Synthesis and Purification of Oligodeoxyribonucleotides

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This thesis is submitted in part fulfilment of the requirements of the degree of Doctor of Philosophy in the University of Edinburgh. Unless otherwise stated the work described is original and has not been previously submitted in whole or in part for any degree at this or at any other university.

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To my parents

and Vanessa

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 fluorescent properties of Tbf-DMTr enable easy detection. The synthesis and purification of long oligonucleotides (> 100-mer) has been undertaken. Additionally a new fluorescent label for oligonucleotides has been developed which enables detection of DNA probes to concentration down to 10^{-11} mol/l.

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Chapter 1: Introduction

1.1 History of DNA Synthesis

The golden age of DNA began with the first important evidence that DNA, an organic phosphate macromolecule, is the genetic material in all living organisms. Reported in 1944, Avery *et al.*[1] discovered that the DNA from one strain of pneumococcus could "transform" another strain. The discovery of the complementary double-stranded (duplex) structure of DNA by Crick and Watson[2] in 1953, and with it the recognition of how the molecule can be replicated was the second important milestone in the history of DNA. These discoveries led to an understanding of the functions of the molecule that holds the key of life. One major function of DNA is to carry the genetic code for each organism. DNA is transcribed into RNA and the RNA is translated into the amino-acid language of proteins. The second major function of DNA is that it carries out a self replication. These discoveries have generated a considerable interest in investigating the DNA molecule and the chemical complexity of such compounds.

The fundamental objective in DNA synthesis is the formation of an ester linkage between an activated phosphoric acid function of one nucleotide and the hydroxyl group of another nucleoside/nucleotide, thus forming the natural phosphodiester bridge between the 5'-OH of one nucleoside unit and the 3'-OH of the next. Early synthetic efforts concentrated on the search for suitable protecting groups for the

bases and the ribose moiety and for proper condensating agents for the phosphodiester bond formation.

The first synthesis of a nucleic acid in the laboratory was reported in 1955 by Todd[3] who successfully synthesized and isolated a dithymidine dinucleotide. Khorana used and developed the phosphodiester approach and finally synthesized the structural gene for tyrosine tRNA, a DNA segment containing 200 bp, highlighting many years of continuous efforts in nucleic acid synthesis[4]. During the same period other organic chemists such as Letsinger,[5] Reese[6] and Eckstein[7] started to explore the phosphate triester approach which was later modified by Narang[8] and Cramer[9] independently who used it to make biologically active material.[10] In the mid seventies, the phosphite triester approach originated in the laboratory of Letsinger[11] who also reaffirmed the solid phase synthesis method which he had introduced earlier with the phosphate triester approach.[5] A new procedure for the preparation of deoxyoligonucleotides was finally devised by Caruthers *et al.*,[12,13] generally known today as the phosphoramidite method. This approach, which is currently widely used, perhaps has been so successful because it is readily adaptable to automated DNA synthesis machines[14] and allows the rapid and inexpensive automated synthesis of oligodeoxyribonucleotides up to *ca.* 150 nucleotides long. Oligodeoxyribonucleotides which would have taken several months to synthesize by solution-phase methods can be synthesized in 24 h by solid-phase methodology.

1.2 Biochemical Application of Synthetic Oligonucleotides

Thirty years ago the chemical synthesis of oligodeoxyribonucleotides did not have any obvious biochemical application, but was rather the interest of the organic

chemist. Since the development of rapid and efficient synthesis and simultaneously, the advent of molecular cloning techniques, synthetic DNA has found use in most fields of biology and biochemistry. The availability of synthetic oligonucleotides transformed the way of thinking of biologists and hence new approaches to problems arose. Some of the applications of synthetic oligonucleotides include the use in the cloning and synthesis of man-made genes[15], as primers for various polymerase chain reaction (PCR) applications,[16] to sequence DNA,[17] for mutagenesis of genes in a site-specific manner,[18] as probes for examining how proteins interact with polynucleotides,[19] in antisense therapy for the treatment of viral diseases and cancer,[20] and for studies on nucleic acid structure.[21] The predominant application of oligonucleotides is in recombinant DNA technology.

Most of the medically important proteins are difficult to obtain from biological specimens, and they are too difficult to synthesize in sufficient quantity by present peptide synthesis technology. As an alternative, the recombinant DNA technology was developed in the late seventies. Genes for a specific protein are chemically synthesised and expressed in micro-organisms. The use of micro-organisms is not a recent innovation since they have been used by man for a long time to produce food, *e.g.* cheese, bread, beer and wine, but until recently such applications were restricted to the use of naturally occurring organisms. With DNA recombinant technology these micro-organisms can be genetically altered to produce a wide range of useful products in agriculture and medicine. The first synthesis of a functional polypeptide product from a gene of chemically synthesized origin was performed by Itakura *et al.*[22] in 1977 with the production of the hormone somatostatin. Later in 1982 the glucose level regulation protein insulin was produced on a large scale after its gene was successfully synthesized and inserted in to *Escherichia coli.*[23] Human growth hormone which is a 191 amino acid protein, a deficiency of which leads to dwarfism, was usually extracted from human cadaver with tragic consequences at the time of

writing. But the alternative source now provided by cloning the recombinant DNA in *Escherichia coli*[24] has taken over. Since then many other hormone proteins and also enzymes have been produced by the recombinant technology making use of synthetic genetic material.

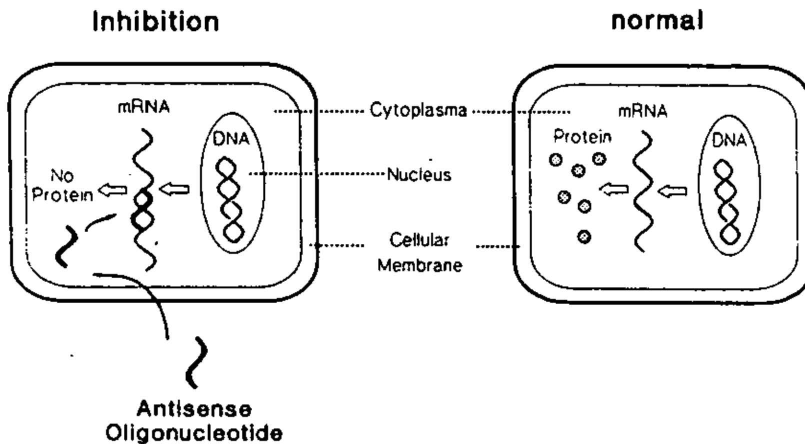


Fig. 1.1: Principle of action of an antisense oligonucleotide

Novel approaches to therapeutics using oligonucleotides have also been developed in the past few years. The transfer of genetic information in most organisms functions from DNA to mRNA to protein. In the case of retroviruses, the information originates in viral RNA. These genetic processes can now be altered by insertion of synthetic oligonucleotides. Oligonucleotide therapeutics include antisense therapy whereby an oligodeoxyribonucleotide (antisense) complementary to a target mRNA (sense) will hybridize and block the translation. Thus, expression of a specific protein can be regulated or inhibited. Zamecnik and Stephenson[25] were the first to propose the use of antisense oligodeoxyribonucleotides for therapeutic purposes. They observed that a 13-mer synthetic oligodeoxyribonucleotide that was

complementary to the RNA of the *Rous sarcoma* virus, could inhibit the growth of this virus in cell cultures. *In vitro* applications that have been successful in the cancer area for example, include inhibition of expression of oncogenes such as *c-myc*;[26] and *in vivo* antisense experiments have been successful in the area of cardiovascular disorder.[27] Applications to viral inhibition, including HIV inhibition, have also been reported.[28] *Fig. 1.1* illustrates simply the inhibition of protein biosynthesis by an antisense oligodeoxyribonucleotide being bound to the mRNA. As yet, few applications of synthetic oligodeoxyribonucleotides of clinical relevance have been reported[29,30,31] and the *in vivo* characteristics of oligodeoxyribonucleotides are only now being investigated.[32,33] Serious problems remain to be overcome before an oligonucleotide reaches the clinic. Nevertheless, it is very probable that such informational drugs will be used against a variety of human diseases in the not too distant future.

1.3 DNA Structure

1.3.1 Primary Structure

The primary structure of DNA was established after the works of Thannhauser and Klein[34] in 1933. DNA is from its constitution a linear polyester with phosphoric acid as the diacid component (the third acidic function has no role in the chain synthesis) and a substituted deoxyribose as the diol component. Thus the primary structure of DNA consists of a chain of 2'-deoxy-D-ribose rings linked by 3'-5' phosphodiesters with each of the sugars having a base attached at the 1'-position in the β-configuration as shown in *Fig. 1.2*. There are no 5'-5' or 3'-3' linkages in the regular DNA primary structure. Carrying out the hydrolysis of a DNA chain by a deoxyribonuclease enzyme, would result in the basic units of DNA called nucleotides.

The four nucleotides Adenosine phosphate, Guanidine phosphate, Thymidine phosphate and Cytidine phosphate can further be cleaved by the nucleotidase enzyme to phosphoric acid and the corresponding nucleosides, deoxythymidine, deoxycytidine, deoxyadenosine and deoxyguanidine, which are the deoxyribose units with one of the four bases: thymine, cytosine, adenine and guanine respectively.

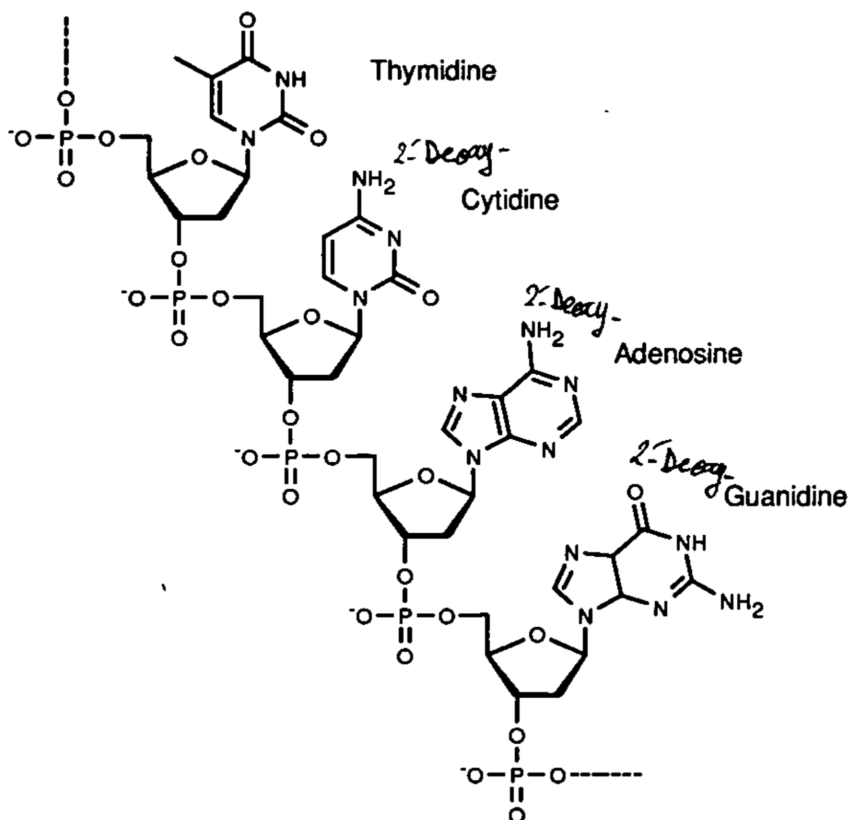


Fig. 1.2: Primary Structure of DNA

Besides DNA the other nucleic acid RNA can occur in different types of cells and differs in its sugar group, being D-ribose instead of 2'-deoxy-D-ribose, and from the occurrence of the base uracil in place of thymine, see *Fig. 1.3*. Molecular weights of DNA molecules are particulary high, with values between one million to one billion Daltons.

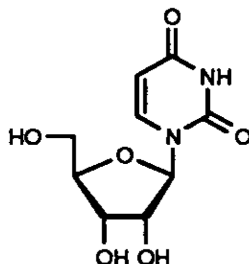


Fig. 1.3: Uridine

1.3.2 Secondary Structure

The presence on three of the bases, adenine, guanine and cytosine of exocyclic amino functions having the potential to form hydrogen bonds and ionisable phosphates groups, make oligodeoxyribonucleotides soluble in aqueous rather than organic solvents. The property of the hydrogen bonding is also very important in the formation of the secondary structure of DNA, it involves specific base pairing between adenine and thymine, and between guanine and cytosine, by the formation of two and three rather stable hydrogen bonds respectively, as shown in *Fig. 1.4*.

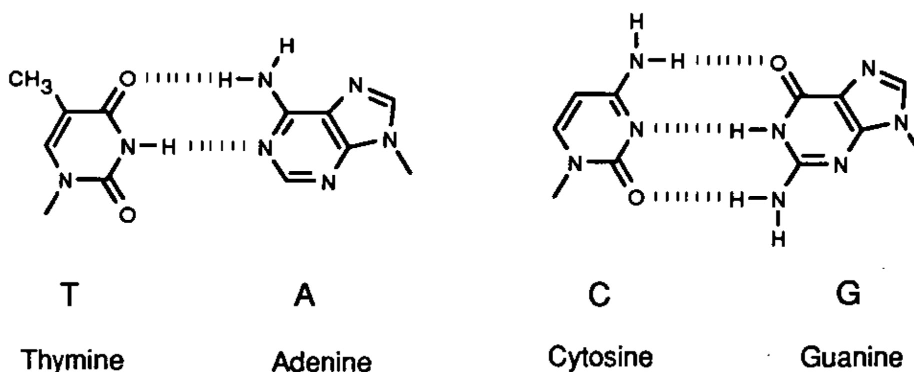


Fig. 1.4: Hydrogen bonding between base pairs in DNA duplexes

Watson and Crick[2] demonstrated by model-building studies that natural DNA consists of an antiparallel duplex held together by hydrogen bonds between A:T and G:C, see *Fig. 1.5*. Since then various DNA conformations have been discovered merely by changing the environment in which the duplex was formed (e.g. salt concentration, humidity) and base sequence. The major regular secondary structures are the A-DNA and B-DNA with right-handed double-helices and Watson-Crick base pairs. These and other DNA secondary structures have been reviewed by Dickerson *et al.*,[35] Jurmak[36] and Saenger.[37] Base pairing can also occur intramolecularly to form hairpin loop type structures, see *Fig. 1.6*.

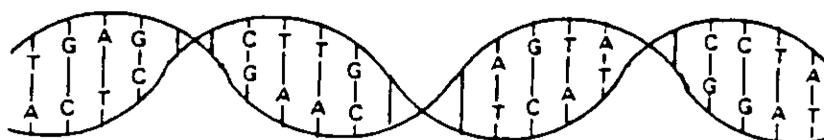


Fig. 1.5: DNA duplex

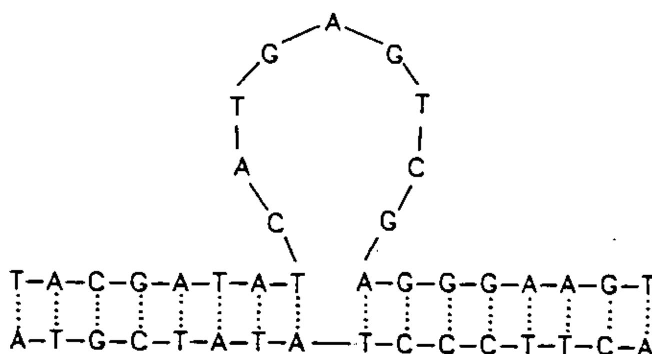


Fig. 1.6: Hairpin loop in DNA strands.

1.4 Chemical Synthesis of Oligodeoxyribonucleotides

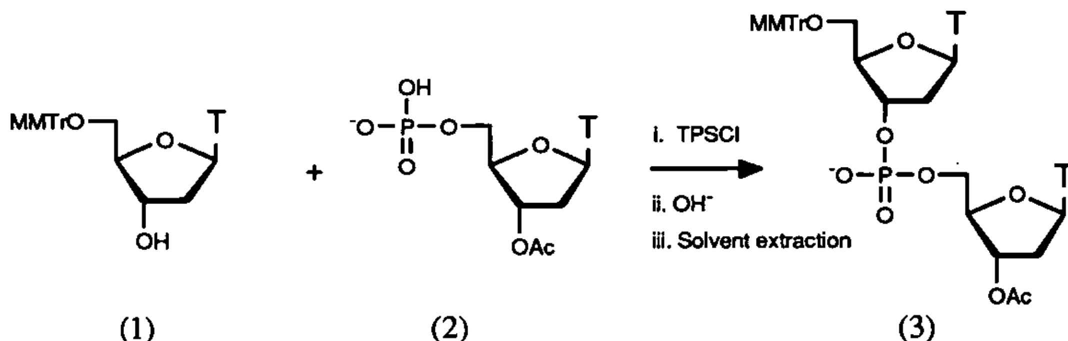
1.4.1 Introduction

From a chemical point of view, oligodeoxyribonucleotides are very sensitive molecules which possess diverse functionalities, unusual solubility and pose difficult purification problems in the conventional chemical sense. Even more difficult is their structure analysis by the standard physico-chemical methods. In spite of these obstacles, their synthesis is a fascinating challenge to organic chemists because of their great importance in living organisms. The aim in oligodeoxyribonucleotide synthesis is to join a 5'-hydroxyl on a nucleoside to a 3'-hydroxyl on another nucleoside via a phosphoric acid linkage to form a correct phosphodiester bridge, forming the backbone of a DNA strand. In order to achieve this, one must use selective blocking and deblocking procedures for primary and secondary hydroxyl (two in the case of ribonucleosides), for primary amino groups and often two of the three oxygens of the phosphate group. One must also be aware of the sensitive glycosidic bonds of the purine and pyrimidine bases with the sugar group, and the variable reactivity of the substituted phosphates. Stereochemical and neighbouring group effects affect noticeably the chemistry as the chain grows. All protecting groups must be removed without causing side-reactions and damage of the DNA sequence. Finally the desired product must be separated from impurities, requiring several purification steps. One can understand that a well thought out strategy has to be formed before starting an oligodeoxyribonucleotide synthesis.

The methods for the chemical synthesis of oligodeoxyribonucleotides have advanced greatly since Todd[3] first made a dinucleotide in the 1950's. Considerable efforts have resulted in the progression from a coupling reaction using a phosphodiester reagent in solution phase to using phosphotriester, H-phosphonate and mainly phosphite triester reagents on solid phase today.

1.4.2 The Phosphodiester Approach

The phosphodiester approach was the first method used in the 1950's and 1960's for the synthesis of di- and polynucleotides. It was developed and championed by Khorana *et al.*[38] who used it first to synthesize dinucleotides with a $C_{5'}$-$C_{3'}$ linkage in 1958 and until the 1970's when they successfully synthesized the genes of an alanine tRNA[39] and of a tyrosine suppressor tRNA.[4]



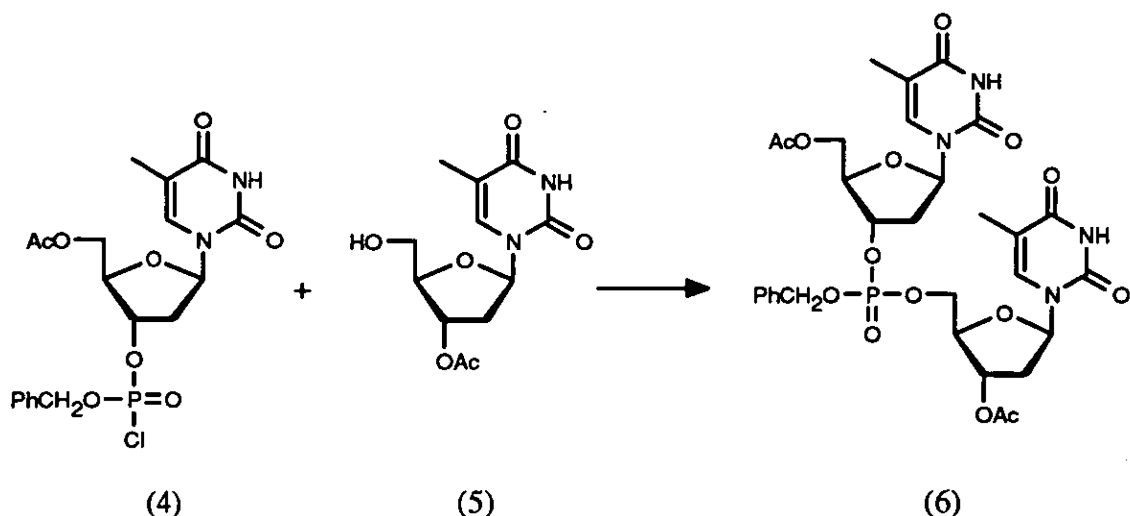
Scheme 1.1: Khorana's phosphodiester approach

The phosphodiester method involves the condensation of commercially available deoxynucleotides; one bearing a 5'-hydroxyl protecting group and the 3'-hydroxyl free to react (1) and the other bearing a 3'-hydroxyl protecting group and a phosphate group at the 5'-hydroxyl (2). The exocyclic amino groups on the nucleobases are also suitably protected. Condensating agents such as dicyclohexylcarbodiimide (DCCI) or triisopropylbenzenesulphonyl chloride (TPSCl) are used to give the dinucleotide (3), see *Scheme 1.1*. The dinucleotide thus obtained, may be elongated by further condensation reactions after prior phosphorylation of the deprotected 5'-hydroxyl. Unfortunately long reaction times, decreasing yields as the chain grows, and time consuming anion-exchange chromatography purification made this method impracticable when synthesising large oligodeoxyribonucleotides.

1.4.3 The Phosphotriester Approach

A possible solution to some of these problems (water solubility, ion exchange chromatography and low yields) inherent in the phosphodiester method could be partially resolved by protecting the free phosphate and thus forming a neutral organic molecule easier to manipulate by standard organic chemistry techniques.

This approach was first introduced by Michelson and Todd[3] in 1955 as shown in *Scheme 1.2*.

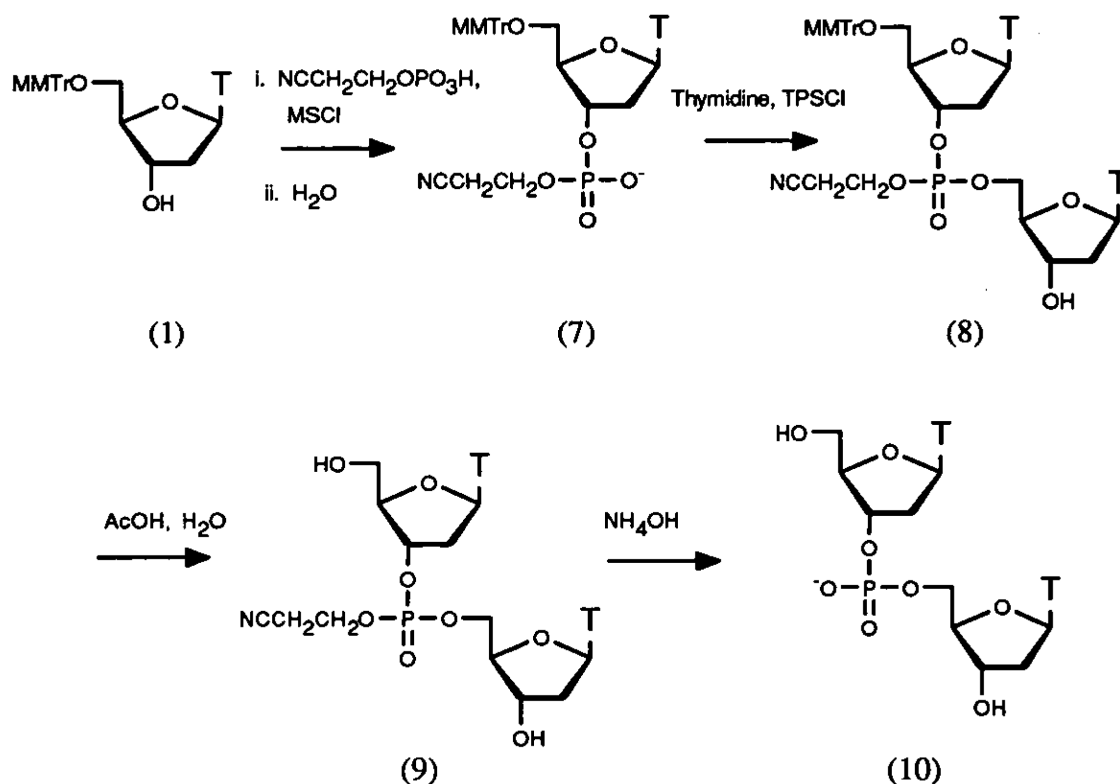


Scheme 1.2: Todd's phosphotriester approach

The basic principle of the phosphotriester method is to mask each internucleotidic phosphodiester function by a suitable protecting group. In Todd's synthesis, 5'-acetyl-3'-benzylphosphochloridate (4) was reacted with 3'-acetyl-thymidine (5) to give the fully protected dinucleotide (6), containing the internucleotidic 3'-5' phosphodiester linkage. This approach was found to be useful for larger scale synthesis and gave shorter purification time periods.

In 1965 Letsinger *et al.*[5,40] introduced the β-cyanoethyl phosphate protecting group using a one pot procedure with the solid phase synthesis principle.

