Chemica Scripta. Vol. 19, pp. 235–237, 1982.

Letter to Chemica Scripta

Flurene-9-methyl-, a Phosphate Protecting Group: Its Application in the Phosphotriester Approach Through the Synthesis of Tetracosathymidylic Acid

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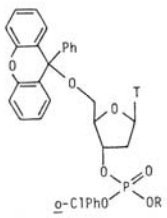
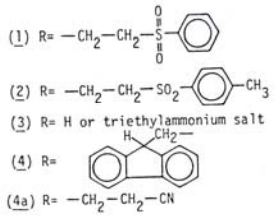
Received April 1, 1982; accepted April 20, 1982

Abstract

The fluorene-9-methyl-(FM) group has been employed for the first time, in the phosphotriester approach, to protect the phosphodiester at the triester level; the FM-group may be conveniently deprotected to generate the diester by treatment for 2 h at 20°C with 10 equiv. of Et_3N. Finally the FM group has been employed in the synthesis of twentyfour unit long polythymidylic acid residue to demonstrate its usefulness as a protecting group in nucleic acid chemistry.

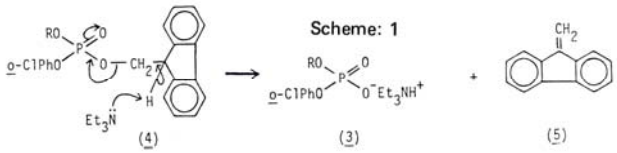
One of the major problems of the chemical synthesis of larger blocks of oligonucleotides by block condensation, through the Catlin–Crammer strategy [1], is centered on being able to protect the 3′-phosphodiester function successfully in the form of a neutral phosphotriester moiety. This 3′-terminal phosphodiester protecting group should be such as that (1) it is stable under normal conditions of manipulation such as saturated $NaHCO_3$ work-up, purification through silica gel column chromatography etc. and thus should be obtainable in high yields and purity; (2) ideally, it should be able to enhance the





lipophilicity of the fully protected oligonucleotide block; (3) it should also be completely removable under mild deprotection conditions to generate the desired phosphodiester without any concommitant removal of any other protecting groups on the molecule; (4) preferably, the generation of the phosphodiester should be performed under a homogeneous reaction condition, which should not contribute to the oxidation or reduction process of the sensitive purine and pyrimidine bases, to facilitate high recovery of the pure product. Most of these conditions are relatively easy to fulfil for shorter oligonucleotides [2]; however, the poorer solubility of fully protected larger oligonucleotide blocks with 3′-terminal phosphotriester (12–14 unit onwards), its purification through standard silica gel column chromatography with high recovery yields and its incomplete conversion to the diester salt pose major obstacles in the synthesis of polymeric units by block condensation reaction. In attempts to find a solution to this problem, we have earlier proposed the 2-phenylsulfonylethyl-(PSE) group (as in 1) [3] as a 3′-terminal phosphodiester protecting group and have demonstrated its usefulness in the synthesis of several biologically important fragments on DNA [4, 5]. However, the introduction of alkyl chain on the phenyl ring of the PSE-group, to

