMODIFIED AND MODIFIED OLIGONUCLEOTIDES

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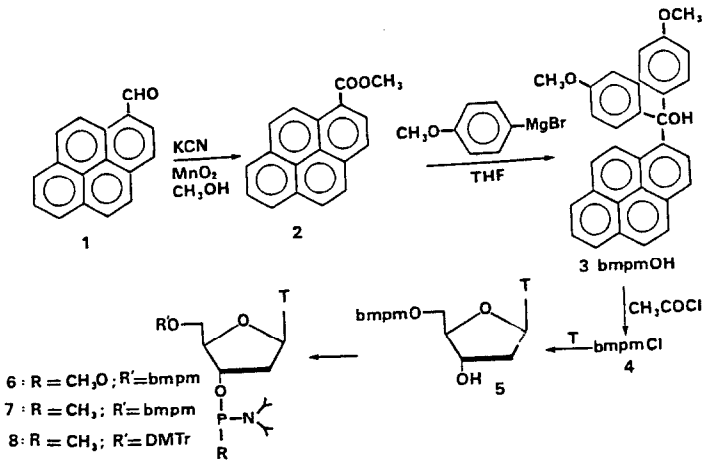
Summary : By replacing the phenyl by a pyrenyl in the dimethoxytrityl (DMTr) group commonly used for 5'-protection in oligonucleotide synthesis, we have obtained a fluorescent acid-labile protecting group which exhibits similar chemical properties to those of DMTr. Here we demonstrate the usefulness of the new protecting group in the purification of both charged and neutral DNA fragments.

The cyclic process leading to a synthetic oligonucleotide normally involves an acid treatment to remove a 5'-protecting group at the beginning of each cycle. The acid labile group most often used is the di-p-anisylphenylmethyl (trivially called dimethoxytrityl, DMTr) developed by Khorana *et al.(1)*. Modifications of this group are very easily achieved but this fact has not been exploited to a large extent. The most notable exceptions are due to Seliger (2) who introduced long chain 4-alkyloxy groups to facilitate HPLC purification and to Caruthers (3) who synthesized a large variety of triarylmethyl groups giving different colours in acid solution. Letsinger and Finan (4) reported a 1-naphthyldiphenylmethyl group, but this requires strong acid treatment for its removal. However, to our knowledge, a modification leading to a highly-fluorescent 5'-protecting group easily removable under mild acid conditions has not been made use of for oligonucleotide synthesis. We chose to substitute a pyrenyl group for the phenyl in DMTr because using its respective excitation and emission wavelengths of 346 and 390nm for detection purposes would enable us to avoid the DNA-damaging wavelengths of 254-260nm currently used.

By 5'-addition of the group to a complete oligomer we would achieve two objectives. Firstly, we would have an alternative detection method to the 254nm UV shadowing procedure routinely used to localize the desired synthetic product on a polyacrylamide gel. Secondly, we would be able to identify easily, and unambiguously, the desired product in a complex mixture resulting from a new, perhaps inefficient synthetic procedure.



The synthesis of 1,1 - bis - (4-methoxyphenyl)-1'-pyrenyl-methyl-chloride 4 and its reaction with thymidine is shown in the Scheme. The aldehyde 1 was converted to the methyl ester 2 in quantitative yield (5). A standard Grignard reaction gave the carbinol 3 in excellent yield (6). The deep purple coloured chloride 4 was obtained by treatment of 3 with acetyl chloride (7). Reaction of 4 with thymidine in pyridine gave the 5'-protected nucleoside 5 (8). The stability towards 80% aqueous acetic acid of bmpmT 5 and DMTrT was compared by tlc analysis. It was estimated that at least 90% deprotection was achieved in 10-20 min for DMTrT and in 30-60 min for bmpmT(9). We could detect quantities of bmpmT down to 10^{-10}M on silica gel tlc plates at 360nm. bmpmT was converted to the methoxy diisopropylphosphoramidite 6 (10) by the method of McBride and Caruthers (11). The same method was adapted to obtain the methyl phosphonamidite derivatives of bmpmT 7 and of DMTrT 8. The products were purified by silica gel column chromatography (10).