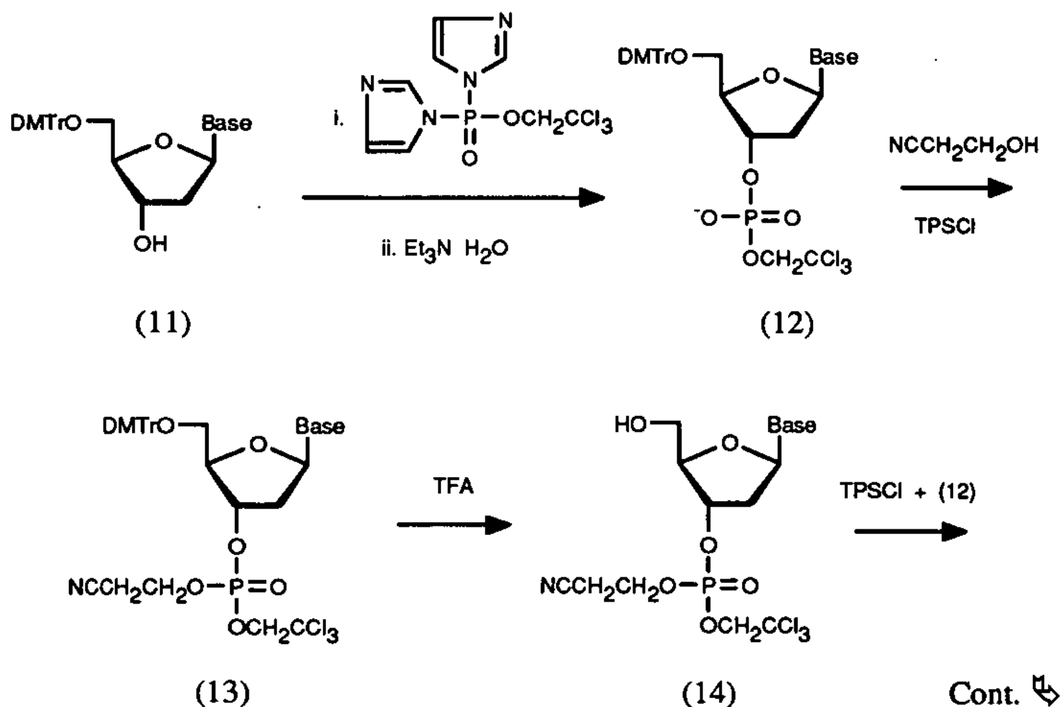
Subsequently the solid phase chemistry was found suitable for small scale synthesis only[41,42] and thereafter Letsinger used the phosphotriester method in solution phase synthesis as pictured in *Scheme 1.3*. First the 3'-OH of a 5'-protected thymidine (1) was phosphorylated with the β-cyanoethyl phosphate in the presence of mesitylene sulphonyl chloride (MSCl) to give the phosphodiester (7). Subsequent condensation with the primary 5'-OH of unprotected thymidine in the presence of 2,4,6-triisopropylbenzenesulphonyl chloride gave the fully protected dithymidine (8). The monomethoxytrityl (MMTr) protecting group and the phosphate β-cyanoethyl protecting group were removed by treatment with acetic acid and aqueous ammonia respectively, to give the thymidine dimer (10) in what is essentially a one pot procedure. Synthesis of oligodeoxyribonucleotides containing nucleosides other than thymidine have also been reported by Letsinger.[43]

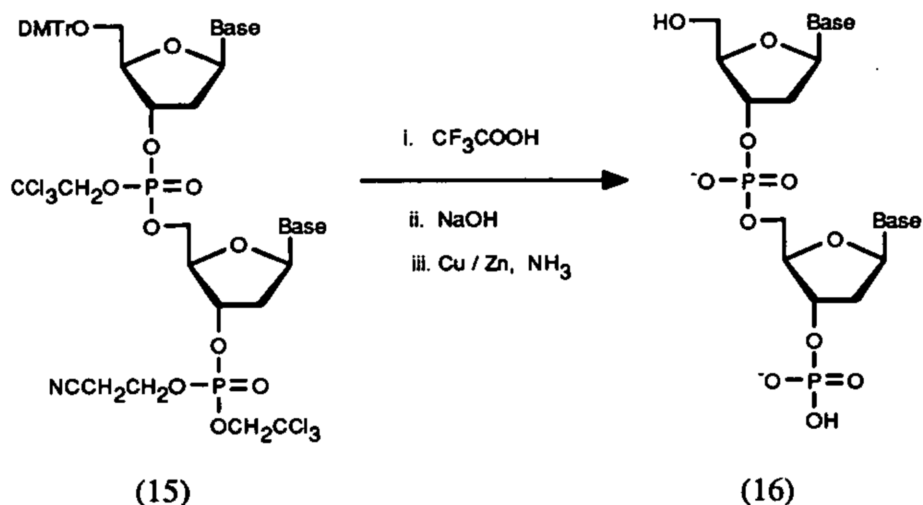


Scheme 1.3: Letsinger's phosphotriester approach

The incomplete phosphorylation in the first step followed by the coupling with unreacted (1) can lead to a complicated reaction mixture including the 3'-3' and the 5'-5' coupled products. The products of these side-reactions can be difficult to separate from the desired product. An attempt to remedy this was undertaken by Letsinger *et al.* when they developed the β-benzoylpropionyl 3'-hydroxyl protecting group.[44] During that period other chemists contributed to the development of the phosphotriester approach essentially by introducing new phosphate and hydroxyl protecting groups. Eckstein *et al.*[45,46,7] investigated the 2,2,2-trichloroethyl phosphate protecting group, Reese *et al.*[6,47] and Van Boom *et al.*[48] proposed aryl phosphate protecting groups with electron-withdrawing substituents in order to increase their base lability. Reese also proposed the methoxytetrahydropyranyl group for the 5'-hydroxy protection.[49]

In order to overcome the problem of side-reactions giving rise to 3'-3' and 5'-5' linkages, a "two step" sequential procedure starting from the fully protected mononucleotide containing fully masked 3'-phosphotriester group was developed by Cramer *et al.*[50] in 1973, see *Scheme 1.4*.





Scheme 1.4: Cramer's phosphotriester approach

A nucleoside protected with dimethoxytrityl (11), an acid labile 5'-OH protecting group, was first phosphorylated with 2,2,2-trichloroethylphosphodiimidazolidate and treated with triethylamine and water to remove the remaining imidazole to afford (12). This nucleotide was then condensed with β-cyanoethanol using triisopropylbenzenesulphonyl chloride (TPSCl) to afford the fully protected phosphotriester (13). After treatment with dilute aqueous trifluoroacetic acid (TFA), to remove the dimethoxytrityl group, the nucleotide (14) is formed. Compounds (14) and (12) are then reacted with the condensing agent triisopropylbenzenesulphonyl chloride to give the protected dinucleotide (15) which can be deprotected using trifluoroacetic acid to remove the dimethoxytrityl group, sodium hydroxide to remove the β-cyanoethyl group and Cu/Zn then ammonia to remove the 2,2,2-trichloroethyl group, to give (16).

Narang *et al.*[8] developed several modified triesters using phenyl, o-chlorophenyl and p-methylthiophenyl protecting groups instead of the 2,2,2-trichloroethyl group used by Cramer. Using this method Narang synthesised oligodeoxyribonucleotides of up to 15 monomer units.

The next development came in 1975 when Narang developed the "modified" phosphotriester method whereby he proposed arysulphonyltriazoles as condensing agents and bis(triazolyl)-p-chlorophenyl phosphate as a phosphorylating agent.[51] Narang used the block synthesis approach in conjunction with this phosphotriester method to produce a hexamer containing guanosine, cytosine and adenosine residues and claimed that these new reagents increased the yields and reduced sulphonation. Later Narang used arylsulphonyltetrazoles which were found to be more reactive than triazoles and triisopropylbenzenesulphonyl chloride.[52] In 1978 Reese *et al.*[53] developed the now widely used 1-mesitylenesulphonyl-3-nitro,1,2,4-triazole (17), MSNT, see *Fig. 1.7*, and Sproat and Bannwarth[54] introduced the 1-methylimidazole which used in conjunction with MSNT forms an intermediate N-methylimidazolium cation possessing a high phosphorylating capability. Coupling times could be reduced to under 10 min. Subsequent research focussed essentially on developing more labile phosphate protecting groups, e.g. Pfleiderer used the β-eliminating 2-(p-nitrophenyl)ethyl, NPE, group;[55,56] and more efficient condensing agents (see *Fig. 1.7*). Ohtsuka *et al.*[57] developed the regiospecific condensating agent 1-mesitylenesulphonyl 5-(pyridin-2-yl)tetrazolide (18), MSPy; Matteucci *et al.*[59] introduced the intramolecular nucleophilic condensating agent attached to the phosphorous atom 1-methyl-2-(2-hydroxyphenyl)imidazole (19) and Efimov *et al.*[58,60] introduced the O-nucleophilic catalysts based on pyridine N-oxides derivatives also attached to the phosphorus atom (20). The latter intramolecularly attached nucleophilic catalyst improved the phosphotriester method further as it jacks up the coupling yield to 98 % and reduces the time taken by a synthesis cycle to 7-8 min. Nevertheless, the principle of the modified phosphotriester approach originally introduced by Narang[51] still remains.

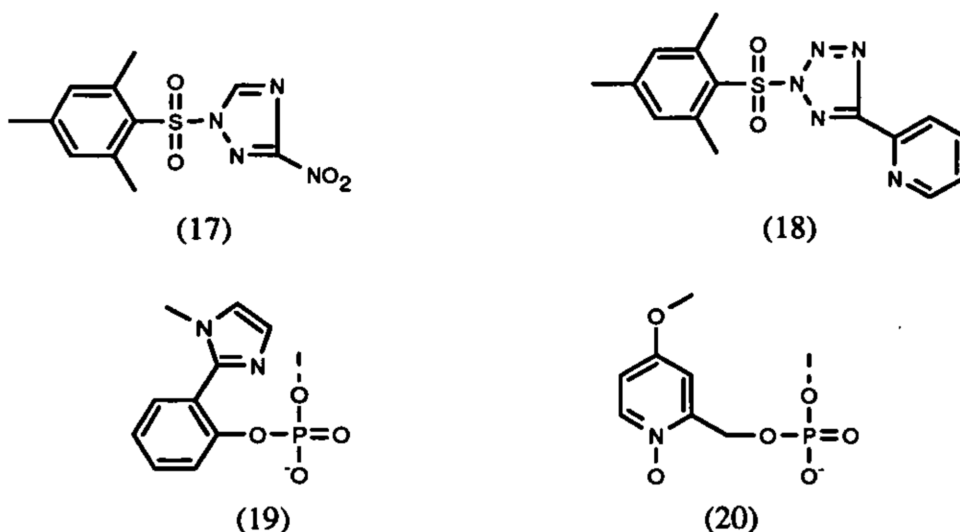
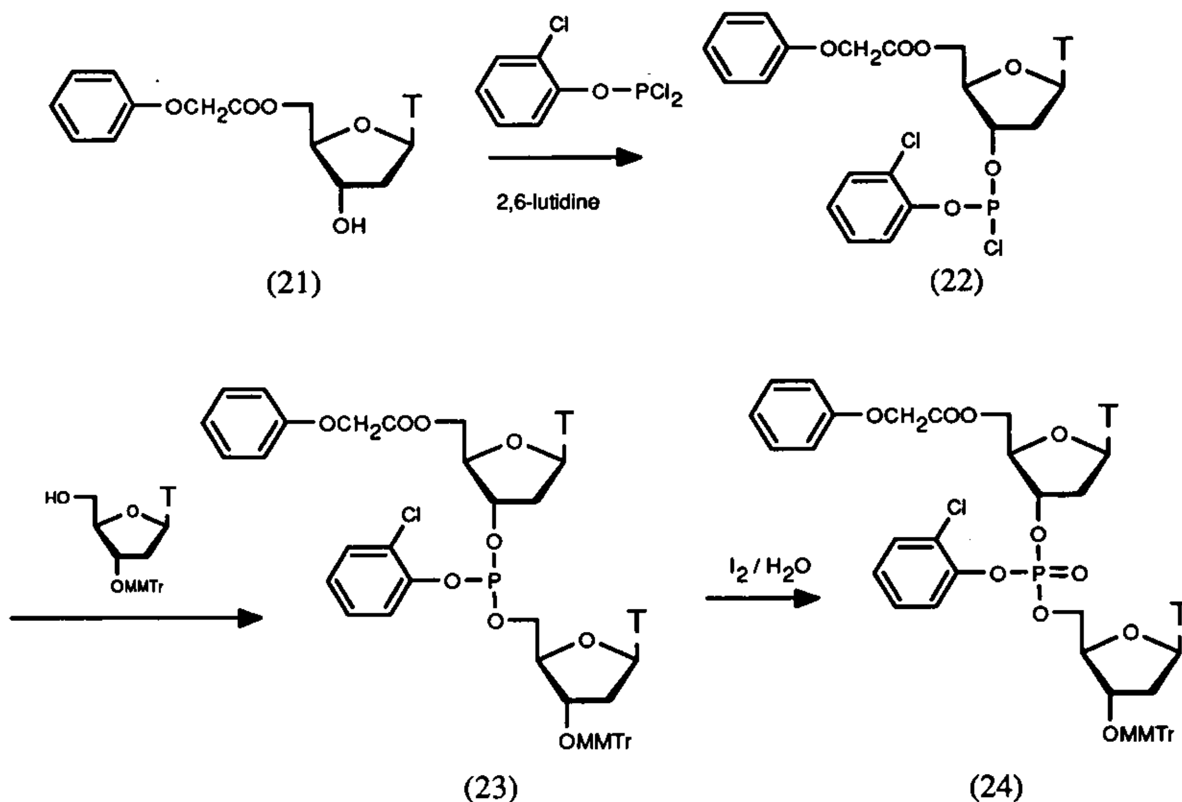


Fig. 1.7: 1-Mesitylensulphonyl-3-nitro-1,2,4-triazole, MSNT

With the development of phosphate protecting groups and efficient condensing agents, coupling steps in the phosphotriester method could be reduced from several hours in solution[3] to less than 5 min on solid phase,[60] enabling the synthesis of oligodeoxyribonucleotides with long chain lengths in satisfactory yields. The synthesis of a 20-residue oligodeoxyribonucleotide can be performed in 8 h.[61] In spite of the improved yields and coupling times, the phosphotriester method does not have the speed and efficiency of the phosphite triester method described in the next section. Moreover two side-reactions give rise to further limitations. Firstly during the coupling the 5'-hydroxyl can be sulphonylated by the coupling agent, therefore reducing the coupling efficiency to 97-98 % which limits the synthesis of oligodeoxyribonucleotides to a maximum of 40 residues.[62] Secondly, deoxyguanosine units can be phosphorylated and substituted with nitrotriazole at the O^6-position unless an extra protecting group is used.[62]

1.4.4 The Phosphite-Triester Approach

In 1975, Letsinger *et al.*[11] introduced the phosphite-triester method which was based on the great reactivity of phosphorochloridite reagents upon alcohols to give phosphite-triesters. Two nucleotide units could be coupled in a matter of minutes instead of hours and could easily be oxidized to the required phosphotriester by aqueous iodine, see *Scheme 1.5*.

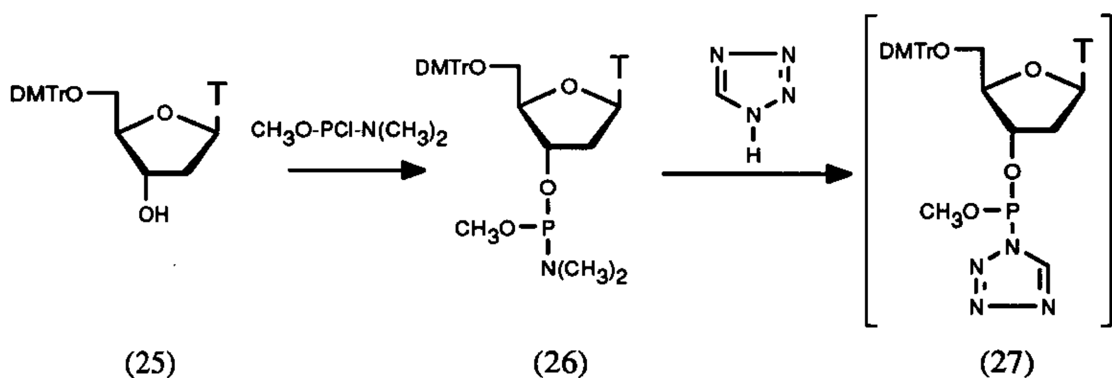


Scheme 1.5: Letsinger's phosphite-triester approach

5'-O-Phenoxyacetyl-thymidine (21) was reacted with *o*-chlorophenyl phosphorodichloridite to give the phosphitylated nucleoside (22), which in turn was coupled with 3'-monomethoxytritylthymidine to give the phosphite dimer (23) which was oxidised by iodine and water to produce the protected thymidine 3'-5' linked dinucleotide (24). Unfortunately further coupling of nucleosides after cleavage of the

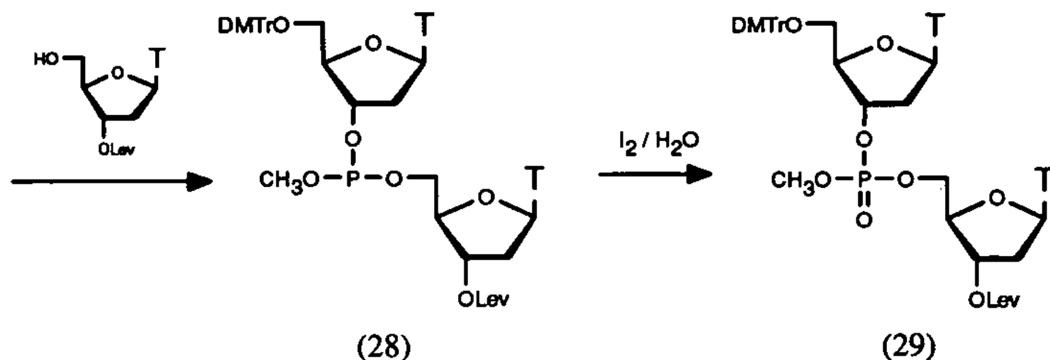
phenoxyacetic ester with ammonium hydroxide resulted in low yields caused mainly by the instability of the protected phosphotriester group towards the phosphitylating agent. The 2,2,2-trichloroethyl group was later shown to be suitable for the synthesis of a thymidine tetranucleotide.[63] Several other phosphate protecting groups were investigated by Ogilvie *et al.*[64] such as 2,2,2-tribromoethyl, benzyl, methyl, *p*-chlorophenyl, 2-phenylethyl, 2-*p*-nitrophenylethyl and β-cyanoethyl. They concluded that methyl and β-cyanoethyl were the most useful and remain as the most widely used phosphate protecting groups. The synthesis of several dimers using the methyl protecting group resulted in coupling times less than two minutes and yields over 90%.[65]

The phosphite-triester method via phosphorochloridites presents some drawbacks, for example introducing the oxidation step can lead to symmetrical as well as unsymmetrical products over which there is no control and this makes purification more difficult and gives lower yields. Also phosphorochloridites are very moisture and oxidation sensitive so that great care has to be taken during manipulations. In 1981, Beaucage and Caruthers[67] introduced the deoxynucleosides phosphoramidites which have the advantage of being more stable at room temperature and less succeptible to hydrolysis. A direct result of this development is that oligodeoxyribonucleotide synthesis was to become transformed from a manual to a semi-automated procedure performed by machines.[66]



(25) (26) (27)

Cont. ↳



Scheme 1.6: Caruthers' phosphite-triester approach

The phosphoramidite approach to oligodeoxyribonucleotide synthesis as described by Caruthers *et al.*[67] is shown in *Scheme 1.6.* 5'-Dimethoxytritylthymidine (25) was reacted with chloro-N,N-dimethylaminomethoxyphosphine to give the nucleotide (26) which was activated by 1H-tetrazole to give the intermediate (27) which in turn was coupled to 3'-O-levulinylthymidine giving the corresponding phosphite-triester (28). After oxidation with aqueous iodine the protected phosphate dimer (29) was obtained.

Further investigations made by McBride and Caruthers[68] on other possible N,N-dialkylaminomethoxyphosphines, included the diisopropylamino (30a), the morpholino (30b), the pyrrolidino (30c) and the 2,2,6,6-tetramethylpiperidino (30d) substituents, see *Fig. 1.8.* In each case they used the chloro phosphytilating agent derivative for its introduction on the 3'-hydroxyl of nucleosides. The diisopropyl and the morpholino phosphoramidite compounds were found the easiest to handle and conferred the most stability. This observations were later confirmed by Adams *et al.*[69] and from then on the N,N-diisopropyl and N-morpholino phosphoramidites were established in most research laboratories.[70,71,72]

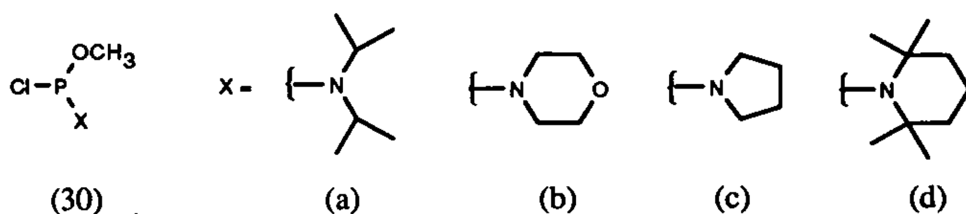
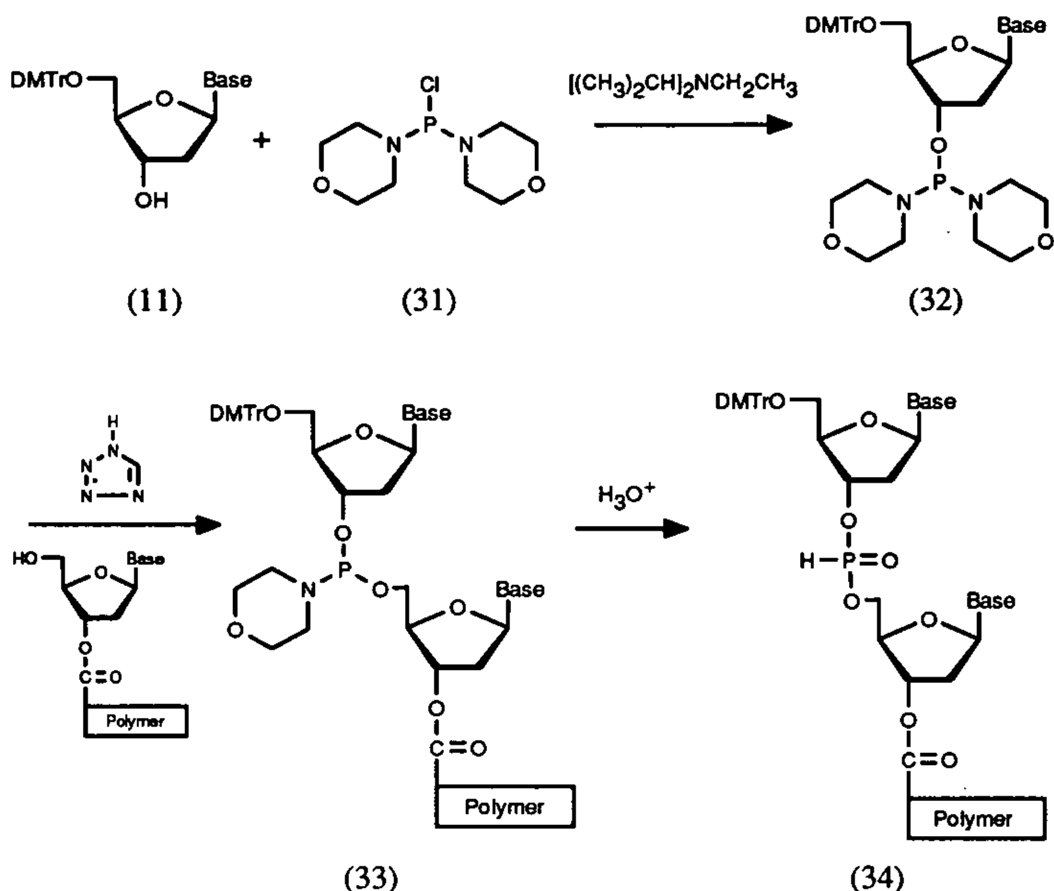


Fig. 1.7: Various phosphitylating agents

In 1983, Sinha *et al.*[73,74] combined the N,N-diisopropylamino group with the β-cyanoethyl group in the preparation of phosphoramidites reagents. The β-cyanoethyl group has the advantage over the methyl group that it is cleaved in concentrated ammonia at the same time as the base protecting groups, whereas the methyl group requires a second treatment with thiophenol in order to obtain a fully deprotected oligodeoxyribonucleotide. Since that time several workers[75,76,77,78,79] have examined other potential phosphate protecting groups but the β-cyanoethyl group in conjunction with the N,N-diisopropylamino group remains unchallenged.

One of the most recent advances in the phosphoramidite method was proposed by Uznanski *et al.*,[80] see *Scheme 1.6*. The difference from the common phosphoramidite approach is that the oxidation of the phosphite-triesters is performed with aqueous iodine at the end of the synthesis instead of at every step. Chlorodimorpholinophosphine (31) was reacted with a 5'-O-dimethoxytritylnucleoside (11) to give a 5'-O-dimethoxytritylnucleotide-3'-O-phosphordimorpholidite (32). The latter phosphoramidite (32) was then coupled with a solid support bound nucleoside in the presence of tetrazole to give a dimer (33). The phosphoramidite P-N bond was subsequently cleaved by mild acid hydrolysis to give (34) which in turn was treated with TCA in order to deprotect the 5'-hydroxyl where further nucleotides can be added.



Scheme 1.6: Uznanski's phosphoramidite method

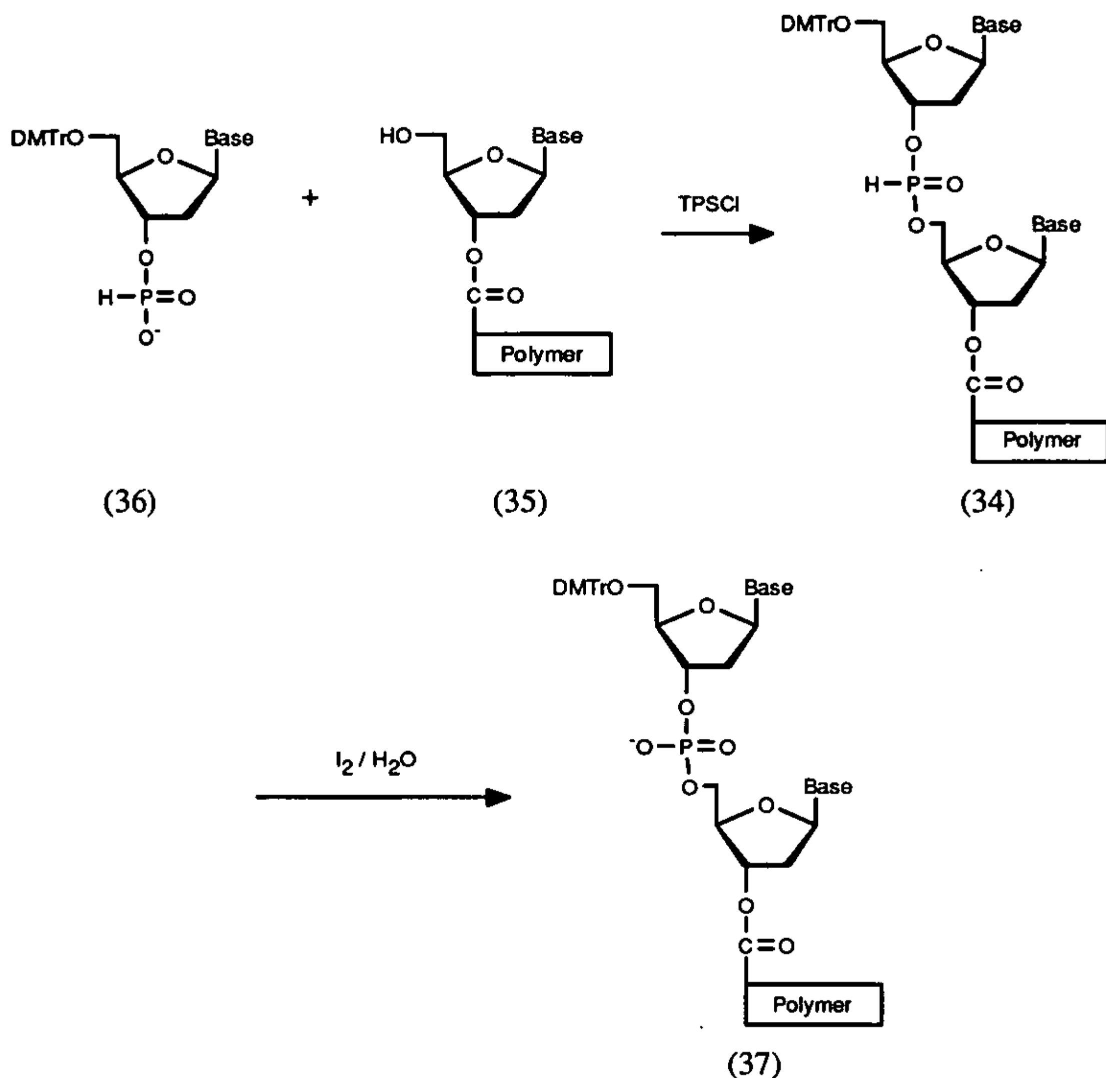
Perhaps the most significant improvement in recent years to increase the quality of synthetic DNA has been the substitution of N-methylimidazole by N,N-dimethylaminopyridine (DMAP) in the capping solution.[81] After each coupling step the unreacted 5'-hydroxyl functions are normally capped with acetic anhydride but when N-methylimidazole was used as a catalyst, a small but detectable amount of 2,6-diaminopurine, a modification of guanine, was formed. None of this side-product was observed when using DMAP during the acetylation steps. The final deprotection step using concentrated ammonia at 55°C for 4 h to remove the exocyclic base protecting groups has been almost unchanged since Khorana introduced it over 20 years ago. The focus of attention has recently been on developing protecting groups removable under much milder conditions; these developments are described in section 1.6.