Scheme: 1



render the fully protected DNA segments more lipophilic, made the substituted PSE-group too stable. Thus, the complete removal of 4-tolylsulfonylethyl-group from (2) with Et_3N (2 equiv.) at 20°C required at least 19 h to generate (3) and we realized from our past experience that the larger fully protected DNA units would almost certainly require longer reaction period and perhaps it would be difficult to drive the deprotection to the phosphodiester to completion. We, therefore, turned our attention to the possibility of employing other categories of base induced β-eliminating phosphate protecting groups. Katagiri *et al.* [6] have earlier reported the use of fluorene-9-methyl-(FM) as a 5′-phosphomonoester protecting group in the diester approach and synthesized di- and trinucleotide blocks in 35% yield. These workers removed the FM-group either by treatment of 0.1 M NaOH at 0°C for 10 min or by the treatment of conc. NH_4OH (28–30%) at 50°C for 2 h. Clearly none of the above removal conditions for the FM-group would be acceptable in the phosphotriester strategy [7] with *o*-chlorophenyl protecting groups at the internucleotide junctions [8]. It was, therefore, imperative that if the FM-group were to be employed as a phosphate protecting group in the triester approach, then one should devise milder, non-nucleophilic deprotection conditions for its removal from the triester (4) and we thought that this might be possible since, in the scheme 1, the phosphodiester should be considered as a better leaving group than the doubly charged phosphomonoester in the diester approach [6]. That this is indeed the case is shown by the complete removal of the FM-group from (4) to generate (3) using 10 equiv. of Et_3N in dry pyridine solution (10 ml/mmol) for 2 h at 20° C. After concentration of the reaction mixture *in vacuo*, the residue was dissolved in $CHCl_3$ (*ca.* 1 ml) and precipitated from a mixture of diethylether-light petroleum (30–50°C), 3:1 v/v to obtain a pure specimen of (2), free of the olefin (5), in a quantitative yield. It is appropriate to

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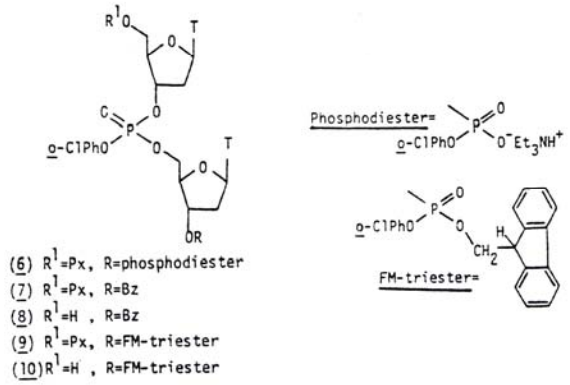
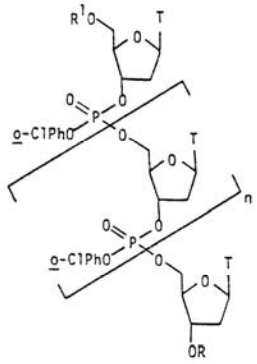
mention here that the removal of 2-cyanoethyl-group from (4a) to generate (3) requires at least 3 h treatment with 15 equiv. of Et_3N at 20°C. This result has prompted us to investigate the application of the FM-group by a synthesis of a twentyfour unit long polythymidylic acid by the block condensation of two appropriately protected dodecamers (17) and (19), and report our study on the properties of the FM-group and to which extent it satisfies the requirements of 3′-terminal phosphodiester protecting group in the triester approach [7].

Thus, the pure fully protected monomer (4) [9] and dimer blocks (9) [9], with 3′-O-*o*-chlorophenyl-9-fluorenylmethyl (FM) phosphate and 5′-O-9-phenylxanthen-9-yl (pixyl) groups [5, 10] were obtained in 79.0 and 87.0% yields (powder) following standard literature condition as reported by us previously [4, 5]. The 5′-hydroxy dimer block (10) and 5′-protected dimer phosphodiester block (6) were obtained in quantitative yields; they were prepared respectively by the deprotection of the pixyl group with 4-toluenesulfonic acid. H_2O in 2% ethanol-



(6) R^1=Px, R=phosphodiester
(7) R^1=Px, R=Bz
(8) R^1=H , R=Bz
(9) R^1=Px, R=FM-triester
(10) R^1=H , R=FM-triester