Two series of oligomers, using either commercial cyanoethyl phosphoramidites or the methyl phosphonamidite 8, were synthesized by normal automated solid phase techniques using tetrazole as activator (12). An acetylation was used after each coupling in order to block all the 5' -hydroxyls which had failed to react. For the final coupling reaction, the 5' -bmpm protected product 6 or 7 was added via a manual injection port. Thus only the desired, complete sequence bore the fluorescent group. Appropriate base treatment, concentrated ammonia solution for the oligonucleotides and ethylenediamine/ethanol for the oligonucleoside methylphosphonates (13), deprotected the oligomers and cleaved them from the solid support.

Fig. 1A shows the reverse phase HPLC profile (14) of a 5'-bmpm 30-base charged oligodeoxynucleotide using a double detection method with, in

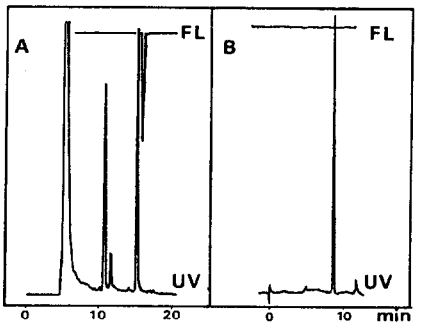


Fig.1. HPLC of bmpm 30-mer

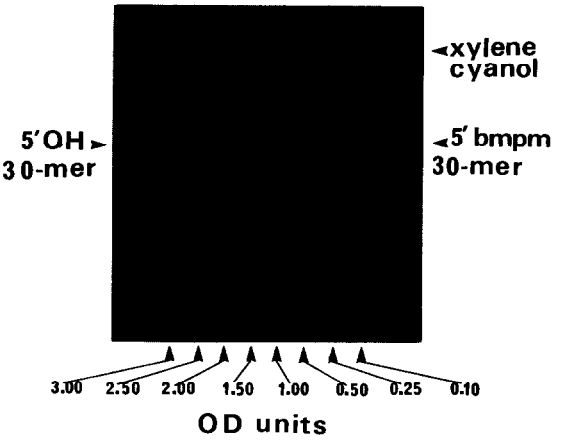


Fig.2. PAGE of bmpm 30-mer

series, a spectrophotometer at 260nm and a spectrofluorimeter with excitation and emission wavelengths of 340 and 390nm. As observed with DMTr-protected oligomers, the bmpm-protected oligomer is well separated from non-fluorescent failure sequences. After deprotection with 80% acetic acid, the product was repurified on the same column (Fig.1B). The 5'-bmpm 30mer was also purified by 8M-urea polyacrylamide gel electrophoresis. The product resulting from a deposit of 0.5 OD units of crude product is clearly visible by fluorescence on a wet gel at 360nm and, because of the retarding effect of the bmpm group, it is well separated from n-1 and other failure sequences (Fig.2).

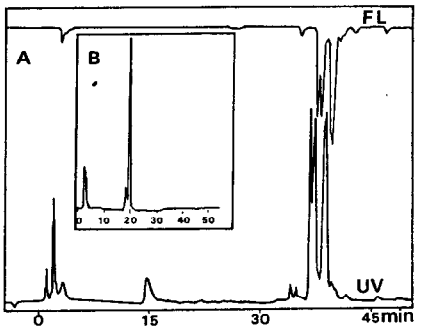


Fig.3. HPLC of bmpmT(pT)$_3$
Me

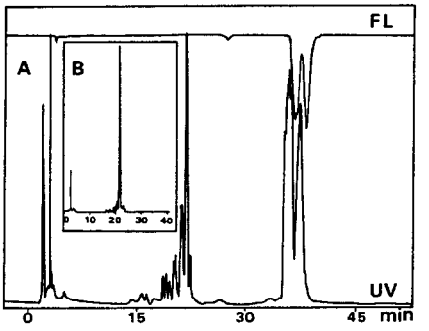


Fig.4. HPLC of bmpmT(pT)$_5$
Me

Fig.3A shows the reverse phase HPLC profile of the oligothymidyl methylphosphonate bmpmT(pT)$_3$ which, because of the presence of two diastereoisomers at each P atom, is a mixture of 2^3=8 diastereomers. In this case, we observe a complex mixture of UV absorbing and fluorescent peaks, indicating that the different diastereomers are retained to differing extents on the column. HPLC analysis of longer sequences shows either a broad pro-