The advantage of the phosphite-triester method or more precisely the phosphoramidite method over the phosphotriester method, is that it offers short reaction times, extremely high coupling efficiencies (> 98 %) and is the only reliable chemistry for synthesis beyond 50 residues. However this method involves relatively unstable starting materials due to the high reactivity of P^{III} reagents.

1.4.5 The H-Phosphonate Approach

During the past few years, the H-phosphonate method for oligodeoxyribonucleotide synthesis, which was first described by Todd *et al.*[82] in 1957, has been re-examined by various laboratories.[83,84,85] This renewed interest is due to the fact that the H-phosphonate chemistry combines the advantages of both the phosphotriester approach, with its stable nucleotides, and the phosphite-triester approach, with its speed of reaction. An example of this approach is shown in *Scheme 1.7*.

A 5'-unprotected nucleoside (35) attached to a solid support is coupled to a 5'-O-dimethoxytrityldeoxynucleoside 3'-H-phosphonate (36) in the presence of an activating agent like TPSCl or sterically hindered carbonyl chlorides (adamantoyl and pivaloyl). The resulting phosphite diester (34) is capped, detritylated with acid and condensed with the next 5'-dimethoxytrityldeoxynucleoside 3'-H-phosphonate. When the synthesis of the DNA segment is completed, the phosphite diester internucleotide linkages are oxidised with *tert*-butyl hydroperoxide or iodine to the phosphate diester (37), and the 5'-hydroxyl and base protection, if any, are removed.



Scheme 1.7: The H-Phosphonate approach

Moreover, the H-phosphonate approach has the advantage that no phosphate triester protecting group is required, nullifying the problems associated with its removal. Furthermore one step in the synthesis cycle is eliminated, as oxidation of all internucleotide linkages is carried out after the complete oligodeoxyribonucleotide assembly. The H-phosphonate procedure is currently cheaper and more efficient for synthesis of many modified backbone oligodeoxyribonucleotides. Indeed, the oxidation step is subject to general base catalysis and this allows nucleophiles other than water to be substituted during oxidation to give a range of oligodeoxyribonucleotide analogues.[86,87]

1.5 Solid Phase Synthesis

As for most organic chemistry reactions, the first workers performing oligodeoxyribonucleotide synthesis carried out the reactions in solution phase; the products of which thus required one or several purification techniques. When in 1963 Merrifield[88] developed a new method of synthesis involving an insoluble solid support which had been developed for peptide synthesis, other workers soon found the principle of solid phase synthesis applicable to many areas of synthetic chemistry. In 1965, Letsinger[5] first applied this method to the synthesis of di- and trinucleotides. The first successful solid phase synthesis of an oligonucleotide by the phosphotriester method was achieved in 1979 by Itakura *et al.*[98] who made dT_{19} in 49 % yield. Then Ogilvie *et al.*[99] made an RNA hexamer with the phosphite-triester chemistry while Matteucci and Caruthers[90] used similar conditions to produce a DNA dodecanucleotide in 30 % yield. Since these pioneering experiments, the solid phase synthesis (SPS) concept has largely been accepted throughout the world for the synthesis of oligodeoxyribonucleotides, using mostly the phosphoramidite method.

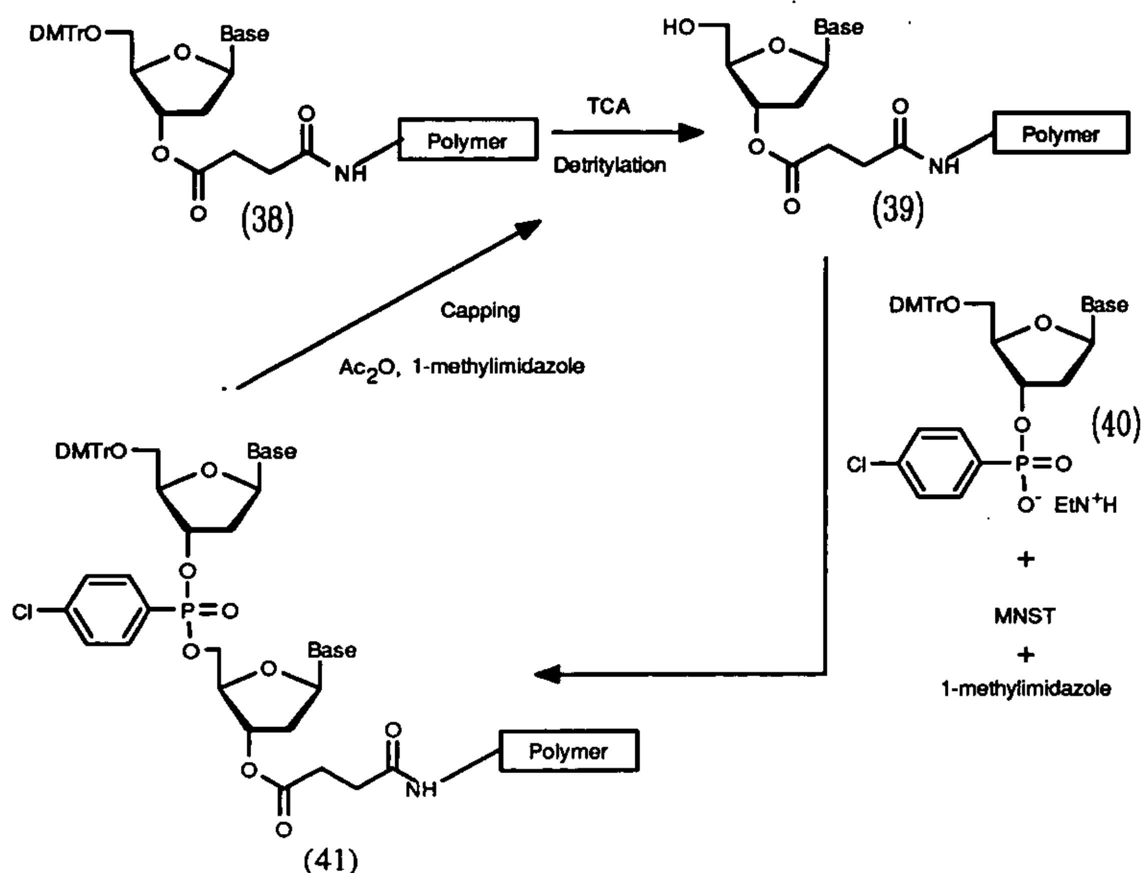
The SPS method is fundamentally a heterogeneous coupling reaction between a deoxynucleotide derivative in solution and another residue bound to an insoluble support. For this, it uses a functionalized support where the oligodeoxyribonucleotide chain attached via a linker can grow during the course of the synthesis. The chemical synthesis of DNA is normally carried out in the 3' to 5' direction to take advantage of the high chemical reactivity of the primary 5'-hydroxyl function. This method has several advantages over the solution phase method:
The removal of reagents etc from the product, which is covalently bound to the solid support, can be performed simply by repetitively washing the solid support and filtering the solution without loss of product. All the reagents can be used in excess, hence achieving high yields. The reactions are all carried out in one flask and therefore

the loss of product is minimized. The operations to be carried out are fairly simple, mixing, washing and filtering, making it possible to have them performed by people unskilled in organic synthesis like biologists. The reaction processes were successfully automated thus reducing the amount of labour and the synthesis time.

Many types of solid support have been investigated for solid phase oligonucleotide synthesis but four are in common use; styrene/divinylbenzene copolymer,[5,89] aminoalkylsilica gel,[65,90,91] polyamide[92,93] and, most importantly, controlled pore glass (CPG) beads[54,94,95] which are found to be ideal as they are rigid and do not swell. Automated syntheses on CPG supports are however limited to scales $\leq$ 10 μmol. Recently Rapp *et al.*[96] have developed a solid support based on a polystyrene polyethylene glycol graft copolymer with improved synthesis efficiency and high loading capacity enabling automated synthesis up to 1 mmole scale. The growing oligonucleotide chain is attached to the solid support *via* a long spacer in order to extend the sites away from the surface and ensure accessibility to all reagents. The length of the linker was found to have an effect on the final yield of the product.[97] Succinate derivatives are generally used and are cleaved at the end of the synthesis by mild alkaline hydrolysis, using ammonium hydroxide which at the same time is used to remove the base protecting groups.[61]

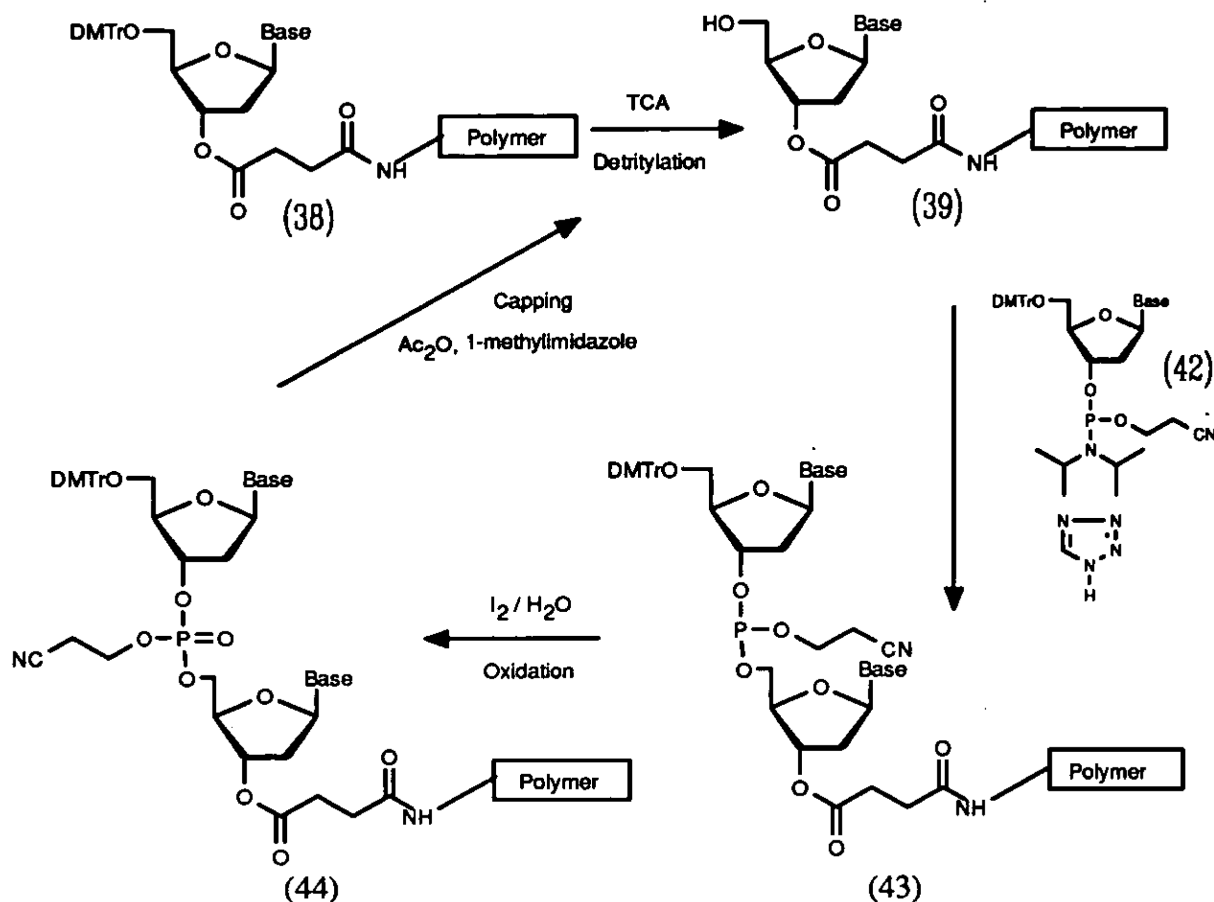
In both the phosphotriester and the phosphoramidite approach, the solid support to be used is first functionalized by addition of the linker and the first nucleoside to give a loaded support. The assembly of the protected oligonucleotide chain is carried out by packing a small column with the deoxynucleoside loaded support (as little as 10 mg of nucleoside loaded CPG can be used for a 0.2 μmol scale synthesis) and allowing solvents and reagents to flow through in predetermined order. The succession of operations is most reproducibly accomplished using a commercial, automated DNA synthesizer; alternatively a manual flow system or a small sintered

glass funnel can be substituted. The outline of the phosphotriester synthesis cycle is shown in *Scheme 1.8*.



Scheme 1.8: The phosphotriester solid phase synthesis cycle

The nucleoside attached to the polymer (38) first undergoes a detritylation step with TCA to deprotect the 5'-hydroxyl affording (39) and is then coupled to a suitably protected nucleotide (40) in the presence of MSNT as a condensating agent and 1-methylimidazole as an accelerating agent to give a fully protected dimer (41). It is then capped with acetic anhydride and 1-methylimidazole as a safety step to block chains which have somehow not reacted. The cycle is repeated until the desired length of oligonucleotide is obtained. At this point all the protecting groups are removed and the linker cleaved with ammonium hydroxide.



Scheme 1.9: Phosphite-triester solid phase synthesis cycle

The synthesis cycle used in the phosphite-triester approach is very similar except it involves an extra oxidation step to obtain the phosphate linkages, see *Scheme 1.9*. The dimethoxytrityl protecting group is removed from the polymer attached nucleoside (38) with TCA, to give a free 5'-hydroxyl (39). A phosphoramidite nucleotide (42) is activated with tetrazole and added to the polymer bound nucleotide (39) to form a phosphite-triester dimer (43). The synthesis cycle then performs the capping of the unreacted free hydroxyl functions and a fortuitous benefit of this step is that phosphitylation of the O-6 position of guanine is reversed. The intermediate phosphite (43) is then oxidized to the phosphotriester (44) with iodine and water. Pyridine or 2,6-lutidine is used to neutralize the hydrogen iodide released during the oxidation step. The cycle is then repeated by detritylation of the dimer (44) and

addition of further nucleotides until the desired chain length is obtained, at which point it is cleaved from the solid support and deprotected with ammonium hydroxide. The orange colour due to the dimethoxytrityl cation, liberated into solution is usually compared in intensity with the detritylation of the previous cycle to obtain a measure of the coupling efficiency. The average coupling efficiency in the phosphoramidite method of DNA synthesis, when carried out on an automatic machine is ~98.5 % and sequences in excess of 100 residues can be prepared.

So far all the chemical steps (described above) involved in the synthesis of oligonucleotides by solid phase synthesis, can be performed with automated DNA synthesizers which are now widely available commercially. The purification of the crude oligonucleotides is the only remaining step which requires manual manipulations; these will be discussed in section 1.8. When programmed with the desired sequence and loaded with the correct reagents DNA machines automatically synthesize DNA without manual intervention. Most machines are able to synthesize more than one oligodeoxyribonucleotide sequence at a time. Recently the solid phase gene assembly has been investigated, using new technologies for multiple chemical synthesis of oligonucleotides and stepwise hybridization on solid-phase support. An economical, rapid and efficient solid-phase gene synthesis is under scrutiny.[100]

1.6 Protecting Groups

1.6.1 Introduction

The problem of DNA synthesis entails the formation of a linear polymeric sugar phosphate ester by the stepwise addition of ester bonds at exactly the correct phosphorus atoms and hydroxyls of the sugar residues. This is accomplished by

selective protection/deprotection of reactive sites in the appropriate sequence. The protecting groups must be easily introduced and removed, the latter being especially important. Due to the sensitive nature of the monomer units and DNA itself, great care must be taken to circumvent two of the biggest problems: internucleotide bond cleavage (breaking of the DNA strand) and depurination (loss of adenine or guanine *via* cleavage of the glycosidic bond). Nucleotides contain many potentially reactive sites. The reactive nucleophilic centres that need protection are amino groups on the bases, sugar hydroxyls (3' or 5'), oxo and imino functions and phosphate groups. As well as the stability of the protecting groups under various synthesis conditions, it is also vital to take into consideration how each group fits into the overall protecting group strategy of the molecule. Thus two classes of protecting groups have been developed:

- Permanent groups that remain intact on the oligomer throughout the course of the synthesis and are only removed at the end. These include the oxo/imido, amino and phosphate protecting groups.

- Transient groups which are removed whenever desired during the synthesis. These include the sugar hydroxyl protecting groups (5'-OH).

In general protecting groups should have the property of being easy to introduce into the nucleosides and nucleotides and provide great stability and lipophilicity, hence facilitating storage and purification. Furthermore they should be cleaved under very mild conditions so as not to affect the oligonucleotide adversely. The reagents used for their introduction should show high regioselectivity. The mutual compatibility of these groups is necessary, and by virtue of their steric and electronic effects, the chemical synthesis should be facilitated even under heterogeneous conditions like solid phase synthesis. Easy detection of the protecting groups is desirable also.

1.6.2 Protection of the Nucleic Bases

The exocyclic amino functions on the heterocyclic bases are permanently protected during DNA synthesis, generally as amides; the most common derivatives being benzoyl (Bz) for N-6 of dA (45), anisoyl (An) (less frequently) (46) or benzoyl (Bz) for N-4 of dC and isobutyryl (iBu) for N-2 of dG (47), see *Fig. 1.8*. The thymidine residue is usually not protected in DNA synthesis since it does not carry any exocyclic amino function.

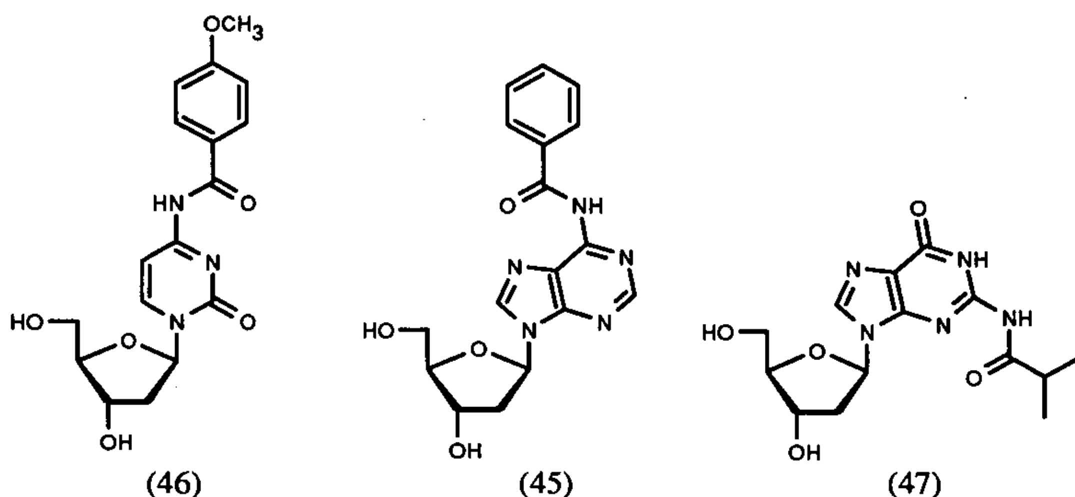


Fig. 1.8: Common protecting groups for dC, dA and dG

These protecting groups are introduced by acylation of the parent deoxynucleosides. The amino groups of adenine and guanine being too weakly basic for selective reaction, two acylation procedures were developed to overcome this problem: the classic method developed by Khorana[101] in 1972 involves prior peracylation of the nucleosides and subsequent removal of the O-acyl groups by treatment with sodium hydroxide in alcohol, see *Fig. 1.9* route A (exemplified with dA N-protection). The selectivity here is due to the greater stability of amides than esters at high pH. This rather tedious process involving a complicated work-up procedure,[102] was later replaced by the transient protection method developed by

Jones *et al.*,[103] see *Fig. 1.9* route B (exemplified with dA N-protection). The term "transient protection" is based on persilylation of the nucleoside, followed by *in situ* N-acylation and silyl ether hydrolysis yielding the N-acylated nucleobases in a one pot reaction. In the example shown above, benzoyl chloride is similarly used with dC but in the case of dG, acylation is effected with isobutyric anhydride; all these protecting groups can be removed using concentrated ammonium hydroxide.

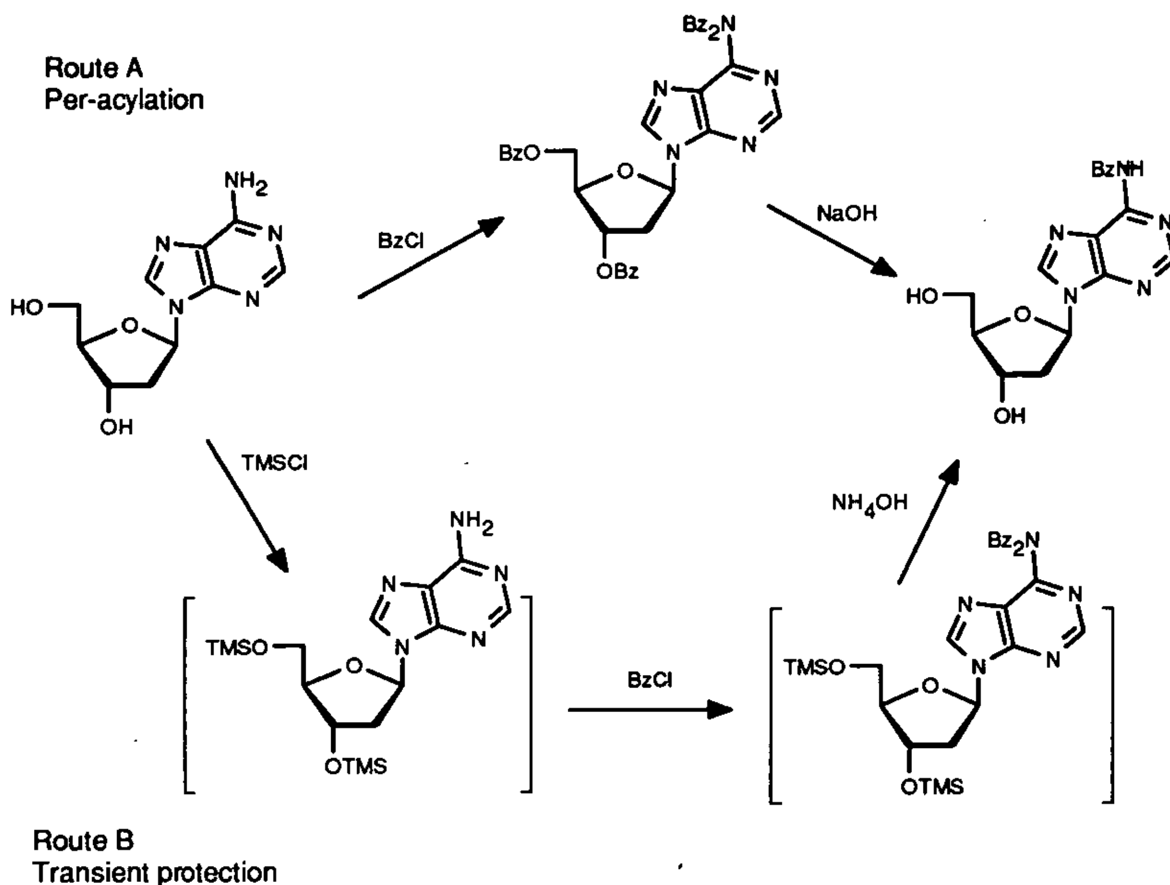
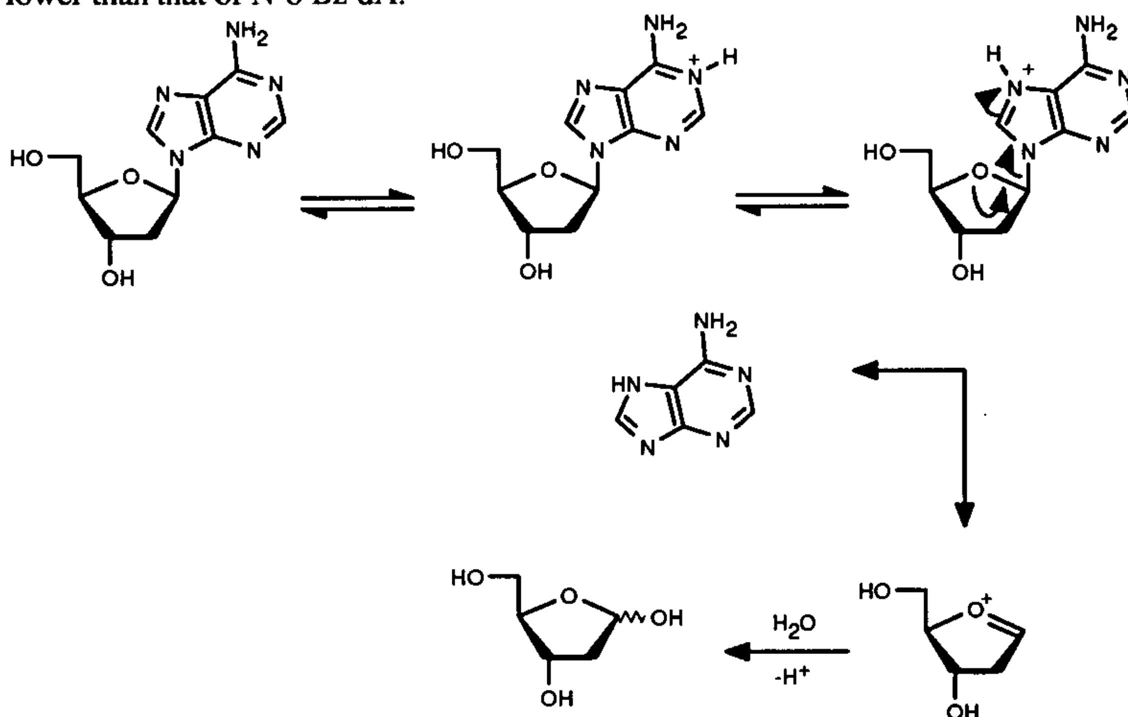


Fig. 1.9: Route to N-6-benzoyl-2'-deoxyadenosine

The protection of deoxyguanidine and especially deoxyadenosine requires special comment, because the chemical stability of the nucleoside is profoundly altered by the protection of the exocyclic amino function. Purine nucleotides and oligonucleotides can loose their purine residue in either strongly basic or moderately acidic conditions, leaving an unsubstituted sugar. Since the growing chain is

repeatedly subjected to acid treatment (during removal of DMTr), the risk of depurination always exists. The mechanism of acid depurination involves a rapid pre-equilibrium of protonation or diprotonation of the purine residue, followed by the rupture of the glycosidic bond that is the rate determining step.[104,105] The acidic depurination mechanism of deoxyadenosine is shown in *Scheme 1.10*. The site of the first protonation is N-1, only a small concentration of the N-7 monoprotonated form may exist at equilibrium. The acidic depurination is thus limited by the rather difficult access to a N-7 protonated form of deoxyadenosine. If, however, the adenine residue is N-6 acylated, the site of first protonation becomes N-7. Since a carbocation is generated at C-1' in the rate determining step, electron withdrawing substituents on the 3'- and 5'-oxygen atoms (like phosphate groups) reduce the extent of depurination. Depurination of deoxyadenosine is lower when it is in the middle of a sequence than when used as a nucleoside. The site of protonation in deoxyguanosine is N-7, and the site of acylation is N-2. The tendency of N-2 iBu-dG to depurinate is lower than that of N-6 Bz-dA.[106]



Scheme 1.10: Acidic depurination mechanism of deoxyadenosine

In an effort to prevent depurination side-reactions to occur, Froehler and Matteucci[72] introduced the N-6 di-n-butylformamidine (N-6 di-n-butylaminomethylene) group (48) (~~commercialized under FOD~~) which greatly reduced depurination of deoxyadenosine and could be removed under mild ammoniacal conditions, see *Fig. 1.10*. Schulhof *et al.*[107] then developed the phenoxyacetyl group for deoxyadenosine (49) and deoxyguanidine (commercialised under PAC), and obtained similar results than with FOD nucleotides. These groups prevent N-protonation through steric factors as well as their electronic environment and thus minimize depurination. More recently the 2-(tert-butyldiphenylsilyloxy methyl)benzoyl group (SiOMB) has been reported by Dreef-Tromp *et al.*[108] to be ideal for the protection of dC, dA and dG, and is rapidly removed by fluoride ion. Another promising protecting group for all three heterocycles is the allyloxycarbonyl group,[109] removable with a palladium (0) complex in formic acid. In the reported assembly of a 60-mer in which the phosphate protection was also changed to allyl, the allyl and allyloxycarbonyl groups were simultaneously removed in nearly 100 % yield.