$CHCl_3$ for 90 s at 20°C [5] and by the action of Et_3N (10 equiv.) in dry pyridine (10 ml/mmol) solution for 2 h at 20°C followed by evaporation *in vacuo* and precipitation of the chloroform solution of the residue from a mixture of diethylether-light petroleum. The fully protected tetramer (13) was then obtained (72.0% yields) in the usual way by reacting a slight excess of (6) with (10) in pyridine solution in presence of 1-mesitylenesulfonyl-3-nitro-1,2,4-triazole (MS–NT) [8]. Earlier, we have reported the preparation of (11) in 71.0% yield and thus a comparison of % isolated yields as dry powders of (11) and (13) adequately show that the FM-group is indeed a stable phosphate protecting group during condensation reaction, work-up procedures and column chromatographic conditions which are normally necessary to synthesize and purify fully protected DNA fragments [3–5]. The FM-group from the tetramer (13) was then removed, following conditions identical to the preparation of (6), to obtain the 5′-protected tetramer phosphodiester component in a quantitative yield. This was then coupled, in presence of MS–NT, to the 5′-hydroxytetramer components (12) and (14) under usual conditions to give the fully protected octamer blocks (16) (R_f = 0.36) [13] and (18) (R_f = 0.44) [13] respectively in 71.0 and 69.5% yields (powders), thus giving further evidence of the stability of the FM-group. It should be emphasized here that the column chromatographic purification of (16) on silica gel required 8% ethanol in $CHCl_3$ phase for elution whilst the purification of (18) required only 6% ethanol in the $CHCl_3$ phase. The pixyl group from (16) and (18) were deprotected using usual conditions and the corresponding hydroxy octamer blocks (16a) (R_f = 0.21) [13] and (18a) (R_f = 0.34) [13] were isolated in 93.0 and 81.0% yields (powders) respectively after work-up and purification through silica gel column chromatography. They were then condensed with a slight excess of (15) under usual conditions to obtain (17) (R_f = 0.29) [13] and (19) (R_f = 0.36) [13] in 72.5 and 69.0% yields respectively as powders after normal work-up and purification by column chromatography. The comparison of % isolated yields, R_f and chromatographic properties of (17) and (19) again provided evidence in support of the stability and enhanced lipophilicity of the FM-group containing oligonucleotide (19). The FM-group from the phosphotriester (19) could be completely deprotected, as for tetramer (13) and octamer (18) blocks, within two hours by the action of 10 equiv. of Et_3N at 20°C to obtain (20) in the form of powder after a usual work-up procedure and was condensed, in presence of an excess of MS–NT in the usual fashion with the 5′-hydroxy dodecamer block (21) to obtain the pure tetracosamer (31 mg, 61%, powder, R_f = 0.26) [13]. The tetracosamer (10 mg) was then depro-



(11) R^1=Px, R=Bz, n=2
(12) R^1=H , R=Bz, n=2
(13) R^1=Px, R=FM-triester, n=2
(14) R^1=H , R=FM-triester, n=2
(15) R^1=Px, R=phosphodiester salt, n=2
(16) R^1=Px, R=Bz, n=6
(16a) R^1=H , R=Bz, n=6
(17) R^1=Px, R=Bz, n=10
(18) R^1=Px, R=FM-triester, n=6
(18a) R^1=H , R=FM-triester, n=6
(19) R^1=Px, R=FM-triester, n=10
(20) R^1=Px, R=phosphodiester salt, n=10
(21) R^1=H , R=Bz, n=10
(22) R^1=Px, R=Bz, n=22

tected using 4-nitrobenzaldoximate ions [8] in aqueous dioxan for 20 h at 20°C followed by a brief aq. NH_3 (d0.9) treatment (24 h at 20°C). The lipophilic materials were then removed by partitioning the aqueous phase with CH_2Cl_2 and diethyl ether. The aqueous phase was collected and concentrated. The residue

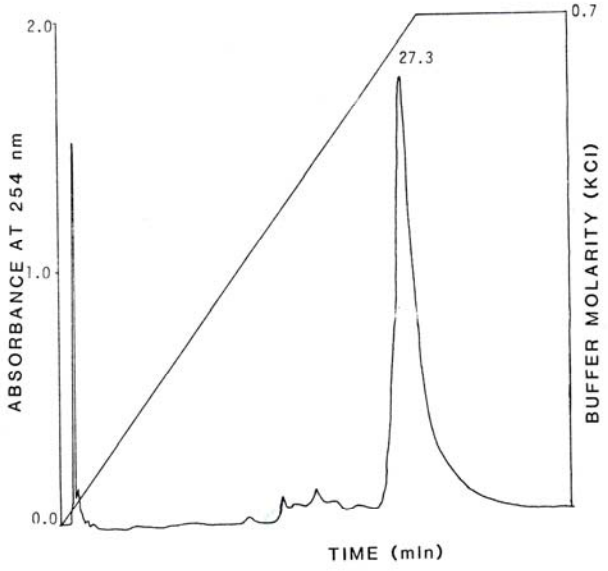


Fig. 1. HPLC elution profile of tetracosathymidylic acid (Permaphase AAX column, linear gradient, 0.01 M KH_2PO_4 to 0.05 M KH_2PO_4 and 0.7 M KCl, pH 4.45, 57°C).