duct peak or a number of more or less well defined peaks as seen for bmpmT $(pT)_5$ (Fig.4A). The fluorescence clearly indicates that the whole peak complex represents products of the correct length, which would not have been evident from the use of the DMTr group. After collection of all the product peaks and removal of the bmpm group, HPLC analysis now shows only one relatively sharp peak (Figs.3B, 4B). Confirmation of the correct structure of these and other oligonucleoside methylphosphonates has been obtained by mass spectroscopy (12) and from thermal stability studies (manuscript in preparation).

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6) The carbinol 3 was prepared by a standard Grignard procedure from magnesium 4-methoxyphenyl bromide and 2, and purified by silica gel chromatography, m.p. 111-115°C, m/z 444($M^{\mp}$); 427 (M-17), H8.5-6.5 (m. 17H,ar); 3.72 (s, 6H,OMe), UV: λ max nm(e x 10^{-4}) (EtOH 95%) 254(6), 268(2.28), 278(3.66), 346(4.98).
7) Acetyl chloride (2ml) was added to a suspension of 3 (1.52g) in cyclohexane (10ml) and the mixture was heated to reflux. Within 10min a clear solution was obtained. Upon cooling, a deep purple precipitate formed which was filtered and washed to give 4 (1.21g, 76%), m.p. 162-164°C.
8) A dry pyridine solution (5ml) of thymidine (240mg, 1mmole) was cooled to 0°C and 4 (500mg, 1.1mmole) was added. After 1hr agitation the solution was concentrated by evaporation and diluted with dichloromethane. The solution was washed with 5% aqueous $NaHCO_3$ solution then saturated NaCl solution before being dried and evaporated. The residue was purified by silica gel chromatography using CH_2Cl_2/MeOH 9/1 as eluant and the product 5 isolated as a foam (448mg, 0.67mmole, 67%). H8.5-6.6 (m, 18H, ar.+H-6), 6.35(t, 1H, H-1'), 4.55(m, 1H, H-3'), 3.97(m, 1H, H-4'), 3.70 (s, 6H, OMe), 3.45(m, 2H, H-5'), 2.57(m, 2H, H-3'), 1.40(s, 3H, Me). UV λ max nm (e x 10^{-4})(EtOH 95%) 245(4.0), 267(2.0), 278(2.7), 331(1.9), 331(1.9), 348(2.6). Fluorescence (EtOH 95%) excitation 346nm, emission maxima at 376, 386 and 396nm. Chemical ionisation ms (positive) m/z = 427 (bmpm), 243(M-bmpm+2H), 127(T+2H). Anal.Calcd. for $C_{41}H_{36}N_2O_7$ C73.64 H5.42 N4.19%; found C73.64 H5.55 N4.13%.
9) The bmpm analogue bearing a single anisyl group was also synthesized (not shown) and 90% deprotection was achieved in 180-360min; this group could be useful in those cases where greater stability is desired.
10) Compounds 6,7 and 8 were synthesized by the method of McBride and Caruthers (11), with bmpm replacing DMTr and chloro-(diisopropylamino)-methylphosphine replacing the analogous methoxyphosphine where appropriate. The compounds were purified by silica gel column chromatography using ethyl acetate/CH_2Cl_2/triethylamine 45/45/10 as eluant. ^{31}p NMR (external 85% H_3PO_4)δ = 6 149.33, 149.01, 7 121.57, 120.76, 8 120.8, 120.4.
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14) Lichrosorb RP-18 250 x 4mm. For bmpm TCCCTCTTGAAGGAAACCAGCCAGTGCCAC linear gradient 0.1M ammonium acetate to 100% acetonitrile in 20min. at 1.5ml/min. For the methylphosphonates H_2O to 100% acetonitrile in 60min.

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