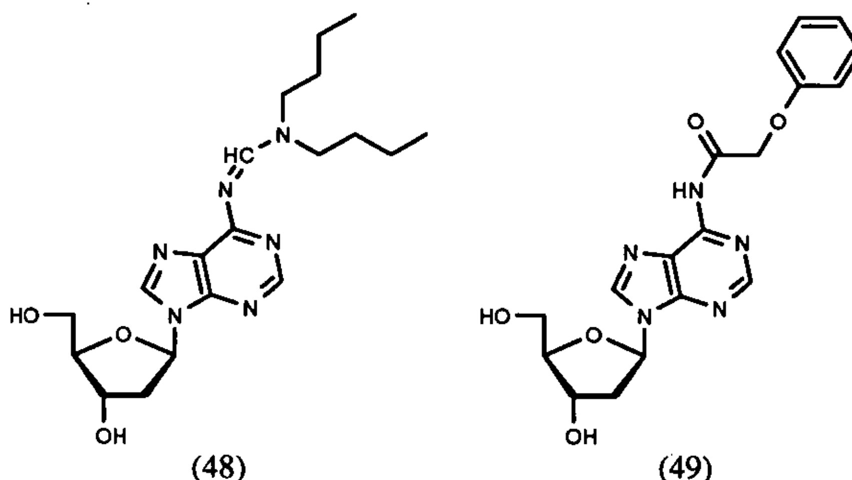


Fig. 1.10: N-6 di-n-butylformamidine protected deoxyadenosine

Hata *et al.*[110,111] investigated the use of the monomethoxytrityl (MMTr) group as an amino protecting group for dC, dA and dG and the dimethoxytrityl (DMTr) group for dA. Both these groups are deprotected with dilute HCl but remain intact when the DMTr group on the 5'-hydroxyl is deprotected with zinc bromide.

A complication with dG is that the O-6 position is vulnerable to reaction with certain reagents used in oligonucleotide synthesis, particularly in the case of the coupling agents (sulphonylation) and phosphorylating agents used in the phosphotriester method.[112,113] One of the best protecting groups was proposed by Pfleiderer *et al.*[114] who used the *p*-nitrophenylethyl (NPE) group to protect dG, dT and U on the oxygen of their amide functions. NPE could be deprotected *via* β-elimination with DBU in pyridine at the same time as it is removed from the phosphate triester. The efficiency of this group was later confirmed when Pon *et al.*[115] synthesized a 36-mer with this dG O-6 protecting group. Another promising O-6 protecting group is the β-cyanoethyl (CNE) group developed by Gaffney *et al.*,[116] cleaved with triethylamine or DBU *via* β-elimination. Several other workers have investigated different oxo and imido protecting groups, e.g. Reese *et al.*[117] described the phenyl, the 2,4-dimethylphenyl and the 2-nitrophenyl groups, which may all be removed by oximate ions; Gaffney and Jones[118] used the trimethylsilyl group which is cleaved under neutral conditions with TBAF; Chattopadhyaya[119] used β-elimination to cleave the Fmoc protecting group and Hata *et al.*[120] developed the (butylthio)carbonyl group cleaveable in aqueous ammonia. These protecting groups often have the second advantage of increasing the lipophilic character of the intermediate products and thus lead to better crystallization and purification.

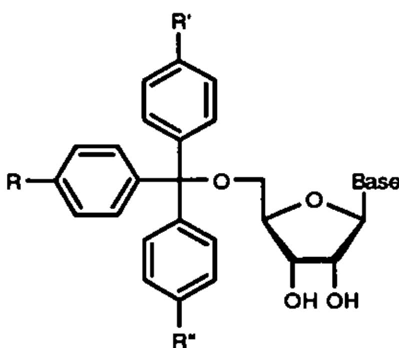
1.6.3 Protection of the Deoxy-Ribose

1.6.3.1 Introduction

The chemical synthesis of oligonucleotides necessitates the proper protection of sugar hydroxyls. The 2'-deoxyribose sugar in DNA and the ribose sugar in RNA have two and three hydroxyl functions respectively. The 5'-hydroxyl, being a primary alcohol function , it is more reactive than both the 3', and where it occurs, the 2'-hydroxyl functions which are both secondary alcohols. Hence the protecting group strategy for RNA is more complex than that of DNA because as well as having one more hydroxyl to protect, it has to differentiate between two alcohols. The first protecting groups used for sugar protection were acetyl[3,38] and benzoyl[121] both cleaved by alkaline hydrolysis, but to fit in with the modern oligonucleotide synthesis strategy, acid labile protecting groups were developed. In the solid phase approach in particular, when frequent deprotection is required, basic media and nucleophiles would degrade the succinate link with the support, the triester and the N-acyl functions of the growing sequence.

1.6.3.2 Primary 5'-OH Protecting Groups

The mono- and dimethoxytrityl groups originally used by Khorana *et al.*,[121] continue to be the most popular 5'-hydroxyl protecting group, *Fig. 1.11*. The trityl group was found to be too stable in 80 % acetic acid[122] and so to make it more labile a methoxy group was introduced on the phenyl ring. The introduction of a *p*-methoxy substituent enhances the rate of acid cleavage by a factor of 10; the dimethoxytrityl group (DMTr) was finally found to have the adequate acid lability for the solid phase synthesis of oligonucleotides.



R=R'=R"=H or R=R'=H, R"=OCH_3 or R=H, R'=R"=OCH_3 or R=R'=R"=OCH_3

Fig. 1.11: The trityl protecting groups

The 4,4'-dimethoxytrityl (DMTr) protecting group presents several advantages: it is a very selective alkylating agent for the primary 5'-hydroxyl; the protected nucleosides are lipophilic, rendering solvent extraction and chromatographic purification easier; upon treatment with an acidic spray on t.l.c, the DMTr substituted compounds are specifically stained; the rate of acid deprotection is high, the deprotection is quantitative and the yield of deprotection can be monitored spectroscopically during synthesis on the solid support. Strong organic acid under anhydrous conditions is usually used for the recurrent deprotection in solid phase oligonucleotide synthesis. The duration of this treatment requires to be strictly controlled, moreover it was found that strong acid as for example benzene sulphonic acid and related acids cause extensive depurination of N-6-benzoyl(dA).[123] A weak concentration of dichloroacetic acid (TCA) in an aprotic solvent is the reagent of choice as its action is rapid (< 2 min) and depurination is kept to a minimum.[61] The most selective reagent for the cleavage of the 5'-O-DMTr group without depurination is zinc bromide in an aprotic solvent, unfortunately this reaction is too slow for a solid supported synthesis.[124] Interestingly, removal of DMTr groups using 80% acetic acid at the end of the oligonucleotide synthesis is easily accomplished with no depurination, provided the base N-acyl groups have been removed.[12]

In 1978, Chattopadhyaya and Reese[125] developed the 9-phenylxanthen-9-yl (pixyl) protecting group, *Fig. 1.12*, whose acid lability is comparable to the dimethoxytrityl group, but whose deoxynucleosides unlike dimethoxytritylnucleosides can almost invariably be induced to crystallise, hence facilitating their purification. Reese *et al.*[126] later developed a number of pixyl structures with the intention of making a wide range of acid sensitive pixyl derivatives which promoted the adaptability of this primary alcohol protecting group to various chemical conditions. Nevertheless, the dimethoxytrityl group remains the most frequently used group, probably because of the general commercial availability of the corresponding chloride. Other acid labile groups were developed, like the *tert*-butyldimethylsilyl group[127] and the *tert*-butyldiphenylsilyl group[116] which could be cleaved not only in 80% acetic acid but also by tetra-*n*-butylammonium fluoride (TBAF) which is particularly useful for acid sensitive nucleosides.

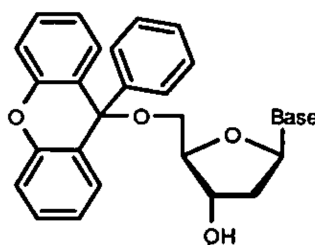


Fig. 1.12: A 5'-O-pixyl protected nucleoside

Several examples of base labile hydroxyl protecting groups have been reported. Khorana and Weimann[128] investigated the pivaloyl group and Letsinger and Ogilvie proposed the *p*-nitrophenyl group[129] and the isobutyloxycarbonyl group[130] which have good selectivity for the primary hydroxyl. Pfleiderer *et al.*[131] used the β-elimination process to cleave the 2,4-dinitrophenylethoxycarbonyl group with tertiary amines. This group was found to be more base sensitive than the β-cyanoethyl group.

Chattopadhyaya[132] introduced the fluoren-9-ylmethoxycarbonyl group (Fmoc) to DNA synthesis from peptide synthesis. This group is also cleaved by β-elimination using a base such as triethylamine or piperidine and its use as a 5'-protecting group was demonstrated by the synthesis of (dT)8. He also showed that this group is compatible with the phosphoramidite method[133] on solid phase by synthesizing an oligonucleotide and using DBU for the recurrent removal of Fmoc.

The concept of a "protected" protecting group was introduced by Reese *et al.*[134] by using the 2-dibromomethylbenzoyl group whose ester is comparatively stable to acid and alkali but when oxidised with silver perchlorate to the aldehyde and then treated with morpholine yields the free alcohol. Seliger *et al.*[135] introduced 4-alkoxytrityl protecting groups as novel type of hydrophobic protection for the 5'-end of oligonucleotides, consequently varying the chromatographic behaviour of oligomers which proved to be useful in their purification. By replacing the phenyl by a pyrenyl substituent in the dimethoxytrityl group, Fourrey *et al.*[136] obtained a fluorescent acid labile protecting group which exhibited similar chemical properties to those of DMTr but enabled easy UV detection of the products. Fisher and Caruthers[137] also developed the idea that the colour of acidic solutions containing triaryl methyl cations is directly influenced by the nature of the aryl substituents attached to the central carbon atom, they demonstrated that almost any colour can be produced by subtle substitution. When the four nucleotide monomers were protected with a different triaryl methyl group, then the collection of the consecutive coloured trityl cation solutions could give a simple and rapid check on the veracity of the synthetic sequence.

1.6.3.3 Secondary 3'- and 2'-OH Protecting Groups

DNA synthesis sometimes requires 3'-protection, this is most frequently achieved with the base labile acetyl group, first used by Todd,[3] and later Khorana.[128] In the case of ribonucleotides the 2'-hydroxyl functions are often protected with a different protecting group from the 3'-hydroxyl functions, for this reason Ogilvie *et al.*[138,139] developed the *tert*-butyldimethylsilyl (TBDMS) group for the selective protection of the 3'-hydroxyl. Pfleiderer *et al.*[131] also developed the *p*-nitrophenylethyl group as a 3'-hydroxyl protecting group. The 2'-hydroxyl occurs only in RNA and must be protected as RNA is sensitive to alkaline hydrolysis. It usually involves the generation of a 2'-oxyanion which subsequently attacks the neighbouring internucleotide linkage leading to migration of the phosphate to the 2'-position or to chain cleavage. For a long time the most popular protecting groups for the protection of the 2'-hydroxyl during RNA synthesis had been the acid labile tetrahydropyran-1-yl (THP) group and the 4-methoxyhydropyran-4-yl (MTHP) group;[48] the former having the disadvantage of forming a mixture of diastereoisomers during the protection reaction of a nucleoside. However these two protecting groups are not completely stable to the recurrent acid treatment used for the deprotection of the 5'-DMTr group. Van Boom *et al.*[140] exchanged the ring oxygen for sulphur to produce a thioether and in a different experiment an SO_2 group to give a sulphone. These groups are achiral, the former being slightly more acid labile and the latter considerably less acid labile than the MTHP group. Two acid stable protecting group were then developed, the *o*-nitrobenzyl group[141] removed by irradiation with UV light in a solution of ammonium formate and the TBDMS group removed by TBAF and first introduced by Ogilvie.[142] 2'-Silyl ribonucleoside phosphoramidites are now commercially available but loss of the 2'-silyl groups and subsequent internucleotide cleavage during ammonia deprotection has been acknowledged; the conditions for ammonia deprotection had to be altered and a new base protecting group removable under milder ammoniacal

conditions had to be used.[143] In the search for a protecting group which would be stable to strongly acidic conditions but cleaved at higher pH, Reese[144] first suggested the 1-(2-chloromethyl)phenyl-4-methoxypiperidin-4-yl (Cpmp) group and subsequent workers[145] justified the use of the more effective 1-(2-fluorophenyl)-4-methoxypiperidin-4-yl (Fpmp) group, see *Fig. 1.13*, which was cleaved faster at pH > 1.0. 2'-Fpmp protected nucleoside phosphoramidites are expected to become widely available commercially and will probably rival their 2'-silyl counterparts in terms of effectiveness.

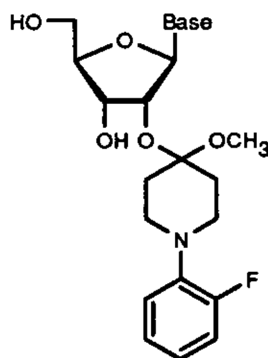


Fig. 1.13: The Fpmp protecting group

Multiple hydroxyl protection has also been achieved, an example of this is the reaction of *p*-nitrophenyl chloroformate and a ribonucleoside which results in the formation of a 2',3'cyclic carbonate[146] which can be removed by alkaline hydrolysis. A bifunctional silylating reagent 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane, known as the "Markiewicz reagent"[147] has significantly improved synthetic routes. It distinguishes between the 2'- and the 3'-hydroxyl on a ribose by selective protection of the 5'- and 3'-hydroxyls leaving the 2'-hydroxyl free to react with another protecting group. The "Markiewicz reagent" can be removed with fluoride ions.

1.7 Purification of Synthetic Oligodeoxynucleotides

1.7.1 Introduction

The deprotection of an oligonucleotide involves removing the various protecting groups and cleavage from the solid support. Incomplete deprotection will adversely affect analysis and purification, as well as biological activity. The traditional automated solid phase synthesis of oligodeoxyribonucleotides usually involves treatment of the final product with concentrated ammonium hydroxide to simultaneously cleave the product from its support and deprotect the cyanoethylphosphate groups. Deprotection of the exocyclic amines is achieved by heating the sample in concentrated ammonium hydroxide for a minimum of 4 h at 55°C. The phosphoramidite synthesis also employs a capping scheme that acetylates the 5'-hydroxyl functions that have failed to undergo coupling. These truncated sequences remain capped during the synthesis but are uncapped during base deprotection in ammonia. The product oligonucleotide which is not acetylated, has a 5'-hydroxyl that is protected by a DMTr group, which can be automatically removed by the instrument or retained if trityl-on purification methods are used. Therefore, the crude mixture dissolved in ammonium hydroxide from the final deprotection contains a variety of impurities, including extraneous ammonium salts derived from the removed protecting groups. It also includes the desired full length oligonucleotide product, shorter failure sequences resulting from incomplete coupling and subsequent capping, a collection of oligonucleotides resulting from other low level side-reactions that were cleaved during ammonolysis and organic by-products resulting from the removal of protecting groups, e.g. benzamides. Therefore one can understand that a rapid and efficient separation method is of great importance to obtain satisfactory purity of the final product.

For most applications of synthetic oligodeoxynucleotides in molecular biology, where only a small amount (≤ 1 O.D. unit, ~40 μg) is required, this amount of material can be easily purified by analytical high-performance liquid chromatography (HPLC). For purer and for higher amounts of oligodeoxynucleotides, it is necessary to apply a combination of two orthogonal methods of purification: (a) either anion-exchange HPLC or trityl-on reverse-phase HPLC (RP-HPLC) and (b) trityl-off reverse-phase HPLC. Method (a) separates the full-length product from failure sequences, and Method (b) separates the desired product from sequences containing base modifications. Polyacrylamide gel electrophoresis (PAGE) is also applied to oligonucleotide purification as a powerful technique; in particular for long sequences beyond 100 residues where single nucleotide resolution is possible, however it is limited to small scales (< 1 mg). A good review on the practical aspect of synthetic DNA purification has been published by Gait[61] and Brown and Brown.[148]

1.7.2 Anion-Exchange Chromatography

Ion-exchange chromatography is a widely used technique for the purification and analysis of charged compounds, such as oligonucleotides. The separation is based largely on charge per unit weight, which is determined by the base length (the number of phosphate groups in the molecule) and the base composition (the respective charges on the bases), the size of molecules to be separated is also an important criteria as the stationary phase is macroporous. The separation of the crude mixture is accomplished by slowly increasing the ionic strength (concentration) of the mobile phase. The desired product is always eluted later than the shorter sequences. Phosphate groups can be masked by secondary structures which will exhibit a different charge ratio, however this phenomenon can be minimized by addition of a

denaturant, such as formamide in the mobile phase. Anion-exchange HPLC should also be carried out with the products of the trityl-off syntheses, as trityl-on oligonucleotides elute very late by this technique and therefore an extremely shallow gradient is necessary. The resolution also decreases as longer oligonucleotides are separated because the relative difference in charge between longer oligonucleotides is less than between shorter oligonucleotides. Moreover peak broadening effects start to appear. This method usually uses standard anion-exchange columns, such as Aquapore AX-300 or Partisil 10 SAX. The mobile phase is 20 % acetonitrile in a potassium phosphate buffer system, pH 6.4. Standard gradient is 100-400 mM of the phosphate buffer over 30-40 min. Oligonucleotides greater than 25 bases usually require a higher concentration of phosphate buffer, however anion-exchange HPLC purification is limited to oligonucleotide lengths up to 50 monomer units.

1.7.3 Reverse-Phase Chromatography

When the reverse-phase, trityl-on chromatography method is used to purify synthetic oligonucleotides, it is necessary for the final 5'-DMTr group to be left attached to the oligonucleotide product. This is usually carried out by simple programming on the synthesizer, the oligonucleotide is then completely deprotected with concentrated ammonia except for the DMTr group. The product is a mixture of the full-length tritylated sequence and a number of untritylated failure sequences. Separation of the desired product by RP-HPLC is made easy as the DMTr group is lipophilic. Normally the first eluted peak contains the oligonucleotides corresponding to the failure sequences (hydrophilic) and the second peak is the desired DMTr bearing product (hydrophobic). A typical RP-HPLC trace of a 6-mer can be seen in section 2.6.2. Analysis and purification of trityl-on oligonucleotides is generally accomplished with a C-8 or sometimes a C-18 RP-column such as, Aquapore RP-300

or Spheri-5. The mobile phase most often used for RP-HPLC analysis and purification of oligodeoxyribonucleotides is an increasing gradient of acetonitrile in 0.1M triethylammonium acetate (TEAA) or 0.1M ammonium acetate, pH 7.0. UV detection of the oligonucleotide products is usually achieved at wavelengths between 260 to 295 nm. Typical gradients useful in the trityl-on and trityl-off method can be seen in the experimental section. Generally the retention time of tritylated oligonucleotides decreases as chain length increases, because of the increasing hydrophilic character introduced by the increasing proportion of nucleotide units over the proportion of the hydrophobic DMTr group.

The trityl-on peak fraction is collected during chromatography and the product is then detritylated to render a biologically active compound. After evaporation of the solution under vacuum, the trityl-product is treated with 80 % acetic acid at room temperature. This acidic treatment does not cause depurination because the bases are much more stable to acid after evaporation. The volatiles are then removed by several co-evaporations with distilled water and the oligonucleotide product is then extracted with ether or precipitated with ethanol or desalted on a gel size-exclusion matrix before being subject to the second stage of purification: the trityl-off RP-HPLC.

There are a number of limitations to the RP-HPLC purification technique for oligodeoxyribonucleotides: if the capping reaction is not efficient, the desired product will coelute with a number of trityl-on impurities. Full length sequences that have undergone some base modification will not be resolved from the desired product as the difference in their hydrophobicity can be minimal. The trityl-on oligonucleotides must be handled carefully as some detritylation can occur due to the acid lability and the sensitivity to heat of the trityl group. It is therefore advised to dissolve the trityl-oligonucleotides in an aqueous buffer between pH 7.0 and 7.5 before and during trityl-on HPLC. In spite of these drawbacks, the trityl-on RP-HPLC purification

method, when carried-out carefully, remains the method of choice for longer oligonucleotides (> 25 residues to beyond 100 residues) and large scale synthesis (g scale).

Trityl-off RP-HPLC with single-base resolution can often be achieved, at least to 25 bases and sometimes longer, depending on the sequence. In that case, the last DMTr group on the 5'-end of the oligonucleotide is removed prior to ammonia deprotection, usually on the DNA synthesizer. The product identification is however not as obvious or predictable as in trityl-on HPLC. The main advantage is that the product recovered after purification is in a ready to use form. However the trityl-off RP-HPLC method is more often used in the final HPLC purification step, following either trityl-on RP-HPLC or anion-exchange HPLC, and therefore attaining the objective of combining two orthogonal approaches to purification.

In trityl-off HPLC, the sample preparation is much simpler than with trityl-on HPLC. The product does not bear a DMTr group and is therefore more stable during handling. The ammonia solution containing the crude mixture is generally evaporated *in vacuo* at 50°C and the residue can be redissolved in distiled water to be injected into an HPLC. Analysis and purification of trityl-off oligonucleotides is generally accomplished with similar equipment as in the trityl-on method; a C-8 or sometimes a C-18 RP-column such as Brownlee Aquapore RP-300, moderately hydrophobic with large average pore size. The mobile phase most often used for RP-HPLC analysis and purification is an increasing gradient of acetonitrile in 0.1 M triethylammonium acetate (TEAA) or 0.1 M ammonium acetate, pH 7.0. UV detection of the oligonucleotide products is usually achieved at wavelengths between 260 to 295 nm. Compounds with shorter length than the product elute faster than the product as they are less hydrophobic. The product peak is collected so as not to include any of the shoulder peaks; an analytical HPLC of this fraction is then carried out in order to confirm the

purity. If some impurity remains, the entire procedure must be repeated. Once the product has been purified it is desalted by precipitation in ethanol or eluted on size-exclusion chromatographic columns. A rapid extraction method using n-butanol was recently devised and obviates the need for prior ammonia or buffer removal.[149]

Problems can sometimes arise with RP-HPLC for example when the sequence to be purified is deoxyguanosine rich[150] or if it contains palindromic regions; it can result in multiple peaks with unpredictable retention times. The ability of reverse-phase adsorbents to resolve oligonucleotides diminishes as oligonucleotide length increases. Longer oligonucleotides that differ in length by a single base exhibit small differences in hydrophobicity, resulting in loss of resolution. The conditions under which RP-HPLC is carried out has little denaturing effect on oligonucleotide that form stable conformations.

1.7.4 Gel Electrophoresis

Resolution of DNA fragments with only one charge difference is possible by electrophoresis on a polyacrylamide gel in the presence of a denaturing agent such as urea. Polyacrylamide gel electrophoresis (PAGE) is now widely used for oligonucleotide analysis and purification. Usually a polymerized, cross-linked acrylamide gel matrix is held between two glass plates and the ends of the gel slab are immersed in buffer chambers containing an electrolytic salt solution through which an electric field of controlled voltage or current is applied. The charged molecules of DNA migrate through the gel matrix, separating on the basis of charge and mass. The rate of migration of molecules to a lesser extend also depend on molecular shape, net hydrophobicity and size.

The dimension and shape of the slab gels are not essential as long as the minimal length is 15 to 20 cm and a thickness of 0.4 to 1.6 mm. The more critical choice is the size of the sample wells as best resolution for preparative samples is obtained by using the sample band as narrow as possible, e.g. 1cm width is useful for sample loads of ~1 O.D. unit. Common electrophoresis buffer consists of a solution of tris base, boric acid and EDTA. A high concentration of urea is maintained in the gel in order to minimize the formation of secondary structures with single stranded oligonucleotides. The polyacrylamide concentration is also varied depending on the oligonucleotide length. Higher concentrations decrease the average pore size of the gel therefore slowing migration and increasing separation of the sample molecules. A polyacrylamide concentration of 20 % is commonly used for oligonucleotides < 25 residues long. Oligonucleotides which are suspected of forming secondary structures, are heated to 90°C for several min in a denaturing buffer prior to loading and immediately cooled on ice. The warming of the sample helps in the denaturation process. After electrophoretic separation, the products can be visualised on the gel slab by UV shadowing whereby the gel is transferred to a fluorescent media and observed under short-wavelength UV light. However the interpretation of UV shadowing is subjective and comparison with oligonucleotides in adjacent lanes of known quality and length is required. The band containing the purified oligonucleotide is then excised and the product extracted into an aqueous buffer (Tris-HCl, sodium chloride and EDTA) to be obtained in a pure form.[61] This method is particularly useful for long oligonucleotides (> 50 residues), but is limited to small scale (up to 1 mg).

MicroGel capillary electrophoresis (CE) separates DNA fragments in a gel-filled capillary according to the same ratio of mass to charge than polyacrylamide gel slab electrophoresis. This powerful technique offers single-base resolution beyond 100 bases in a quantitative manner so that the resulting electropherograms can be

displayed, stored and integrated like HPLC chromatograms. The combination of gel materials and heating of the capillary confers a significant denaturing effect to the method. However CE remains in the domain of analytical techniques as the loading scale is limited to 10 μg.

1.8 Labelling of Oligonucleotides

1.8.1 Introduction

Oligodeoxyribonucleotides tagged with various ligands have been widely used as research tools for genetic analysis, to assign gene function and to elucidate mechanisms in molecular biology.[151,152] In addition to their use as hybridization probes[153] they have been used in diagnostic procedures.[154] Labelled oligodeoxyribonucleotides carrying a reporter group now have widespread use for automated sequencing,[155,156] fluorescence microscopy[157] and hybridization affinity chromatography.[158] With the advent of the "Human Genome Project"[159] large-scale automated DNA sequencing has taken on an increased importance.