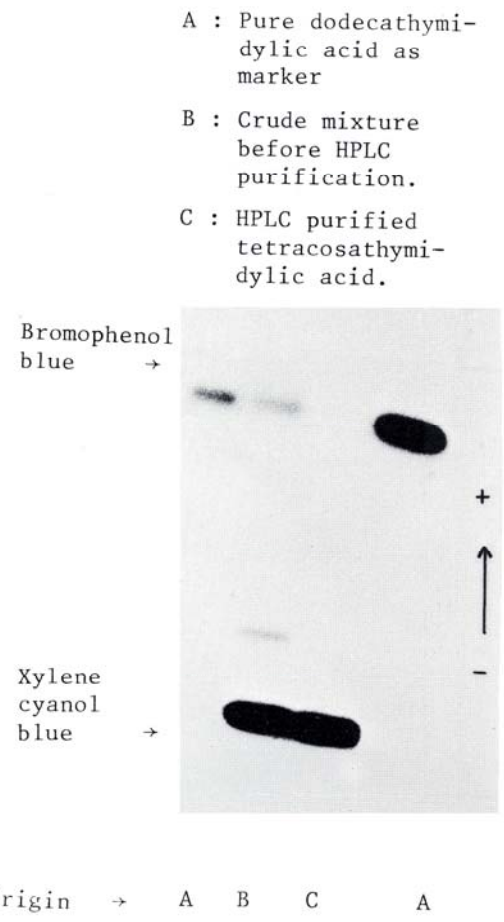


Fig. 2. 20% polacrylamide gel electrophoresis of ^{32}P-labelled oligothymidylic acids.

Fig. 3. 20% polyacrylamide gel electrophoresis of ^{32}P-labelled HPLC purified material after it has been digested following Maxam and Gilbert's procedure [11].

was then subjected to a treatment with 80% aqueous acetic acid for 15 min and concentrated. The residue was co-evaporated with water several times and was directly examined by HPLC (Permaphase AAX, 55°C, linear gradient, 0.01 M, KH_2PO_4, 0.0 M KCl to 0.05 M $KH_2PO_4$4, 0.7 M KCl, pH 4.45) as shown in the elution profile in Fig. 1. The main peak contained 89.5% of the total A_{260} OD units. An aliquot of this material was ^{32}P-labeled using γ-^{32}P–ATP and kinase along with the crude material before HPLC and they were electrophoresed on a 20% polyacryalmide gel as shown in Fig. 2 to examine their purities. The ^{32}P-labeled pure material (collected by HPLC) was sequenced by Maxam–Gilbert's procedure (Fig. 3) and was enzymatically completely digested by Snake venom phosphodiesterase to establish the exclusive presence of $3' \rightarrow 5'$ linkages in the tetracosathymidylic acid.

Acknowledgements

The authors thank Professor L. Philipson for encouragement and the Swedish Board for Technical Development for generous financial support.

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9. The fully protected phosphotriester (4) was prepared over 85% yield (powder), using fluorene-9-methanol, basically employing a literature procedure (Katagiri *et al., J. Am. Chem. Soc.* **97**, 7332 (1975)). The dimer block (9) has been prepared following our procedure [4].
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12. The retention times of DNA fragments with old Permaphase AAX column (*ca.* 70 runs) were not repeatable; thus, older columns retained DNA segments much longer time than anticipated.
13. Merck silica gel 60 F_{254} pre-coated plates have been used in 10% ethanol–$CHCl_3$ system.