DNA labelling with radioactive atoms is commonplace in molecular biology, as it offers very sensitive detection of the labelled probes. Radioactive atoms such as ^{32}P are introduced in oligonucleotides by the action of polynucleotide kinase and γ-^{32}P-dATP. DNA radioiodinated probes are required in some diagnostic applications. For this purpose a pyrimidine phosphoramidite is substituted at the 4-position[160] or at the 5-position[161] by a phenyl group via a suitable spacer arm; and after incorporation of the derivative in the DNA synthesizer, the deprotected oligonucleotide can be iodinated by the chloramine T procedure.[160] ^{125}I labelled primers have also been used in polymerase chain reactions in conjunction with a biotin label. Various radio-

isotopes such as ^{35}S, ^{14}C and ^{3}H are currently being used.[162] Although these techniques are very sensitive, they have several major drawbacks. The half-life of a radio-isotope, 14.2 days for ^{32}P, can be inconveniently short, thus requiring repeated labelling of the DNA probe in long term experiments. Radioactive labelling techniques are time consuming, expensive and involve hazardous exposure to ionising radiation for the technician. It was also found that the specific activity of the radio-labelled DNA varies with time and batch. Moreover, these techniques require special training and special equipment to ensure proper shielding, monitoring, containment and waste disposal. For these reasons non-radioactive labelling techniques for oligonucleotides have been developed in preference to radioactive labels.

1.8.2 Non-Radioactive Labelling Groups

In the class of directly detectable labels, the fluorescent dyes are the most prominent; they can be detected visually by means of fluorescence microscopy or fluorimetry. A wide range of fluorescent dyes have been used in the covalent labelling of oligonucleotides, these include among others: fluorescein,[163] 4-chloro-7-nitrobenzo-2oxa-1-diazole (NBD),[164] Texas red,[164] dansyl[165] and bathophenanthroline-ruthenium (II) complexes[166] derivatives, see *Fig. 1.14.* Fluorescently-labelled oligonucleotides are finding considerable application in DNA sequencing and more especially as diagnostic reagents. A set of phosphoramidite reagents useful in preparation of 5'-fluorescence-tagged oligonucleotides for DNA sequencing has been developed by Du Pont.[167] The fluorescent labels can be chosen with regerd to their fluorescence wavelength maxima, allowing simultaneous detection of different oligonucleotides labelled with different fluorophores. This approach is applied to Sanger's enzymatic method of DNA sequence analysis.[164,168]

The use of a single fluorophore in automated DNA sequencing has also been reported. Fluorescent *in situ* hybridization (FISH) techniques[169] use labelled primers for the mapping of genes in cancer diagnosis, where fluorescence microscopy is used to detect the position of the hybridized fluorescent oligonucleotide.

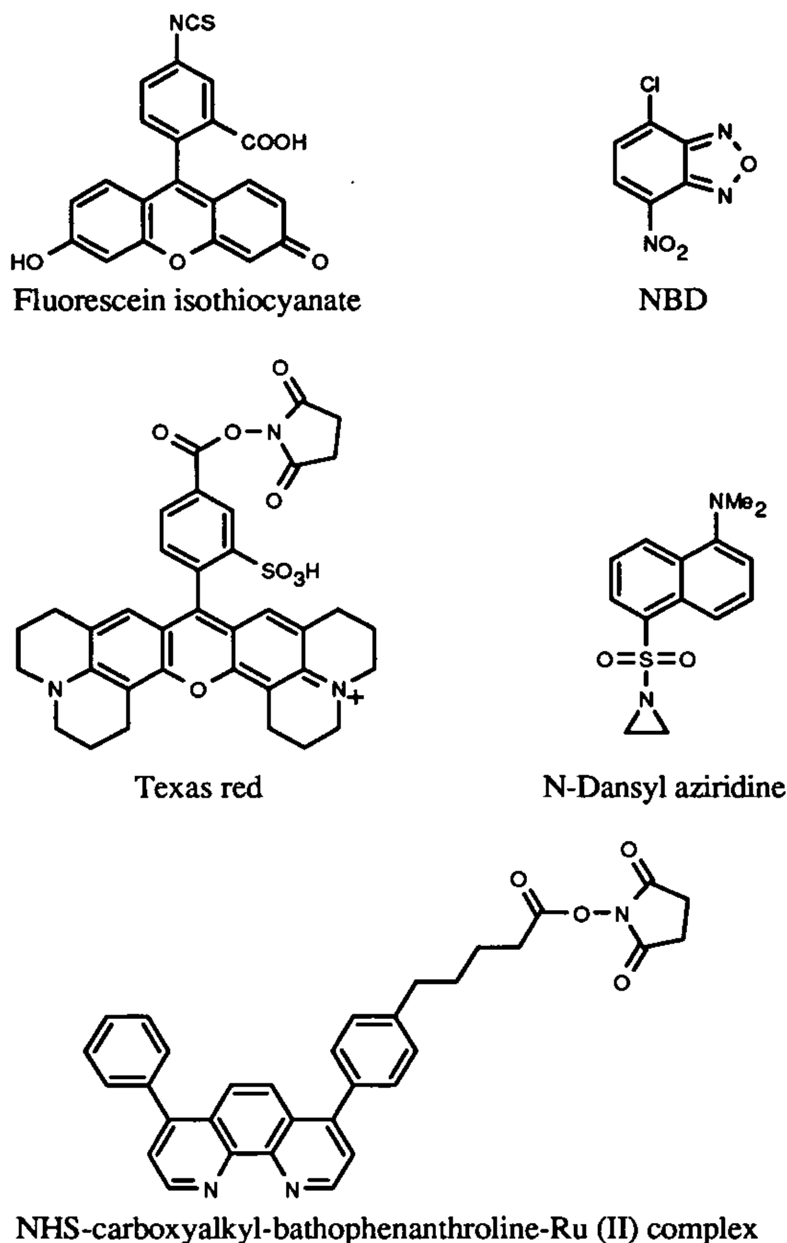


Fig. 1.14: Fluorescent labels

Enzymes have also been attached covalently to oligonucleotides, and are detected by addition of an enzyme substrate which produces either a coloured product or a light signal *via* a chemiluminescent reaction. Such enzymes include alkaline phosphatase (AP) or horseradish peroxidase (HRP).[170,171] The colorimetric detection of AP is usually carried out using a mixture of 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium, resulting in the production of a blue-purple precipitate.[172] The use of the enzyme HRP offers a wider range of substrate available, for instance 3,3'-diaminobenzidine which produces an insoluble coloured precipitate. Other substrates produce soluble conjugates which are useful in the quantitative measurement of binding affinity by spectrometry. When HRP is bound to an oligonucleotide, it can oxidize 3-aminophthalhydrazide (luminol)[173] in the presence of hydrogen peroxide to produce chemiluminescence. The intensity of the chemiluminescence can be increased by addition of some enhancers such as *p*-hydroxycinnamic acid or *p*-iodophenol. Alkaline phosphatase (AP) has also been found to produce chemiluminescence when reacted with the dioxetane generated by photooxygenation of 3-phosphate-9H-xanthen-9-ylideneadamantane monopyridinium salt, see *Fig. 1.15*.[174] Chemiluminescent detection offers several advantages over colorimetric detection: the background is theoretically zero, detection is very rapid, measurement of the observed signal is relatively easy using a charge-coupled device and the labelled probe can be washed off allowing several hybridizations to be carried out. On the other hand the synthesis and purification of the enzyme-oligonucleotide conjugate is expensive and time consuming, only one enzyme can be attached per oligonucleotide and the activity of the enzyme may be impaired.[170,173]

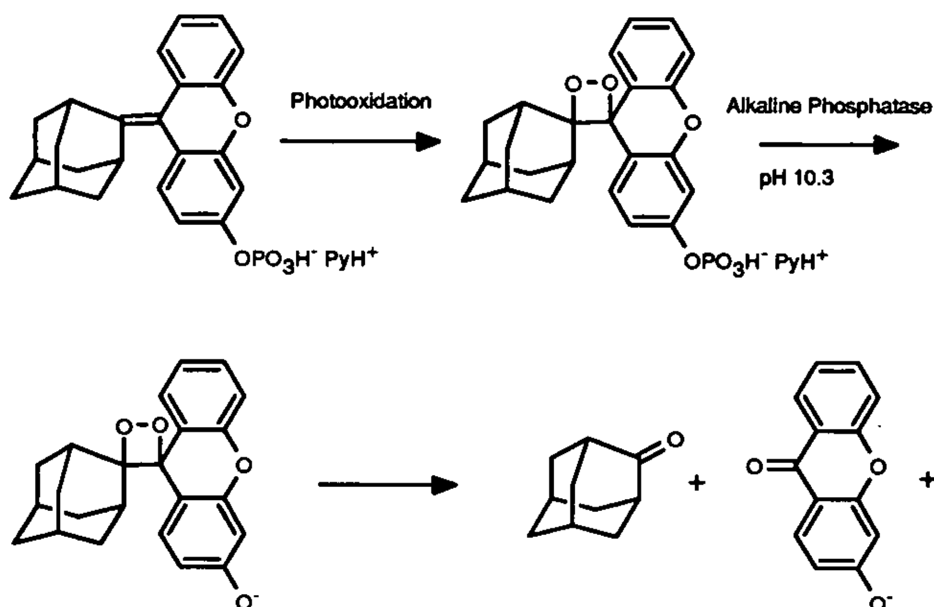


Fig. 1.15: Chemiluminescence for Alkaline Phosphatase

Another class of labelling group involve those that are detected indirectly. Biotin is in fact the non-radioactive labelling agent most frequently used to tag oligonucleotide probes primarily because of its exceptionally strong binding to the egg white glycoprotein avidin and with its non-glycosylated bacterial counterpart streptavidin, both of which have four biotin binding sites.[174] For the detection of the biotinylated oligonucleotide, this is incubated with an avidin (or steptavidin) -enzyme conjugate which results in the formation of an oligonucleotide-biotin-avidin-enzyme complex which in turn is incubated with the appropriate enzyme substrate to give a colorimetric or chemiluminescent signal.[175] Anti-biotin antibodies have also been used for detection.[176] However biotin is a expensive hapten and is unsuitable for use in certain *in situ* hybridization applications due to the high level of endogeneous biotin present in some tissues. Various other indirectly detectable labels have been incorporated into oligonucleotides whose antibody had been raised and is obtainable in reasonable quantities. Phosphotyrosine phosphoramidite for example has been reacted with an oligonucleotide and subsequently detected using a commercially available anti-phosphotyrosine antibody followed by a secondary antibody-HRP

conjugate.[176] Even the fluorescent reporter group fluorescein can be detected using anti-fluorescein antibodies.[177]

1.8.3 Introduction of Labelling Groups

The common methods to introduce non-radioactive labels into oligonucleotides are either post synthetically, where the label is attached by an enzymatic or by a chemical reaction after the complete synthesis and purification of the oligonucleotide; or during the solid phase synthesis of the oligonucleotide. The former method is not suitable for automation and is therefore time-consuming whereas the latter can easily be automated and is therefore more convenient.

The enzymatic labelling of oligonucleotide generally involves a modified nucleoside triphosphate carrying the labelling group which can be incorporated into oligonucleotides *via* enzymes such as deoxynucleotidyl terminal transferase which attaches a tail of ribo- or deoxyribonucleotides at the 3'-end.[177] A number of modified nucleoside triphosphates are now available, the most common being 5-(N-(N-biotinyl-ε-aminocaproyl)-3-amino-allyl) uridine 5'-triphosphate (Bio-11-UTP) and 5-((N-biotinyl)-3-amino-allyl) uridine 5'-triphosphate (Bio-4-UTP) and their 2'-deoxyuridine analogues.[177,178] DNP derivatives of 8-aminohexyl adenosine 5'-triphosphate[179] and digoxigenin-O-succinyl-ε-aminocaproyl-[5-(3-aminoallyl)-2'-deoxyuridine-5'-triphosphate] (Dig-11-dUTP)[180] have also been successfully incorporated into DNA. However, enzymatic labelling remains a time-consuming exercise which is used in small scales only.

The post-synthetic approach to the chemical labelling of oligonucleotides involves the attachment of a reactive group (usually a nucleophile such as an aliphatic amine or thiol) to the oligonucleotide either during or after the solid phase synthesis, and subsequent reaction of the reactive group with an activated labelling molecule. These reporter groups tethered to the base residue can therefore be introduced site-specifically (depending upon the sequence location of the base). Typically these methods employ a linker arm containing a primary aliphatic amino function attached to the C-5 position of thymine or the N-4 position on cytosine which subsequently can be reacted with an amino-reactive labelling molecule, such as an active ester or an isothiocyanate. A variety of useful procedures for the attachment of amine reactive labels to base modified oligonucleotides have been described by Urdea *et al.*[171]

A large number of examples where primary amino functions have been introduced onto the 5'-end of an oligonucleotide, exist at the present time. The most prominent example of a 5'-amino linker that has been commercialized under the name "Aminolink 2" by ABI, is the 2-cyanoethyl phosphoramidite of 1-trifluoroacetylaminohex-6-ol. Using this linker, a four step procedure is required to label an oligonucleotide, see *Fig. 1.15*. This useful amino linker is reactive with a wide variety of amine-reactive labelling groups, such as fluorescein isothiocyanate and other fluorophores active esters or isothiocyanates. Other examples of post-synthetic labelling of oligonucleotides involving aliphatic amino-hydroxy-alkyl linkers include the phosphoramidite and H-phosphonate monomers of N-trityl 6-amino-hexan-1-ol developed by Sinha and Cook[181] and the 2-cyanoethyl phosphoramidite of N-Fmoc-aminoethanol among others.[182] Aliphatic amines have also been introduced into oligonucleotides by methods other than phosphorylation. For example at the N-4 position of cytidine via a bisulfite-catalysed transamination reaction[183] or at the C-5 position of uridine via a palladium mediated olefination reaction.[184] But this approach can lead to destabilization of the helix structure as the incorporation of such

functional groups can interfere in the Watson-Crick hydrogen bonding.[185] In addition, this approach to labelling is expensive, mainly due to the cost of the deoxynucleotide precursors.

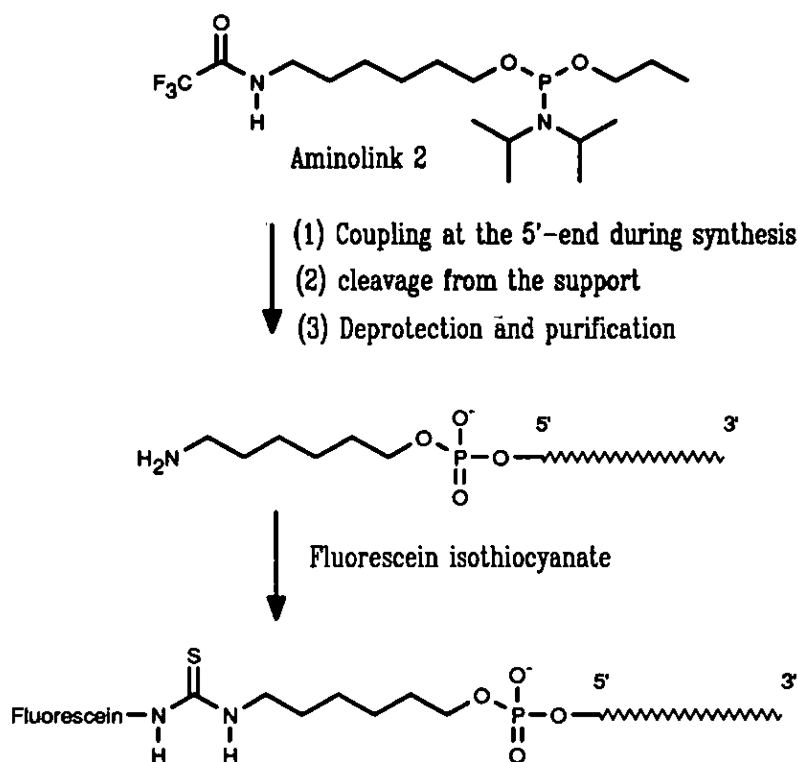


Fig. 1.15: Chemical fluoresceination of amino-modified oligonucleotides

Instead of chemical labelling using a amino linker, some workers have examined the possibility of using thiol linkers followed by reaction with sulphur reactive labelling molecules such as iodoacetamides, γ-bromo-α,β-unsaturated carbonyls and aziridinyl sulphonamides. Thiol linkers have been attached to the 3'-end of oligonucleotides during solid phase synthesis with functionalized supports.[186,187] Usually the thiol function is protected in the form of its disulphide during the synthesis and deprotected after synthesis with dithiothreitol. Alternatively 5'-thiol modified oligonucleotides have been used, based on S-trityl-mercaptoalkanols and H-phosphonate monomers.[181] The trityl group is removed after synthesis using silver

ions to give the free thiol which can be reacted with sulphur reactive fluorophores, such as biotin maleimide. Agrawal and Zamecnik[188] developed the synthesis of oligonucleotides containing two different labels, whereby they first synthesized an oligonucleotide containing both a phosphorothioate-diester internucleotidic linkage and an aminoalkyl phosphoramidate link. An heterobifunctionally labelled oligonucleotide was subsequently obtained by successive reaction of the modified oligonucleotide with a sulphur reactive labelling group and then with an amine reactive labelling group.

A more convenient way to introduce a non-radioactive labelling group onto an oligonucleotide is achieved during the synthesis of the oligonucleotide on the solid support. Generally labelling groups have been used in the form of their phosphoramidite derivatives. This enables the labelling reaction to proceed in an entirely automated way and, additionally, the purification of the labelled oligonucleotide after cleavage from the support is made easier as the product is likely to behave differently in HPLC and PAGE than the unlabelled failure sequences. The attachment of multiple labels can be achieved in a controlled manner, in order to increase the signal and thus improve sensitivity of detection. The biotin phosphoramidite monomer has been developed by Pon[189] and is now the standard way of introducing biotin into oligonucleotides, see *Fig. 1.16*. DNP phosphoramidite monomers[190] as well as fluorescein phosphoramidite monomers[191] have also been developed and successfully applied using the standard automated DNA synthesis conditions, see *Fig. 1.16*. These labelled oligonucleotides were found to be stable to ammonia deprotection and did not show any adverse effect on hybridization with their complementary sequence.

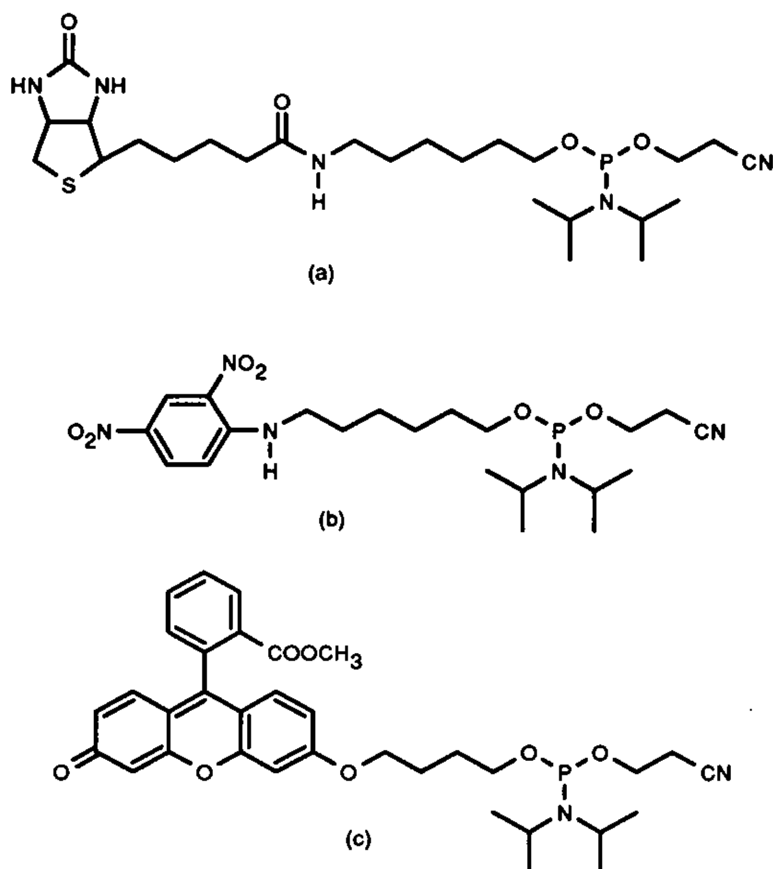


Fig. 1.16: Phosphoramidite labelling monomers; (a) Biotin, (b) DNP, (c) Fluorescein

In order to increase the signal sensitivity of labelled oligonucleotides, Misiura *et al.*[176] introduced the multiple biotin, see *Fig. 1.17*, and phosphotyrosine labelling using a non-nucleoside phosphoramidite based on a 3-carbon glyceryl backbone. The synthesis of the biotin phosphoramidite monomer was achieved by reacting the anion of solketal with acrylonitrile followed by the reduction of the resulting 2-cyanoethyl solketal to give 3-aminopropyl solketal. This amine was then reacted with the N-hydroxysuccinimide ester of biotin, followed by the removal of the isopropylidene protecting group with aqueous HCl and dimethoxytritylation of the primary hydroxyl function, and phosphitylation of the secondary hydroxyl function. The presence of the DMTr group enables the coupling efficiency to be monitored after deprotection and the free hydroxyl function can then react with a further phosphoramidite label. Recently Theisen *et al.*[192] achieved multiple addition of fluorescein using 5-

carboxyfluorescein phosphoramidite and the same backbone as that used by Misiura *et al.*,[176] see *Fig. 1.17*. These labelling group derivatives have the advantage of being soluble in acetonitrile and require minimum changes to the standard protocol used in the phosphoramidite DNA synthesis.

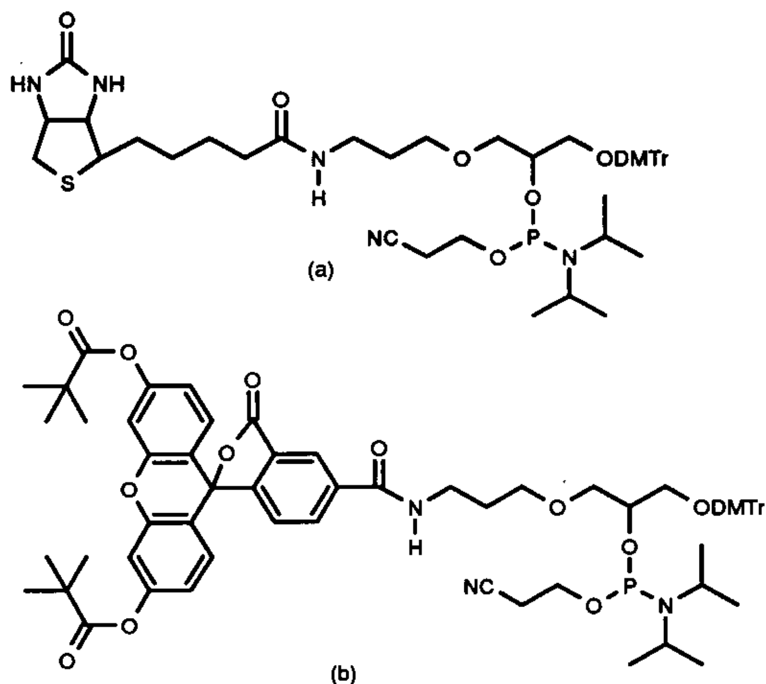


Fig. 1.17: Multiple addition phosphoramidites; (a) Biotin phosphoramidite, (b) Fluorescein phosphoramidite

Chapter 2: Discussion

2.1 Basis for the Design of a Hydrophobic 5'-Protecting Group

In the chemical synthesis of oligonucleotide fragments, chromatographic methods are very important for their purification, as described in section 1.7. Two powerful methods are routinely used, ion-exchange chromatography and the perhaps more widely used reverse-phase HPLC (RP-HPLC) which enables the efficient purification of quantities up to 50 mg.[62] The main chromatographic separation criterion for reverse-phase HPLC is a difference in hydrophobicity of the components in a crude mixture. More commonly, the identification of the desired product is achieved by leaving the 5'-DMTr (di-*para*-anisylphenylmethyl) group on the final monomer so that the product can be resolved from non-5'-DMTr terminated sequences by reverse-phase HPLC.[148] The fraction containing the product can be separated, detritylated and obtained in a pure form following an additional chromatography step.

The chromatographic behaviour of oligonucleotides can be varied within certain limits by attachment of a hydrophobic group at the 5'-terminus; generally an unspecific change in the overall polarity of the molecule is produced. Unfortunately the change in the total polarity introduced by the addition of a single DMTr group is greatly reduced when synthesizing longer oligonucleotides (> 30-mer). Consequently when a

long oligonucleotide is purified by reverse-phase HPLC, the retention time of the 5'-DMTr terminated product is near to the retention time of the failure sequences and separation is not easily achieved.

Several workers have attempted to modify the DMTr group in order to increase its hydrophobic character, see *Fig. 2.1.* Seliger and Görtz[135] introduced *para*-alkyloxy groups on triphenylmethylcarbinol (50). Letsinger and Finnan[193] substituted one phenyl ring by a naphthyl group (51) but strong acidic conditions were required for deprotection, hence producing depurinated by-products. Later Fourrey *et al.*[136] introduced the fluorescent pyrenyl group (52). However in all three cases only the 5'-protected phosphoramidite monomer of thymidine has been successfully prepared. Because of the steric bulk of these protecting groups, the reaction with the 5'-hydroxyls of purine nucleosides proved to be difficult.

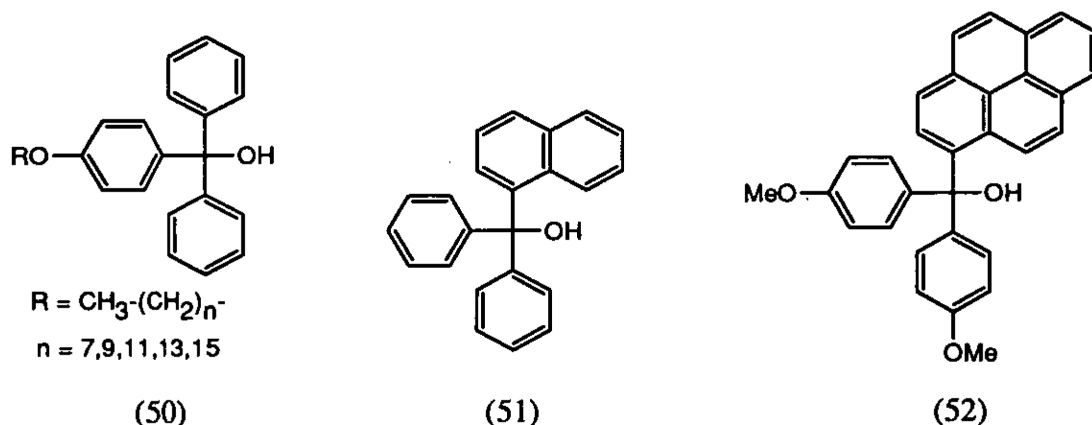


Fig. 2.1: Hydrophobic 5'-OH protecting groups

Recently, Ramage and Raphy[194] demonstrated that amino acid and peptide derivatives of tetrabenzo[*a,c,g,i*]fluorene (53), see *Fig. 2.2*, possess strong hydrophobic properties and were retained on porous graphitised carbon (PGC). However these derivatives were base labile and therefore incompatible with the strategy used in the phosphoramidite chemistry for oligonucleotides. Tetrabenzo[*a,c,g,i*]fluorene, Tbf, (53) nevertheless exhibited some interesting

properties, e.g. strong hydrophobicity and fluorescence both of which could be usefully exploited in oligonucleotide synthesis. Subsequently we investigated tetrabenzo[*a,c,g,i*]fluorene (53) for the design of a highly hydrophobic 5'-hydroxyl protecting group useful in the purification of both short and long oligonucleotides.

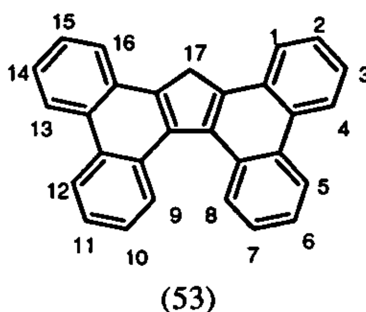


Fig. 2.2: Tetrabenzo[a,c,g,i]fluorene (Tbf)

The strong hydrophobic character of tetrabenzo[*a,c,g,i*]fluorene (53) can influence the polarity of its adducts but a 5'-hydroxyl protecting group requires further properties for practical use in the purification of oligonucleotides:

- The protecting group would be regioselective for primary alcohols in order to leave the secondary 3'-hydroxyl of nucleosides unprotected.
- Removal of the protecting group would be carried out under mild acidic conditions in order to minimize the depurination side-reactions. Hence the protecting group should be very acid labile.
- The introduction of a chiral centre would complicate the purification of derivatives, therefore the protecting group should be achiral.
- To avoid any chemical interaction with the oligonucleotide strand, the protecting group should not contain any reactive functionality.
- The protecting group should be introduced under simple reaction conditions which do not affect the oligonucleotide, in order to be of practical use.

- The protecting group should be accessible in reasonable quantities, using optimised synthesis conditions.
- Finally it would be a great advantage if the protecting group would confer crystalline properties to the protected nucleosides and therefore would ease their purification.

2.2 Tetrabenzo[*a,c,g,i*]fluorene, Historical Background

The design of a new hydrophobic protecting group for oligonucleotide synthesis was primarily based on a benzofluorene structure, see *Fig. 2.3*, because of their strong hydrophobic properties and their reactivity at the methylene bridge, moreover they are known to have fluorescent properties. Polycyclic aromatic compounds containing fluorene have been extensively described in the chemical literature.[195,196,197,198] Monobenzofluorenes and dibenzofluorenes in particular have been reviewed[199,200] and their preparation and chemical reactivity discussed. Perina *et al.*[201] investigated the adsorption properties of benzo[c]fluorene type derivatives on chromatographic supports. However the tetrabenzo[*a,c,g,i*]fluorene system (53) has not been considered in those respects.

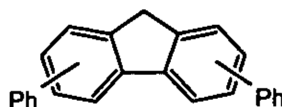
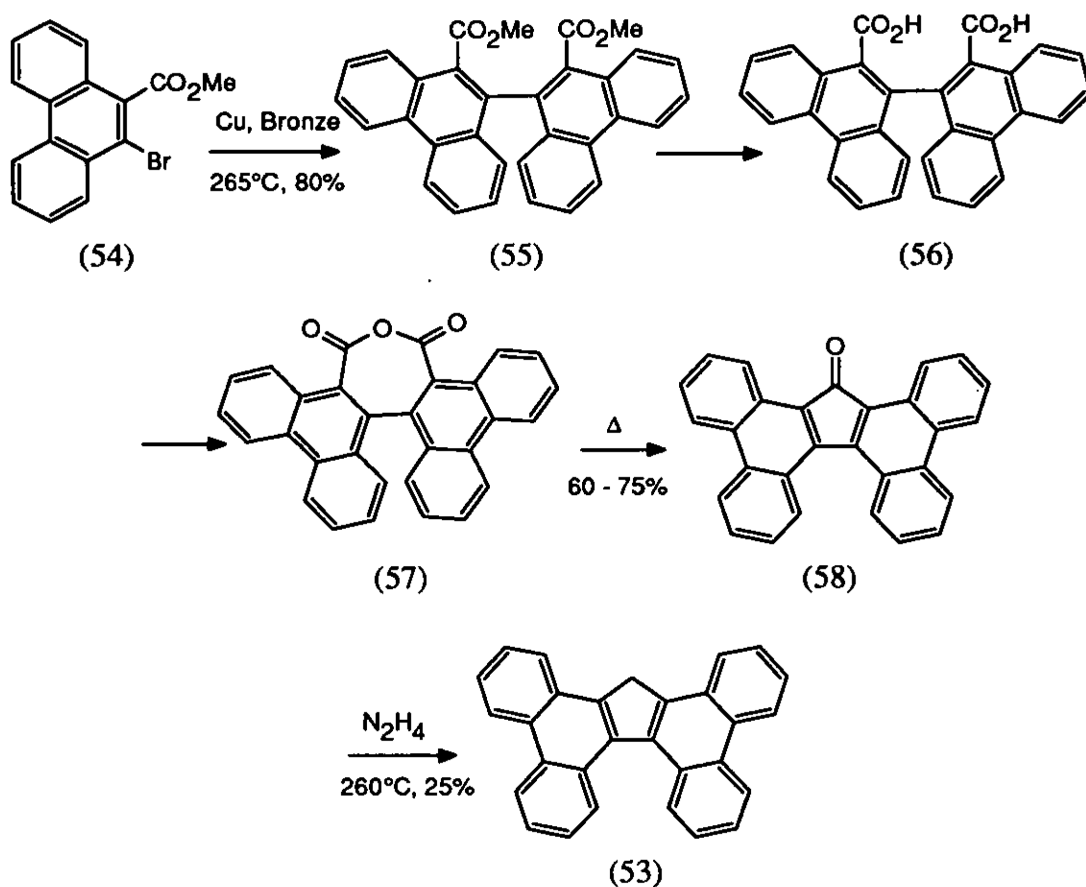


Fig. 2.3: Benzofluorenes

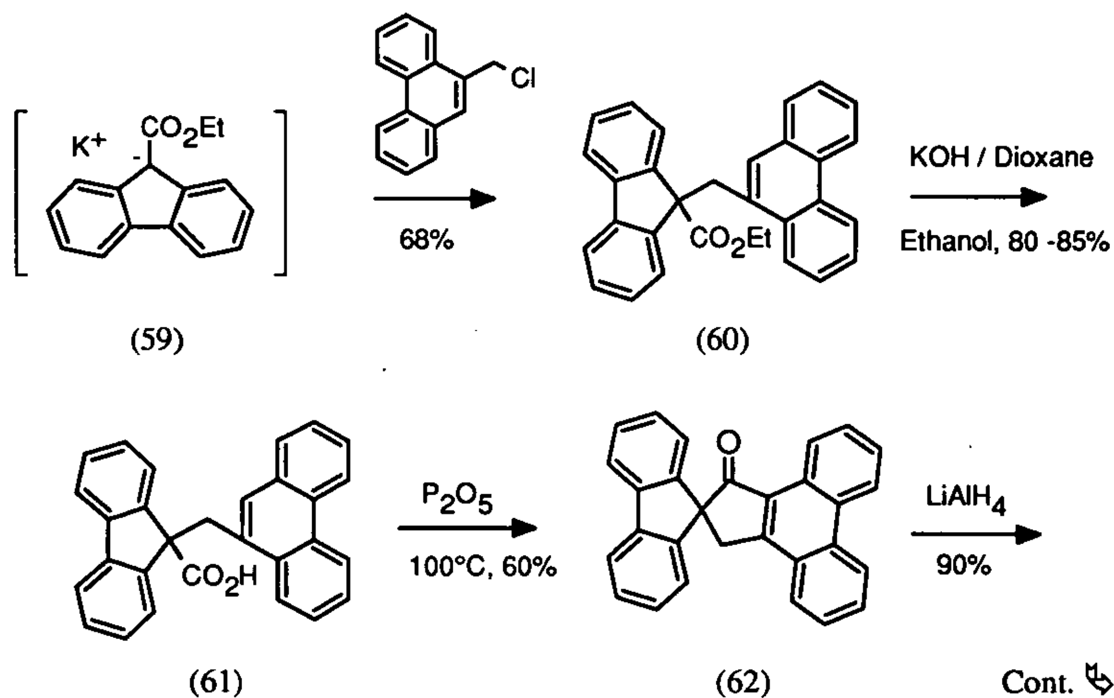
De Ridder and Martin reported the first synthesis of tetrabenzo[*a,c,g,i*]fluorene (53) in 1960[195]. The synthesis, shown in *Scheme 2.1*, required harsh conditions (e.g.

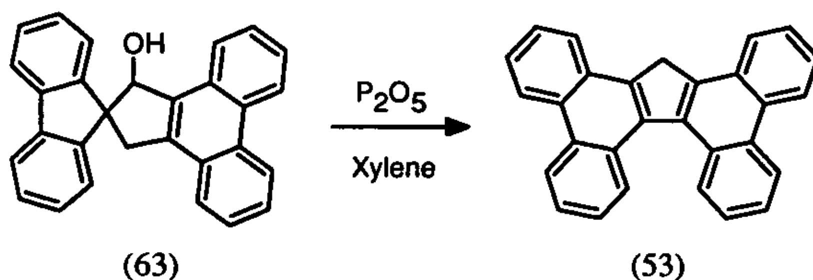
several hours > 260°C) and gave a poor yield (< 12%). Methyl-9-bromo-10-phenanthroate (54) underwent an Ullmann reaction at 265°C in the presence of copper to give dimethyl-9,9'-biphenanthryl-10,10'-dicarboxylate (55) in 80% yield. Base hydrolysis gave the corresponding diacid (56) which on treatment with acetic anhydride gave the anhydride (57) which underwent pyrolysis to give tetrabenzo[*a,c,g,i*]fluorenone (58) in 60 to 75% yield. Tetrabenzo[*a,c,g,i*]fluorene (53) was finally obtained by reduction of the ketone with hydrazine in sealed tubes at 260°C.



***Scheme 2.1: Synthesis of Tbf by De Ridder and Martin*[195]**

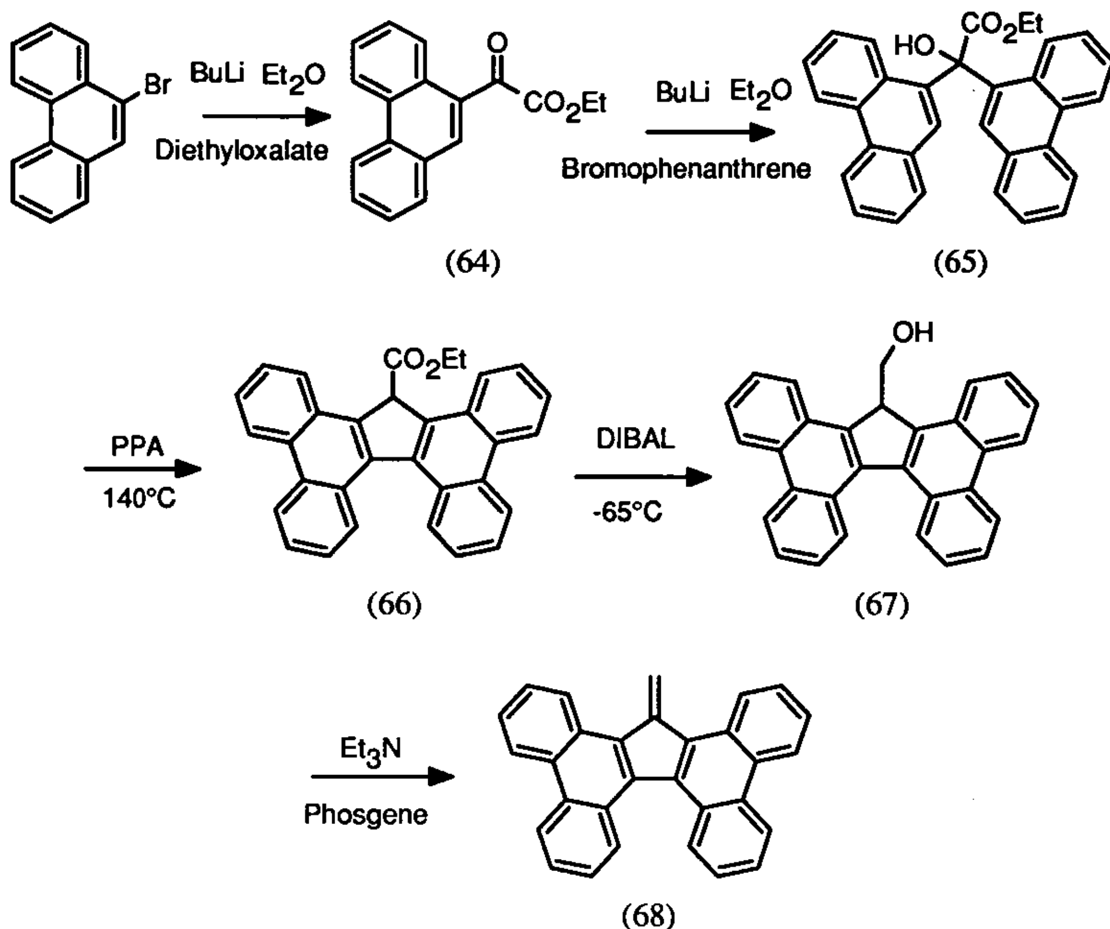
In a second synthetic route, shown in *Scheme 2.2*, Martin *et al.*[202] used milder reaction conditions. They prepared various benzofluorenes *via* the Wagner-Meerwein rearrangement of the fluorene structure. The synthesis of (60) was achieved by condensation of the potassium salt of ethyl-9-fluorenecarboxylate (59) and 9-chloromethylphenanthrene in 68% yield. The ester (60) was hydrolysed by potassium hydroxide in dioxane-methanol to give the corresponding acid (61) which in turn was cyclised in the presence of phosphorus pentoxide under reflux in toluene to give the spiro ketone (62) in 60% yield. The ketone (62) was then reduced to the secondary alcohol (63) with lithium aluminium hydride in 90% yield. Finally tetrabenzo[*a,c,g,i*]fluorene (53) obtained in 70% yield by dehydration and rearrangement of (63) with phosphorus pentoxide under reflux in xylene. Both routes involved several steps with moderate yield and difficult conditions, making it somewhat impractical for a large scale synthesis.





***Scheme 2.2: Synthesis of Tbf by Martin et al.*[202]**

Since these two synthetic routes were published, ca. 30 years ago, neither tetrabenzo[*a,c,g,i*]fluorene (53) nor any of its derivatives have been reported in the literature in spite of their interesting properties. Recently, Ramage and Raphy[194] synthesized three derivatives of tetrabenzo[*a,c,g,i*]fluorene: 17-ethoxycarbonyl tetrabenzo[*a,c,g,i*]fluorene (66), 17-hydroxymethyltetrabenzo[*a,c,g,i*]fluorene (67) and 17-methylenetetrabenzo[*a,c,g,i*]fluorene (68), see *Scheme 2.3*. For the synthesis of these molecules they used the procedure developed by Hopkinson *et al.*[203] for the synthesis of benzofluorenes *via* the rearrangements of α-alkoxycarbonyldiarylmethyl cations. Ethyl, 2-oxo-2-(phenanthren-9'-yl)acetate (64) was obtained in 63% yield by treating diethyl oxalate with 9-phenanthrenyl lithium at 0°C. Compound (65) was subsequently synthesized in a similar fashion using (64) as the keto ester component. The tertiary alcohol (65) was then treated with polyphosphoric acid at 140°C which led to the formation of the cyclic ester (66) in 49% yield. Reduction of ester (66) to the corresponding alcohol (67) was carried out at -65°C in DCM using DIBAL. Finally, the alkene (68) was obtained in 15% yield when treating the alcohol (67) with phosgene in the presence of a strong base such as triethylamine. Clearly the acidic proton at position 17 on tetrabenzo[a,c,g,i]fluorene means that basic reaction conditions produce the elimination product (68). Although a successful synthesis of the target compound (67) was achieved, a low overall yield (10%) after several chromatographic separations, made it unattractive for large scale preparations.



Scheme 2.3: Synthesis of tetrabenzo[a,c,g,i]fluorenyl-17-methanol

Base labile derivatives of tetrabenzo[*a*,*c*,*g*,*i*]fluorene (53) such as described above were designed to be compatible with the peptide synthesis methodology. N-tetrabenzo[*a*,*c*,*g*,*i*]fluorenyl-17-methyloxycarbonyl (Tbfmoc) derivatives of α-amino-acids and peptides, see *Fig. 2.4*, were subsequently used in affinity purification on porous graphitised carbon (PGC). Oligonucleotide synthesis in general requires several base treatments (eg. phosphate deprotection), therefore Tbfmoc adducts would not be suitable; rather compounds which are base stable and acid labile are required.

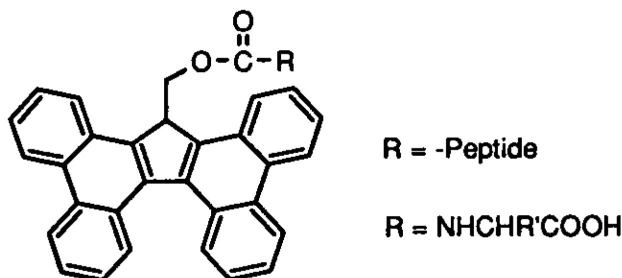
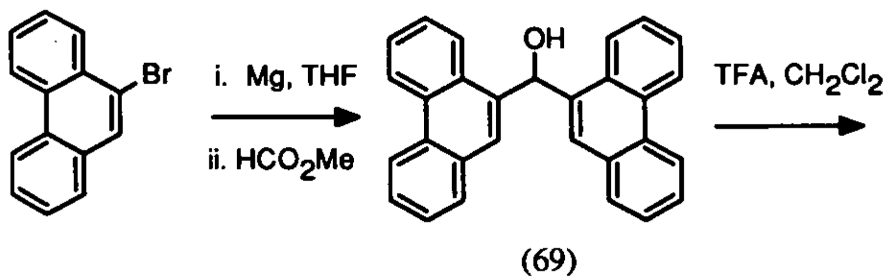


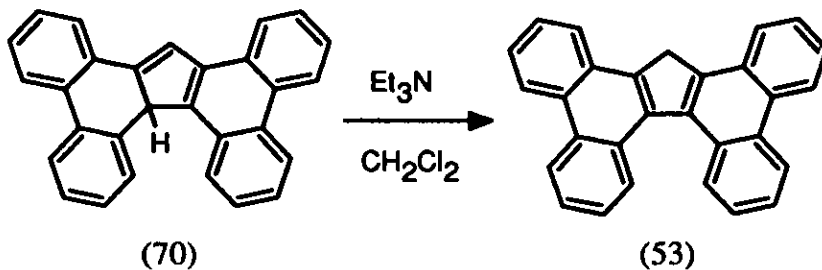
Fig. 2.4: Tbfmoc derivatives of amino acids and peptides

2.3 Synthesis of Tetrabenzo[*a,c,g,i*]fluorene and Derivatives

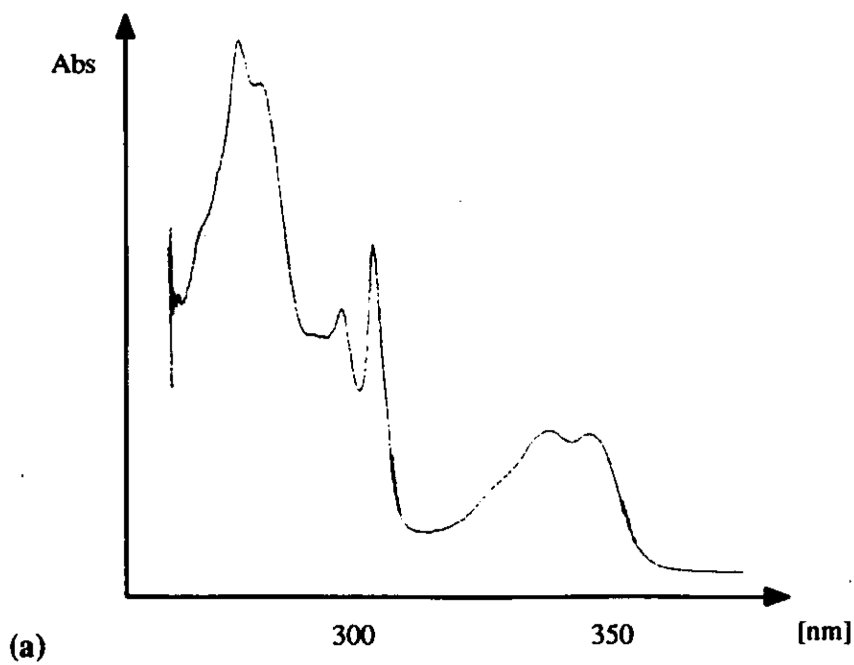
2.3.1 Novel Synthesis of Tetrabenzo[*a,c,g,i*]fluorene

The only two available syntheses of tetrabenzo[*a,c,g,i*]fluorene (53), described above, possess several drawbacks since they involve many low yield steps and somewhat harsh conditions. The need for a new synthesis was obvious. A more efficient synthesis of tetrabenzo[*a,c,g,i*]fluorene (53) was achieved involving organomagnesium reagents and the Hopkinson rearrangement of diarylmethyl cations. A Grignard reagent derived from 9-bromophenanthrene was generated in THF. Following the addition of 0.5 equivalent of ethyl formate, the secondary alcohol (69) was isolated in 52% yield. When using methyl formate instead of ethyl formate, a slight improvement of the yield (60%) was observed. Under these conditions the carbonyl carbon is more accessible to the attacking nucleophile. The cyclisation of alcohol (69) was attempted with various acids such as polyphosphoric acid, conc. sulphuric acid and aluminium chloride and thermally by heating the solid to melting; but proved to be unsuccessful. Finally a [2+2] cyclisation of di(phenanthren-9-yl)methanol (69) was achieved with trifluoroacetic acid in dichloromethane to give the fluorescent compound (70) in 90% yield, see *Scheme 2.4*.





Scheme 2.4: Synthesis of tetrabenzo[a,c,g,i]fluorene



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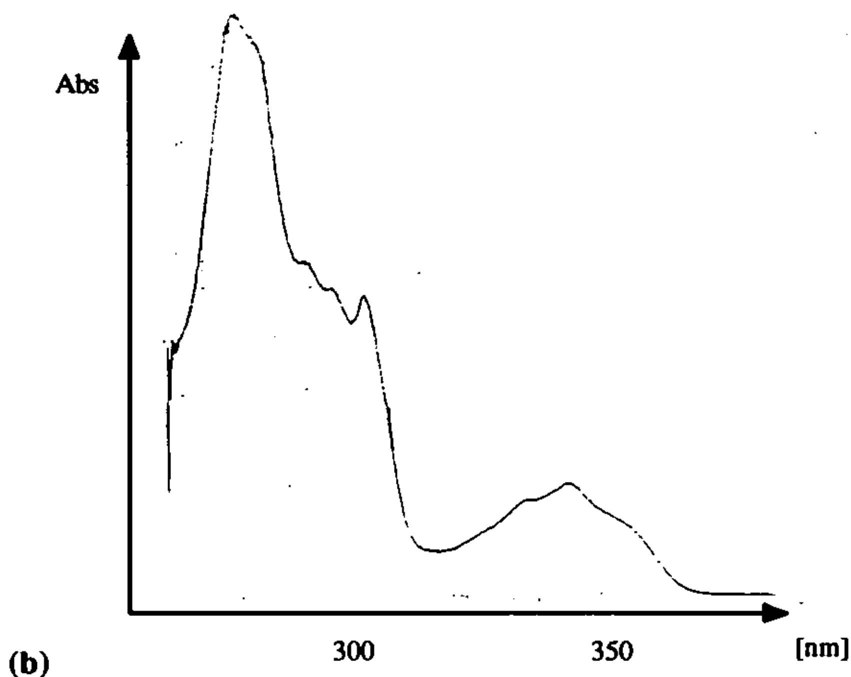
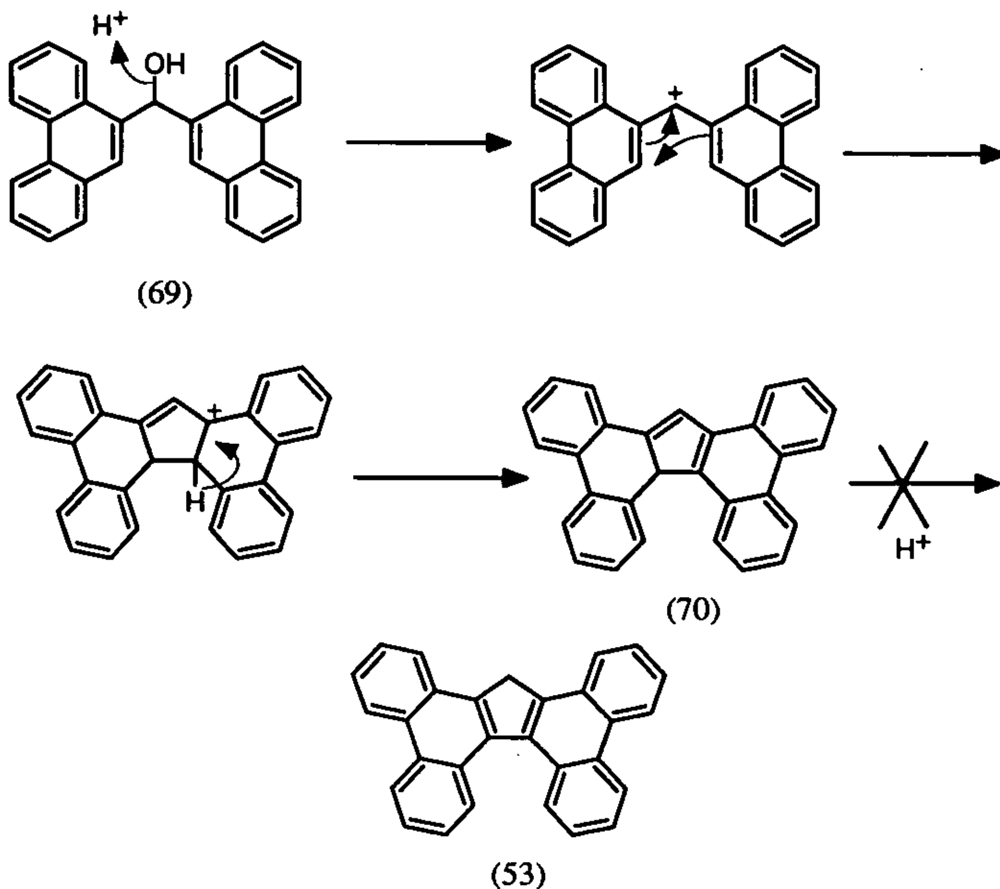


Fig. 2.5: UV spectra of (53) (a) and (70) (b) in DCM

Although compound (70) had a similar melting point to the literature value for (53), 280-282°C,[195] and the mass spectrum showed the expected molecular ion at 366, the proton nmr spectrum showed only one proton signal at δ = 5.40 ppm whereas (53) would require two. Moreover the aromatic signals in the proton nmr had a complex pattern when a simple pattern was expected for symmetrical tetrabenzo[*a*,*c*,*g*,*i*]fluorene (53). Also, the UV spectrum of (70) run in DCM was different from the spectrum of (53), see *Fig. 2.5*. The structure of (70) was finally assigned after taking into account the various possible intermediates that would be formed had the reaction proceeded according to the mechanism shown in *Scheme 2.5*. The isomerisation to (53) did not occur during the cyclisation reaction because it would require an intermediate anti-aromatic cation. Tetrabenzo[*a*,*c*,*g*,*i*]fluorene (53) was formed in 90% yield through a base catalysed isomerisation. The overall yield was 49% starting from 9-bromophenanthrene and involved three simple chemical steps.



Scheme 2.5: Mechanism of cyclisation of di(phenanthren-9-yl)methanol (69)

2.3.2 X-Ray analysis of Tbf and the Tbf/picric acid complex

Tetrabenzo[*a,c,g,i*]fluorene (53) is insoluble in polar solvents, slightly soluble in DCM, chloroform and THF and soluble in benzene and toluene. Suitable crystals for X-ray analysis were obtained by recrystallisation from benzene. The crystal structure of Tbf reveals several interesting features see *Fig. 2.6* and *Table 2.1*.

The molecule is symmetrical about the C2 axis. A significant twist between the two phenanthrene moieties indicates that the molecule is not planar. Responsible for

the non-planarity is the interaction between H_8 and H_9. Both protons are about 2.14Å apart whereas the Van der Waal's radius for hydrogen is 1.2Å. The twist can be defined by the dihedral angle between C_8, C_{8a}, C_{8b} and C_{8c} which has a value of 14.5°. When the torsion angles between atoms H_8 , C_8, C_{8a}, C_{8b}, C_{8c}, C_{8d}, C_9 and H_9 are 0° then theoretical calculations indicate a H_8-H_9 distance of 0.62Å which is nearly half the radius of one hydrogen atom. The molecule of tetrabenzo[*a*,*c*,*g*,*i*]fluorene is therefore chiral when it adopts a fixed orientation as in the solid state.

Molecular formula	$C_{29}H_{17}$
Formula weight	365.4
Crystal dimensions [mm^3]	0.5 x 0.4 x 0.15
Crystal system	Monoclinic
Cell dimensions a [Å]	18.446(6)
Cell dimensions b [Å]	5.101(3)
Cell dimensions c [Å]	19.155(9)
Cell dimensions α [°]	90.00
Cell dimensions β [°]	90.86(3)
Cell dimensions γ [°]	90.00
Melting point [°C]	281
Density $_{calc.}$ [g/cm^3]	1.35
Z	4

Table 2.1: Crystallographic information of tetrabenzo[a,c,g,i]fluorene

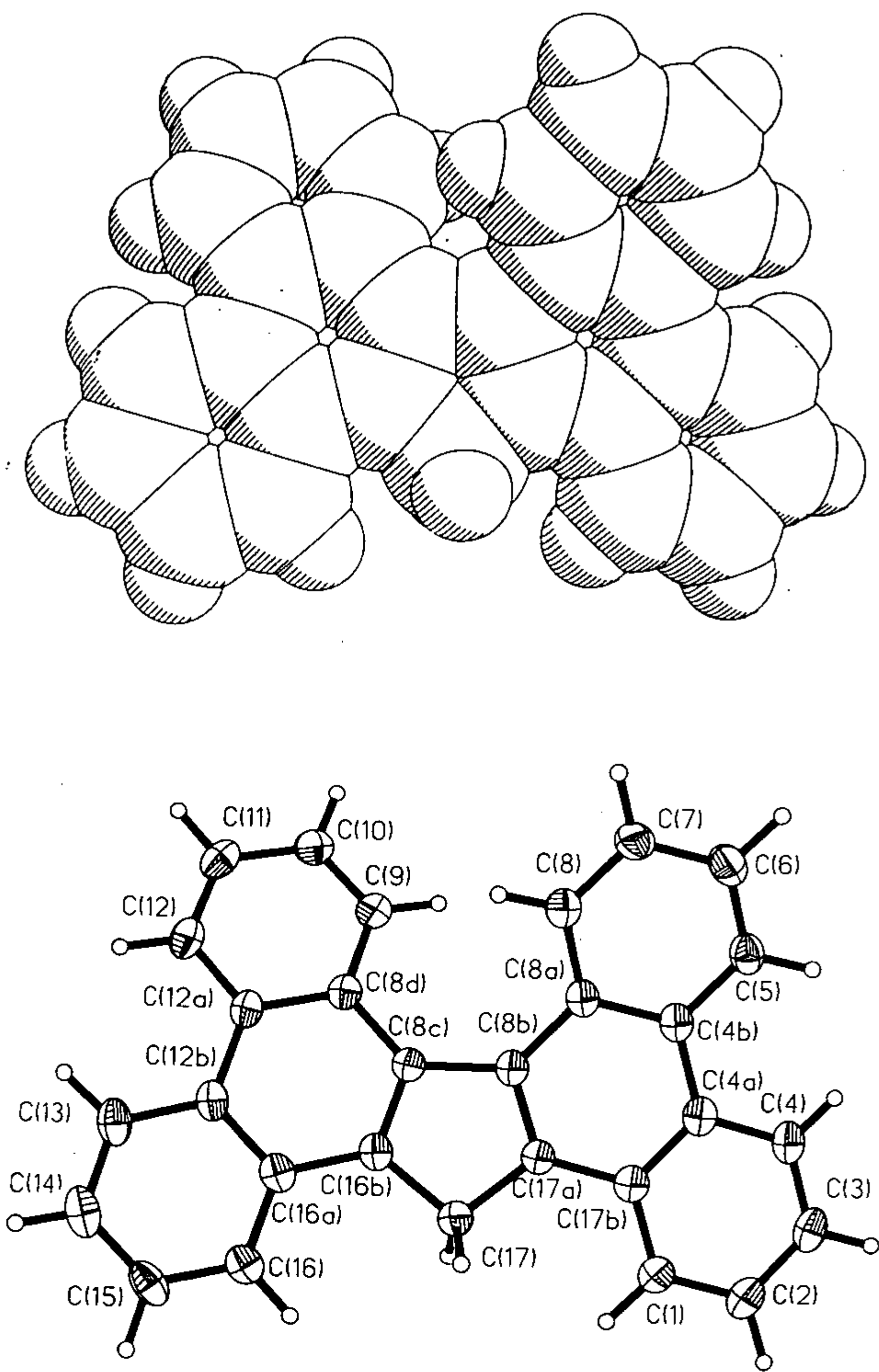


Fig. 2.6: X-Ray structure of tetrabenzo[a,c,g,i]fluorene (Tbf)

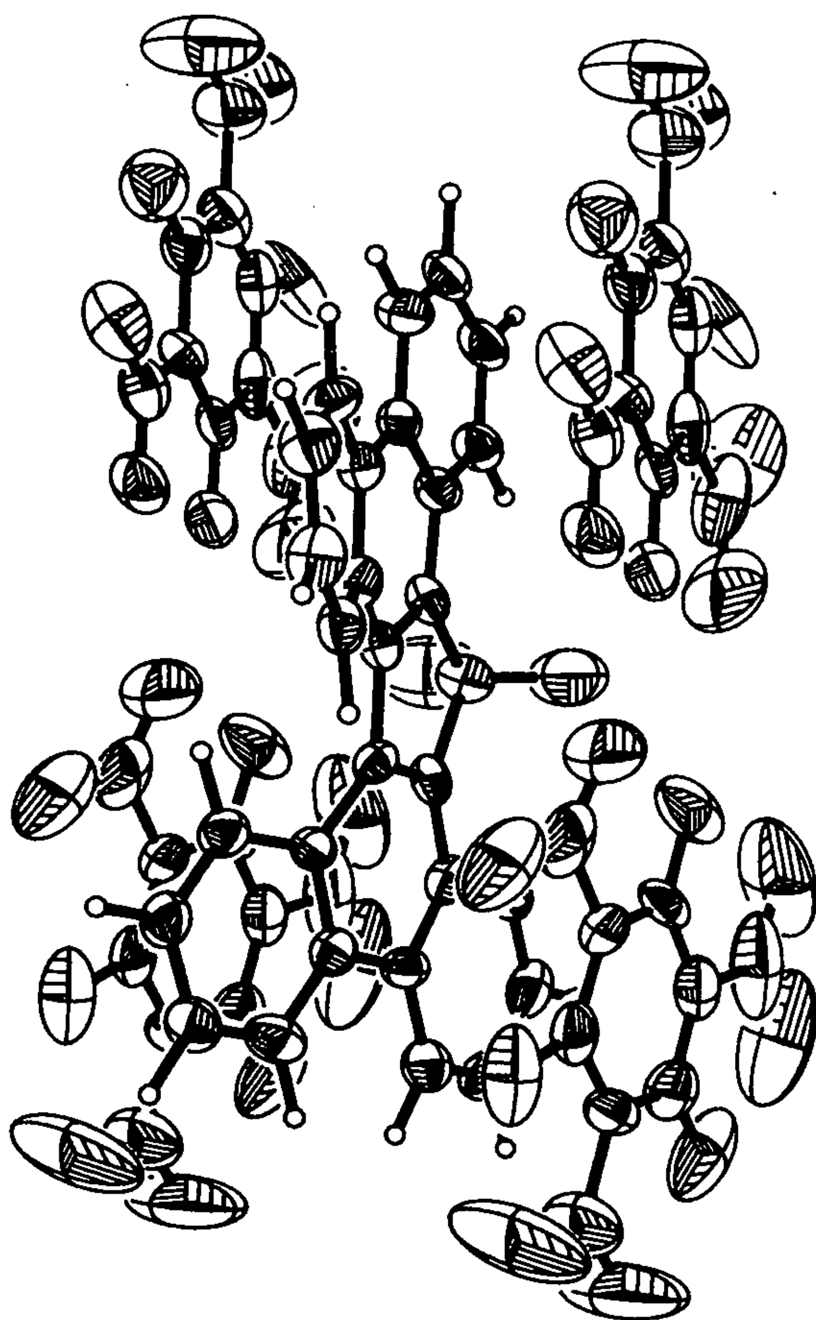


Fig. 2.7: X-Ray structure of the Tbf/Picric Acid Complex

The charge-transfer complex between phenanthrene and picric acid is known and its crystal structure has been determined by X-ray analysis.[204] Phenanthrene and picric acid molecules are stacked alternately along the C-axis making their molecular planes parallel. Given the fact that polycyclic aromatic hydrocarbons are active π-donors[205] in general, Tbf and picric acid were co-crystallized in benzene and the crystals examined by X-ray analysis, see *Fig. 2.7* and *Table 2.2*. The evidence of complex formation was visible; when a yellowish solution of Tbf in benzene and a yellowish solution of picric acid in benzene were mixed together a dark red coloured solution appeared. The same observation was made between Tbf and 2,4,6-trinitrobenzene although their complex did not give satisfactory crystals for X-ray analysis. The crystal structure resolution started with the assumption that there was one picric acid molecule associated with each Tbf molecule but this proved to be wrong. During the crystallization in benzene, yellowish crystals and dark brown crystals were observed, the former being an excess of Tbf crystallising and the latter being the crystals of the complex; this also indicated that the complex did not crystallise in equimolar proportions. The dark brown crystals were then subsequently recrystallised separately to give the crystals finally used in the X-ray analysis which confirmed that two molecules of picric acid bind to one molecule of Tbf. From *Fig. 2.7*, it appeared that Tbf and picric acid molecules are both severely disordered in the crystal. The solvate, benzene, was also found in the crystal. It was found that the planes of Tbf and picric acid have an intermolecular distance of 3.3Å. The angular rotation about the axis perpendicular to the ring plane in the Tbf molecule and between the two picric acid molecules are ±20°. The picric acid molecules do not lie directly in the same plane as their opposite phenanthrene moities in the Tbf molecule but appear to have an angular twist of ±5.0°. The hydroxyl groups of the picric acid molecules were subject to pseudo three-fold symmetry which caused random delocalisation over the three possible sites; no significant intermolecular contact could be determined.

Two large electron intensities were located in position 17 of Tbf, but neither could be modelled as hydrogens but instead appeared to fit in with two oxygen atoms which raised some questions. As outlined in section 2.3.3.3, position 17 is particularly reactive towards oxidation reaction even with air. The reactivity of methylene-bridged polyarenes and in particular their oxidation has been described.[206] Also X-ray analysis accounts for the average possible structural unit in reactive molecules. In this case the position of the two oxygen atoms may suggest that they belong to an intermediate of the oxidation reaction leading to tetrabenzo[*a,c,g,i*]fluorenone (58) (the C-O distances are ±1.20Å). Besides, the high anisotropic values of the nitro groups indicated to a possible reaction in the crystal which was occuring between a nitro group and one of the 17-oxygens which moreover are closely separated by about 2.4Å. However, the fact that the site occupancies of the nitro groups were not likely to go beneath 1.0 was contradictory and lead us to conclude that there was no reaction proceeding in the crystal but that possibly a stable oxidation intermediate, was captured. Further confirmation of these observation might have been obtained with the same crystal being subject to another X-ray analysis, but this could not be achieved. UV studies were unsuccessful as the charge-transfer complex is dissociated by greater solvation forces in the highly dilute conditions for UV measurements. Nevertheless we could prove that Tbf can act as a π-donor in the formation of organic molecular complexes or charge-transfer complexes and that it presents the potential to be investigated in the search for organic semi-conductors perhaps .

Molecular formula	$C_{44}H_{23}N_6O_{14}$
Formula weight	859.63
Crystal dimensions [mm^3]	0.43 x 0.27 x 0.04
Crystal system	Triclinic
Cell dimensions a [Å]	6.891(5)
Cell dimensions b [Å]	16.070(9)
Cell dimensions c [Å]	18.304(12)
Cell dimensions α [°]	67.91(5)
Cell dimensions β [°]	82.18(5)
Cell dimensions γ [°]	82.72(5)
Melting point [°C]	210
Density $_{calc.}$ [g/cm^3]	1.54
Z	2

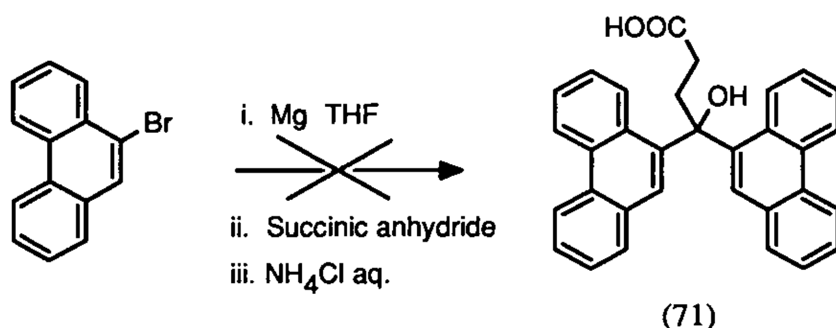
Table 2.2: Crystallographic information of the Tbf/Picric Acid Complex

2.3.3 Chemical Reactions of Tetrabenzo[*a,c,g,i*]fluorene

2.3.3.1 Formation of 17-Alkylated Tetrabenzo[*a,c,g,i*]fluorene

In order to introduce additional properties to the Tbf group, detailed in Section 2.1, an investigation of its chemical reactivity was undertaken. The first idea was to modify the Tbf structure at the position 17 by introducing an alkyl chain containing a functionality during the formation of the ring system. Richter[207] used the cyclodehydration of benzilic acid and its derivatives with aluminium trichloride to synthesize fluorene-9-carboxylic acids. We undertook a similar approach when hydroxy-esters were synthesized *via* a Grignard reaction in a first step and 17-tetrabenzo[*a,c,g,i*]fluorenylalkyl esters by cyclodehydration in a second step. The first attempt shown in *Scheme 2.6*, involved the reaction of 0.5 equivalent of succinic anhydride with 1 equivalent 9-bromomagnesiumphenanthrene. But the γ-hydroxy-propanoic acid derivatives (71) could not be obtained; only the γ-keto acid intermediate could be isolated in 8% yield. Keto-carbonyl band at 1670 cm^{-1} distinct

from the acid carbonyl band at 1710 cm^{-1} and a mass spectrum peak at 278 confirmed the structure of the γ-keto acid. The Grignard reagents were reacted with diethylmalonate but this also proved unsuccessful. Diethyl succinate has a more accessible carbonyl carbon and contains less acidic methylene protons than the malonate esters but its reaction with 9-bromomagnesiumphenanthrene failed to give a product either. Instead of the magnesium compound, 9-lithium phenanthrene was reacted with dimethylsuccinate but only phenanthrene could be isolated. Subsequently the synthesis of Tbf derivatives was investigated with the Tbf ring structure already formed.



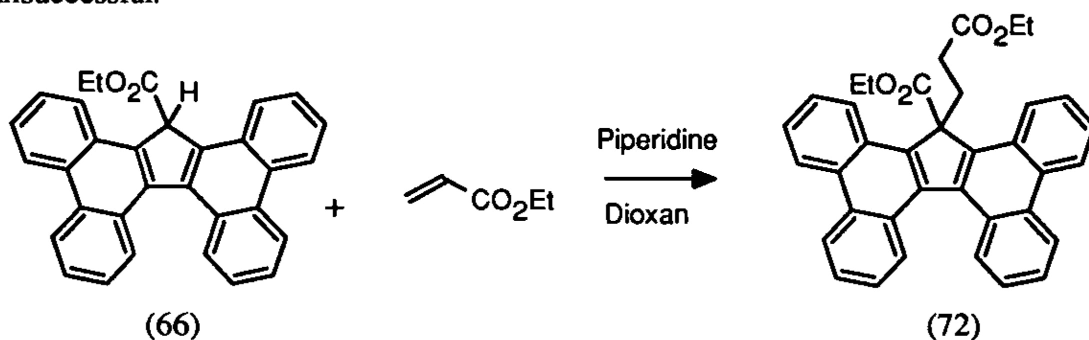
Scheme 2.6

2.3.3.2 Nucleophilic Substitution

The most significant feature is the attractive reactivity of the methylene group at position 17 where the hydrogen atoms are strongly acidic. This reactivity resembles the one observed for the methylene in position 9 of fluorene.[208,209] The acidity is attributed to the high stability of the cyclopentadienyl anion. This anion exhibits an aromatic character as the five-membered ring has two double bonds and the fifth atom possesses an unshared pair of electrons in an orbital perpendicular to the ring-plane.

The four π electrons of the double bonds and the unshared two electrons form an aromatic sextet. The relatively strong acidity of the methylene group was first used for Michael-addition reactions:

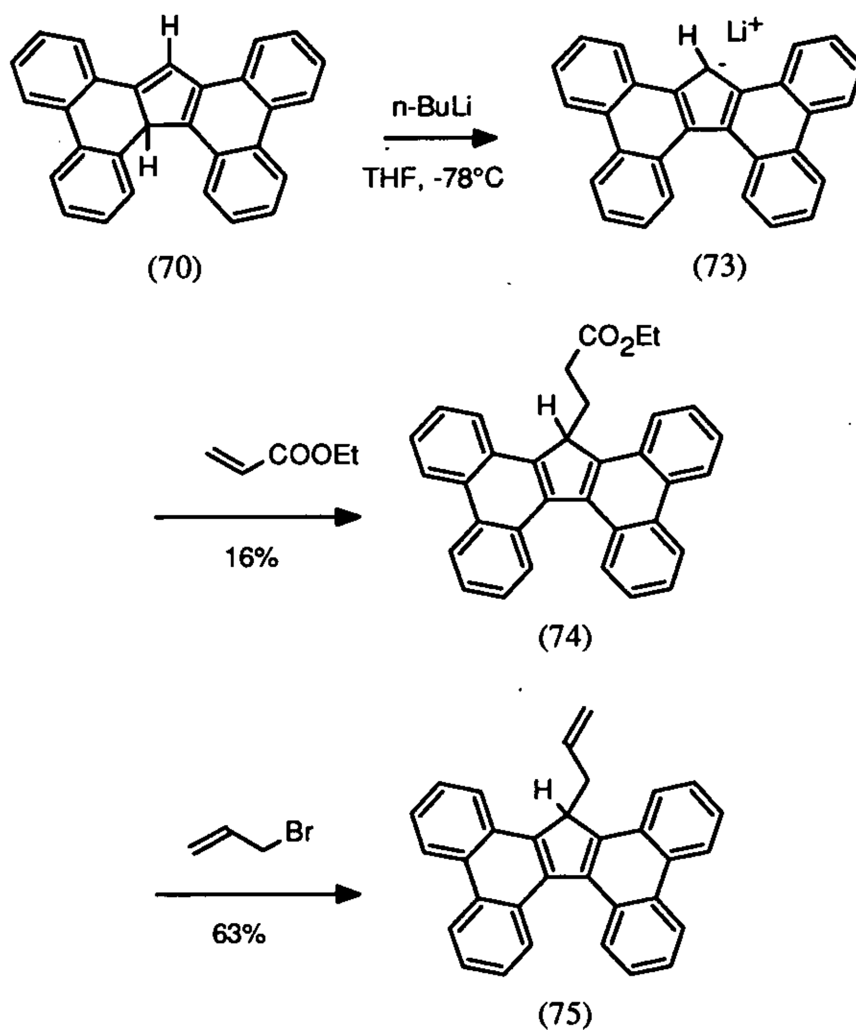
17-Ethoxycarbonyltetrabenzo[*a,c,g,i*]fluorene (66), synthesized according to Ramage and Raphy,[194] was reacted with ethyl acrylate in the presence of a base, see *Scheme 2.7*. When potassium hydroxide was used as a base, the reaction did not proceed. It is possible that the hydrolysis of the ester interfered. However, the expected product (72) was obtained after 4 days in the presence of piperidine to give yellow crystals in 20% yield. Subsequent hydrolysis and decarboxylation was unsuccessful.



Scheme 2.7

The Tbf anion can also be generated from the reaction of tetrabenzo[*a,c,g,i*]fluorene (53) or 8bH-tetrabenzo[*a,c,g,i*]fluorene (70) with n-butyllithium at -78°C in THF. The subsequent reaction of ethyl acrylate with the organolithium compound (73) gave the adduct ethyl 3-(17'-tetrabenzo[*a,c,g,i*]fluorenyl) propanoate (74) in 16% yield, see *Scheme 2.8*. When using an excess of butyl lithium in the formation of (73), ethylacrylate reacted in unimolecular proportions without forming any *bis*-17,17-(ethylpropionate) tetrabenzo[*a,c,g,i*]fluorene, therefore indicating a steric hindrance around carbon 17. In order to improve the yield we attempted to reduce the possibility of a 1,2-addition on the carbonyl group. The more hindered *tert*.-butylacrylate was used for this

purpose but failed to react. One should draw attention on the fact that the available 17-hydrogen is rendered more reactive in compound (66) due to the stabilising effect of the neighbouring ester group on the anion intermediate. Hence a better yield was obtained in the synthesis of ethyl-17-ethoxycabonyl-(17-tetrabenzo[*a,c,g,i*]fluorenyl)-3'-propionate (72) over ethyl 3-(17'-tetrabenzo[*a,c,g,i*]fluorenyl) propanoate (74) in spite of a greater steric hindrance.



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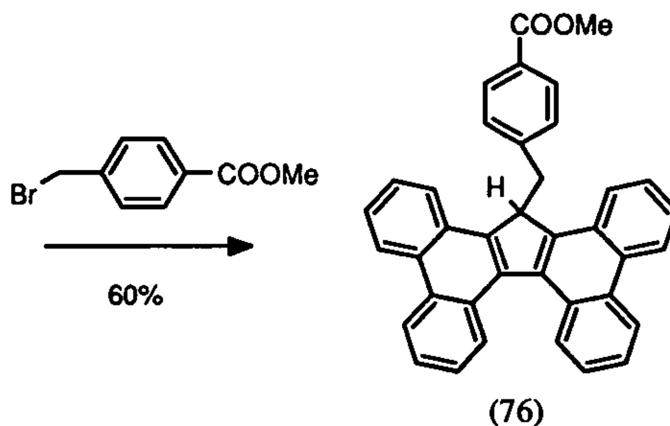
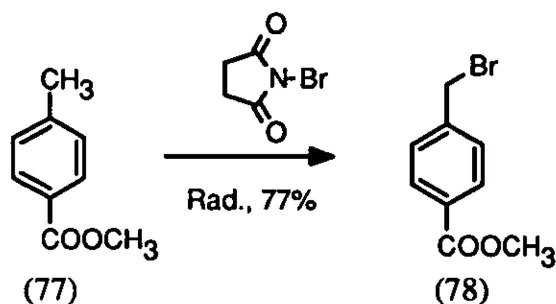


Scheme 2.8: Alkylation reactions with Tbf

Oxetane, acrolein and 1,3-dibromopropane were also used as substrates in alkylation reactions with (73); neither of them gave any product. It was therefore decided to investigate more reactive substrates such as allyl bromide or benzyl bromide.

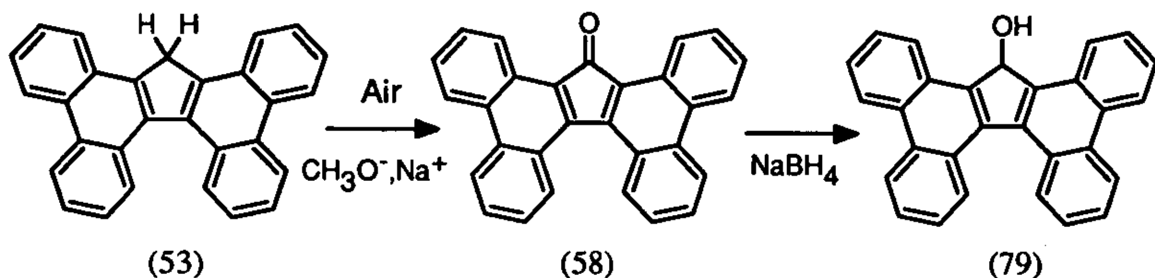
The rate of formation of allyl or benzyl cations is relatively high and steric hindrance is low therefore nucleophilic attack is favoured. This point was confirmed when the tetrabenzo[*a,c,g,i*]fluorenyl anion (73) was treated with allyl bromide and methyl-(*para*-bromomethyl)-benzoate (78). 1-(17'-Tetrabenzo[*a,c,g,i*]fluorenyl)-prop-2-ene (75) was obtained in 63% yield and methyl 4-(17'-tetrabenzo[*a,c,g,i*]fluorenylmethyl) benzoate (76) in yields ranging from 60 to 71% after chromatographic purification, see *Scheme 2.8*. These two products are accessible in three steps with reasonable yields and possess reactive functionalities which can be used in further chemical reactions. Methyl-(*para*-bromomethyl)-benzoate (78) was synthesized from methyl-(*para*-methyl)-benzoate (77) *via* a free radical bromination reaction according to *Scheme 2.9*. Because of the electrophilic character of the bromine radical, the reaction almost exclusively proceeded at the electron richer methyl α to the the aromatic ring.



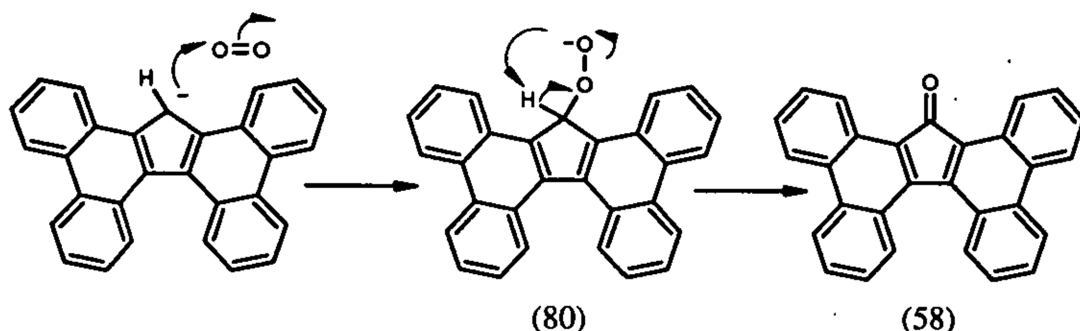
Scheme 2.9: Bromination of methyl-2-methylbenzoate

2.3.3.3 Oxidation

When the anion (73) is generated at -78°C with n-butyllithium, extreme care must be taken to remain under inert atmosphere. In the first experiments one could observe an intense red coloration in the reaction mixture. After isolation of the product by flash chromatography and analysis by mass spectrometry (M+ = 381) and infra-red spectroscopy (carbonyl band at 1700 cm^{-1}) tetrabenzo[*a,c,g,i*]fluorenone (58) was identified. A more efficient deliberate synthesis of the ketone was subsequently developed, by stirring a solution of tetrabenzo[*a,c,g,i*]fluorene (53) in THF with sodium methoxide overnight in an open flask, see *Scheme 2.10.* Tetrabenzo[*a,c,g,i*]fluorenone (58) was isolated in 52% yield after purification as dark red crystal with poor solubility in most organic solvents. In the same manner as fluorene, oxygen in the air can oxidise Tbf in the presence of a base to form (58). The mechanism for the formation of the ketone rather than hydroperoxide products from the reaction of the 17-Tbf anion with molecular oxygen presumably involves intra- or intermolecular abstraction of a 17-proton of the intermediate (80) by the peroxy anion leading to loss of hydroxide ion and formation of a carbonyl group as outlined in *Scheme 2.11*.



Scheme 2.10



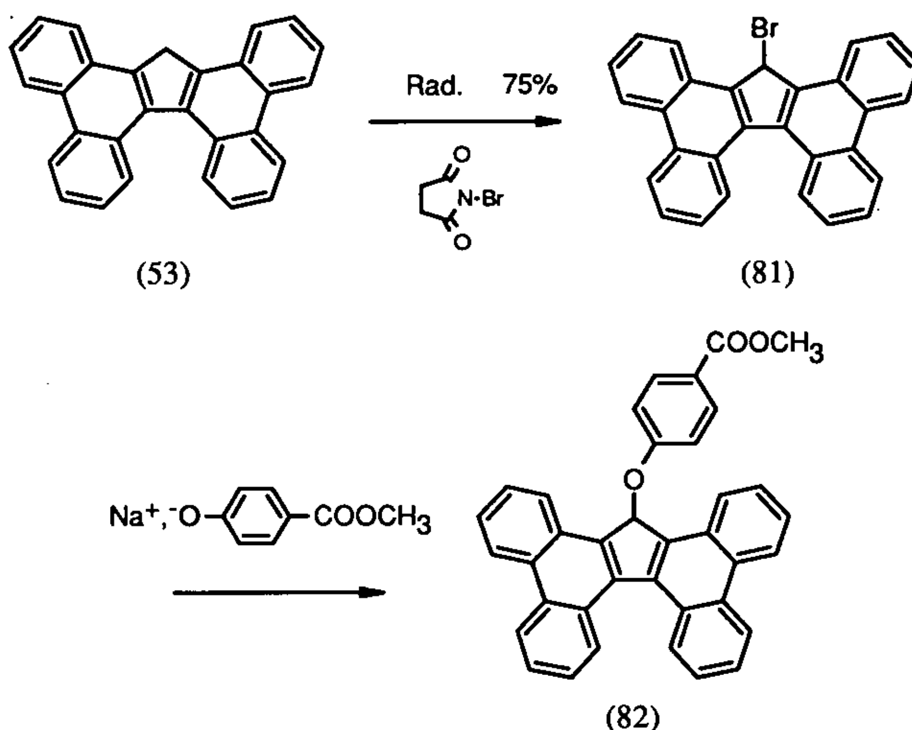
Scheme 2.11

The reduction of tetrabenzo[*a,c,g,i*]fluorenone (58) was carried out with sodium borohydride in THF to produce a yellow solid, 17-hydroxytetrabenzo[*a,c,g,i*]fluorene (79) in 71% yield, see *Scheme 2.10*. Neither compound (79) nor (58) fluoresced in contrast to Tbf.

2.3.3.4 Bromination

Free radical bromination of the methylene group was readily achieved with N-bromosuccinimide, a radical initiator and irradiation with a halogen lamp, see *Scheme 2.12*. The reaction afforded 17-bromotetrabenzo[*a,c,g,i*]fluorene (81) as a bright orange solid in high yield (75%). The fact that neither 17-bromotetrabenzo[*a,c,g,i*]fluorene (81), tetrabenzo[*a,c,g,i*]fluorenone (58) nor 17-

hydroxytetrabenzo[*a,c,g,i*]fluorene (79) were fluorescent, enables us to conclude that substitution of Tbf by a heteroatom results in the disappearance of fluorescence. The attempt to prepare the Grignard reagent of (81) in either diethylether or THF failed, therefore one can summarise that the space available around the methylene group does not allow the somewhat bulky formation of the magnesium-solvent complex. Instead bromine was used for its good leaving group properties in nucleophilic substitution. 17-Bromotetrabenzo[*a,c,g,i*]fluorene (81) was reacted with methyl-*para*-hydroxybenzoate according to *Scheme 2.12*. The phenoxyether methyl 4-(17'-tetrabenzo[*a,c,g,i*]fluorenyl)oxybenzoate (82) was obtained in 26% yield as a bright yellow solid that did not fluoresce.

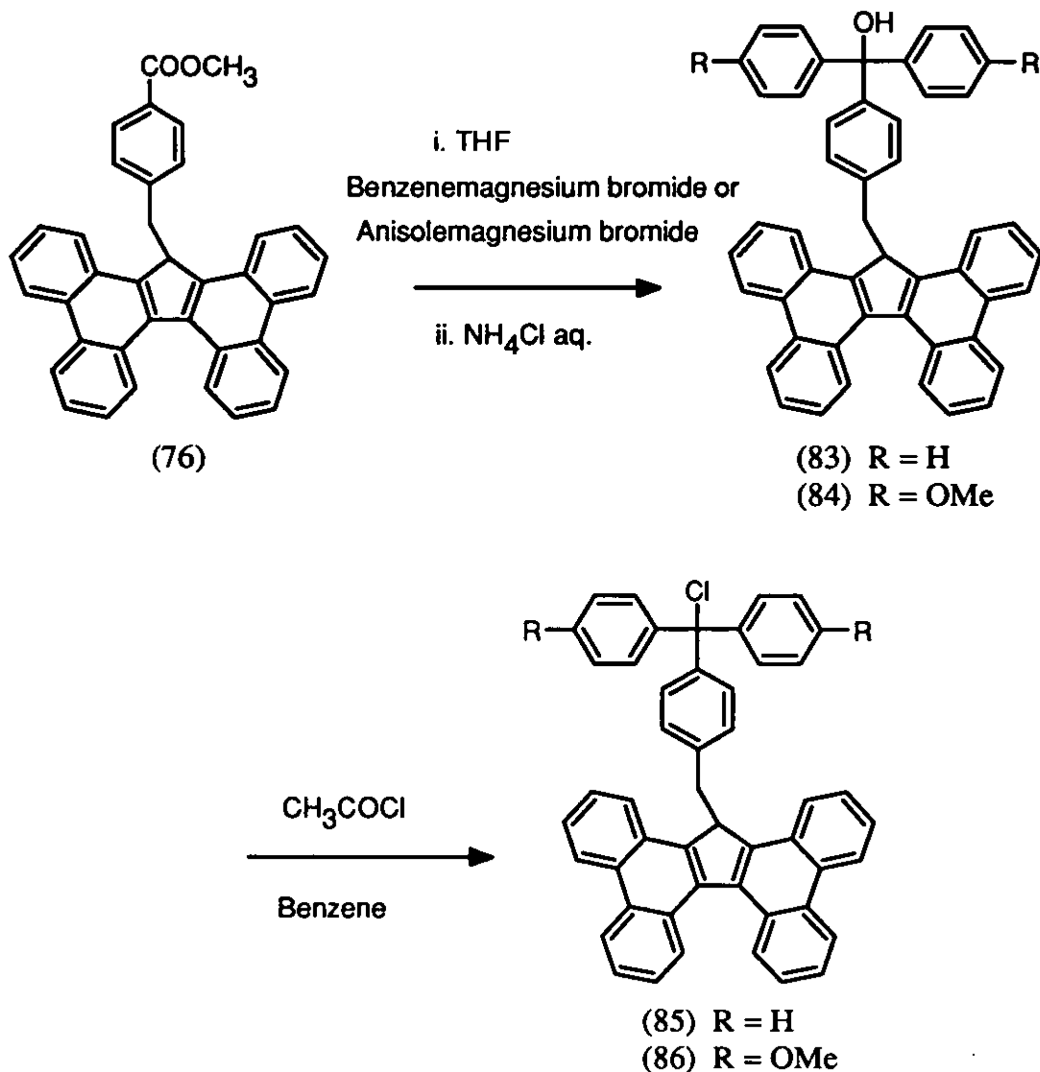


Scheme 2.12

2.4 Synthesis of Tetrabenzo[*a,c,g,i*]fluorenyltrityl Derivatives

As a general starting material in the design of a 5'-hydroxyl protecting group for nucleosides, methyl 4-(17'-tetrabenzo[*a,c,g,i*]fluorenylmethyl) benzoate, compound (76) was chosen. Its trityl derivatives can be directly formed *via* a Grignard reaction. Tetrabenzo[*a,c,g,i*]fluorene-trityl compounds would fulfil the requirements outlined in section 2.1 for the design of a 5'-hydroxyl protecting group, in particular the selectivity for primary alcohols. Moreover the well known chemistry of the trityl group as a protecting group can be adapted to these new nucleosidic derivatives.

The Grignard reaction of ester (76) with 2 equivalents of bromobenzene was first investigated, see *Scheme 2.13*. The triphenylcarbinol derivative, 4-(17'-tetrabenzo[*a,c,g,i*]fluorenylmethyl)-trityl alcohol (83), was obtained in 63% yield as a white, crystalline, fluorescent compound. Before the trityl alcohol derivative (83) could be used as a protecting group, the trityl chloride equivalent had to be generated. The latter compound, 4-(17'-Tetrabenzo[*a,c,g,i*]fluorenylmethyl)-trityl chloride (85), was synthesized according to the methodology used for trityl chloride.[210] From (83) and an excess of acetyl chloride in benzene or cyclohexane 4-(17'-tetrabenzo[*a,c,g,i*]fluorenylmethyl)-trityl chloride (85) was isolated in 88% yield after recrystallisation in benzene. In order to increase the reactivity two electron donating methoxy groups were added to form dimethoxytrityl derivative (86) which was synthesized in a similar fashion from the ester (76) and 4-(17'-tetrabenzo[*a,c,g,i*]fluorenylmethyl)-4",4"'-dimethoxytrityl alcohol (84) in 16% yield overall starting from 9-bromophenanthrene. [Compounds (83) and (85) have been abbreviated Tbf-Tr-OH and Tbf-Tr-Cl respectively; compounds (84) and (86) Tbf-DMTr-OH and Tbf-DMTr-Cl respectively; derivatives of this type will be generally called Tbf-Trityl]



Scheme 2.13

The crystallization of Tbf-DMTr-OH (84) enabled single crystals, satisfactory for X-Ray analysis, to be prepared. *Fig. 2.8* represents molecule (84) in the solid state; the crystallographic information is presented in *Table 2.3*. The crystal structure presents the same twist between the two phenanthrenyl groups as in the molecule of tetrabenzo[*a,c,g,i*]fluorene (53) along the C2 axis. The dihedral angle measured between C_8, C_{8a}, C_{8b} and C_{8c} with a value of 15.5° is 1° greater than in the tetrabenzo[*a,c,g,i*]fluorene molecule. The value of the angle between C_{17}, C_{18} and C_{4p} is 113.2°, thus greater than a sp^3 carbon bond angle. The trityl group is

characteristically fixed in a propeller shaped orientation, "endo" to the tetrabenzo[*a*,*c*,*g*,*i*]fluorene moiety.

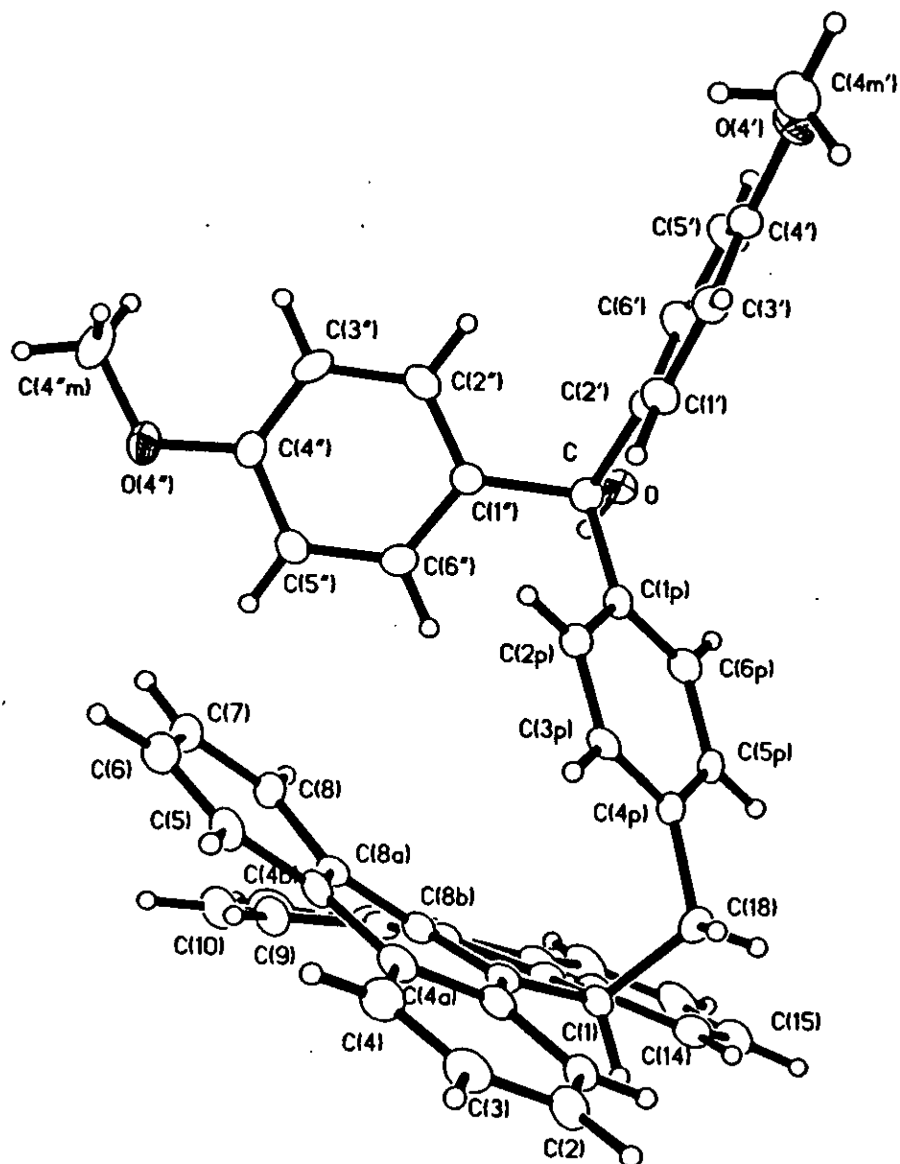
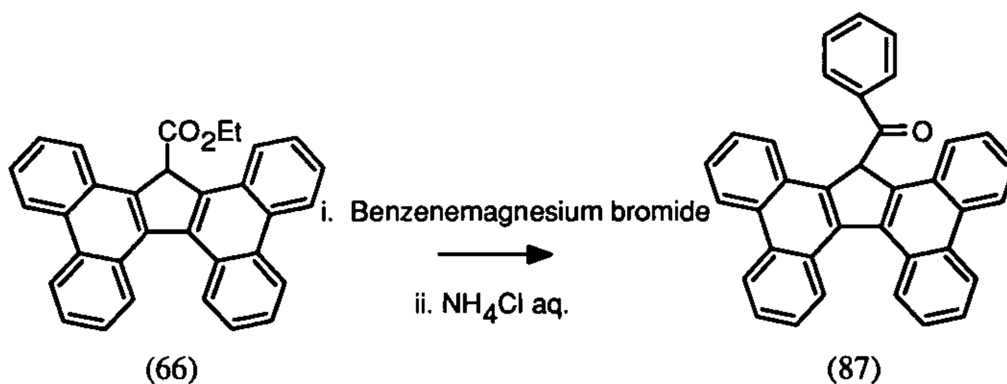


Figure 2.8: Crystal structure of Tbf-DMTr-OH (84)

Molecular formula	$C_{51}H_{38}O_3$
Formula weight	698.3
Crystal dimensions [mm^3]	0.08 x 0.08 x 0.39
Crystal system	Monoclinic
Cell dimensions a [Å]	13.6213(22)
Cell dimensions b [Å]	14.530(6)
Cell dimensions c [Å]	18.422(11)
Cell dimensions α [°]	90.00
Cell dimensions β [°]	99.71(3)
Cell dimensions γ [°]	90.00
Melting point [°C]	210
Density $_{calc.}$ [g/cm^3]	1.291
Z	4

Table 2.3: Crystallographic information for Tbf-DMTr-OH (84)

Later, we attempted to generate a Tbf trityl derivative starting from 17-ethoxycarbonyl tetrabenzo[*a,c,g,i*]fluorene (66) originally synthesized by Ramage and Raphy[194] and kindly donated by S. Irving.[211] Compound (66) underwent a Grignard reaction with 2 equivalents of benzenemagnesium bromide but failed to form the trityl derivative, 1,1-*bis*(phenyl)-1'-tetrabenzo[*a,c,g,i*]fluorenyl-methanol (88), *Scheme 2.14*. Analytical data, mass spectra (peak at 470) and IR (C=O band 1705 cm^{-1}) indicated the presence of the intermediate ketone (87) in the reaction mixture. The elimination product that could be formed from compound (88), was not detected in the mass spectra. Consequently the compound is probably too hindered for a second addition of a benzene ring onto the carbonyl function to occur.



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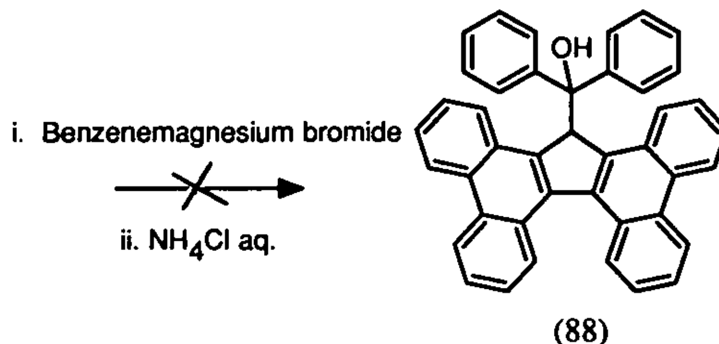


Scheme 2.14

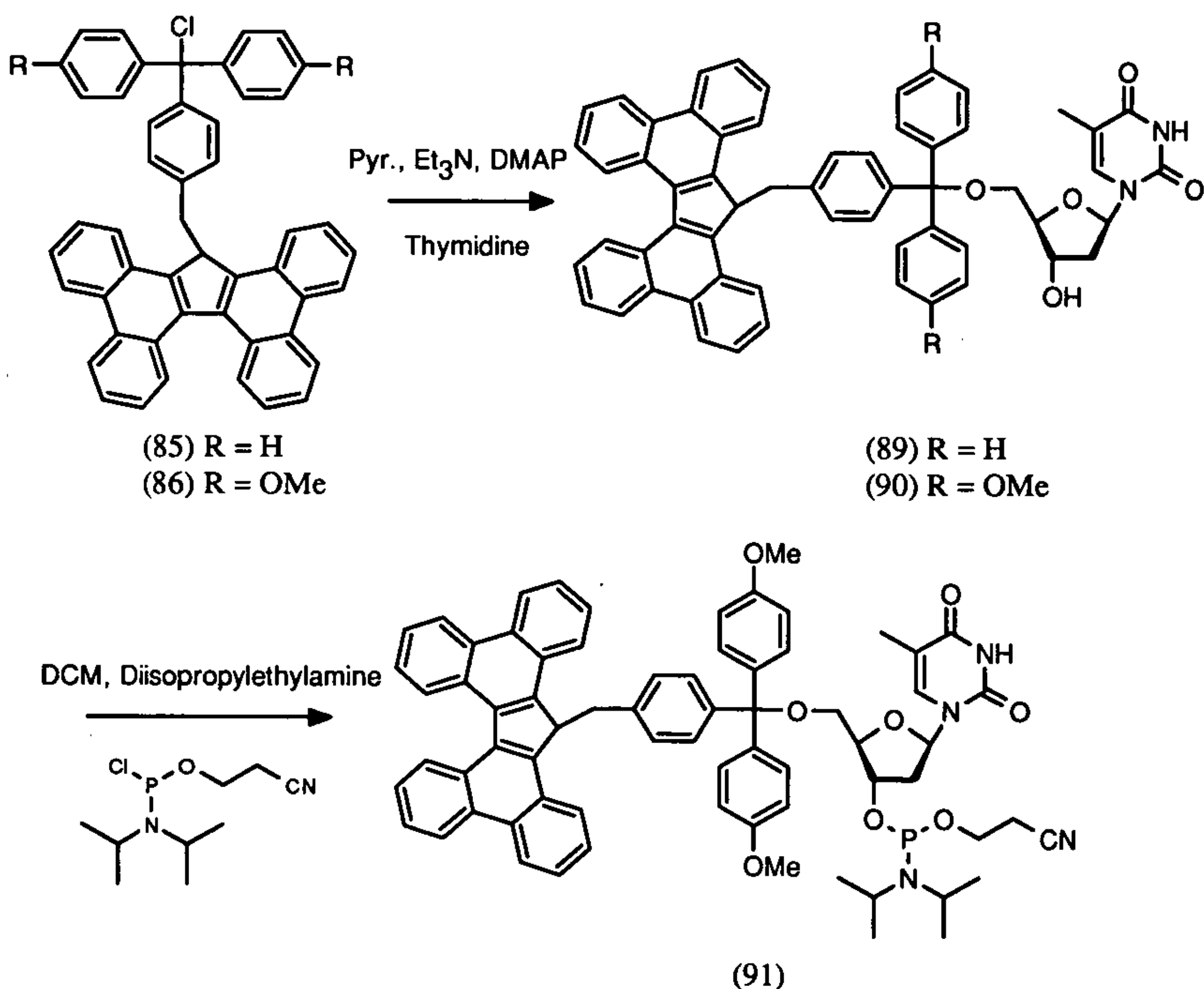
2.5 Tbf-Trityls as 5'-Hydroxyl Protection for Nucleosides

2.5.1 5'-Hydroxyl Protection

In order to investigate the possibility of using the Tbf-Trityl group for 5'-hydroxyl protection, it was necessary to synthesize a nucleotide monomer which would then be converted into an oligonucleotide. It was decided that thymidine would be the most convenient nucleoside to employ for these studies as it could be used without having to consider exocyclic-amine protecting groups.

The Tbf-Tr and Tbf-DMTr groups were introduced into the nucleoside *via* their chloride. These reacted with the 5'-hydroxyl function to form an ether linkage, following a S_N1 mechanism. The trityl cation intermediate is mesomerically stabilized due to the delocalization of the positive charge over the aromatic system. For sterical reasons a Walden inversion[212] following an S_N2 mechanism is improbable, furthermore the reaction seem to proceed better in polar solvents (stabilization of the ionic intermediates). The primary hydroxyl reacted much faster than the secondary 3'-hydroxyl and so very little of the 3'-protected or 3',5'-protected products were

observed. This result was expected as primary hydroxyl groups are more nucleophilic than secondary hydroxyls. The yields of the 5'-protected thymidine obtained from this reaction were moderate when compared to that obtained when 4,4'-dimethoxytrityl chloride DMTr-Cl is used because of the difference in the steric bulk. Following this, the phosphoramidite thymidine monomer was prepared in high yields from N,N-diisopropyl β-cyanoethyl phosphoramidyl chloride, according to the procedure developed by McBride and Caruthers.[68] The reaction scheme leading to the phosphoramidite monomer is shown in *Scheme 2.15*.



Scheme 2.15

2'-Deoxythymidine was protected using Tbf-Tr-Cl (85) and Tbf-DMTr-Cl (86) in pyridine with the presence of triethylamine as a base and DMAP as a nucleophilic catalyst,[213] to give the corresponding 5'-protected nucleoside (89) and (90). After optimisation of the reaction conditions, a yield of 35 and 40% after chromatographic separation for 5'-O-(4-(17"-tetrabenzo[*a,c,g,i*]fluorenylmethyl)-trityl)-thymidine (89) and 5'-O-(4-(17"-tetrabenzo[*a,c,g,i*]fluorenylmethyl)-4"',4""-dimethoxytrityl)-thymidine (90) respectively was obtained. The 5'-protection reaction had to be carried out under strict anhydrous conditions in order to avoid hydrolysis of the reactive species. The original reaction was carried out in pyridine, but very low yields were obtained. When heated up the reaction mixture turned dark and gave several by-products (3'- and 3'-5'- substitution). The yield was improved by the addition of a stronger base, triethylamine, and a catalyst DMAP[213] but the most significant improvement was observed when the reaction was carried out in a concentrated medium, therefore increasing the absolute rate of the reaction. In the latter reaction over 90% of the unreacted protecting group reagent could be regenerated from the chromatographic fractions. Thus, despite of the lower yields obtained for the 5'-protection reaction, compared to DMTr-Cl (80-100%), Tbf-Tr-Cl or Tbf-DMTr-Cl can act as suitable protecting groups for the selective protection of primary hydroxyls. The synthesis of the phosphoramidite (91) was performed under dry, oxygen free conditions in order to obtain the product in the highest purity possible. N,N-diisopropyl β-cyanoethyl phosphoramidyl chloride was reacted with the 5'-protected nucleoside (90) with hydrogen chloride being eliminated, to form a phosphite bond onto the 3'-hydroxyl function. The phosphoramidite, 5'-O-(4-(17"-tetrabenzo[*a,c,g,i*]fluorenylmethyl)-4"',4""-dimethoxytrityl)-thymidine-3'-O-N,N-diisopropyl-β-cyanoethyl phosphoramidite (91), was isolated as an oil which could be precipitated with n-hexane to give a white powder that can be stored for several months in a freezer without decomposition. ^{31}P nmr of this product gave two peaks at 148.51 and 148.03ppm corresponding to the diastereoisomeric mixture.

Decomposition products in particular phosphonate by-products were observed in the region of +15ppm. Compound (91) is only partially soluble in acetonitrile at the concentration normally used on DNA synthesizers (0.1 M) but was found to be very soluble in DCM. The phosphoramidite derivative of the less acid labile protected nucleoside (89), was not prepared in this instance but its synthesis would proceed under similar conditions. The coupling of Tbf-DMTr-Cl (86) to deoxycytidine and deoxyadenosine was attempted under similar conditions than thymidine. Some adduct could be seen by t.l.c of the crude but was too little to be recoverable.

2.5.2 Deprotection Study

The deprotection rates of the newly protected thymidine, compound (89) and (90) were compared with the deprotection rate of the commonly used 5'-DMTr-thymidine nucleoside. The deprotection was carried out under acidic conditions. The acid labile Tbf-Trityl group generates a colourful (deep orange) carbocation the absorbance of which at 500 nm was measured against time, see *Fig. 2.9*. The deprotection study was carried out at 50°C in 80% acetic acid/acetonitrile. Under these conditions, the kinetics of deprotection could be examined over a realistic time scale. The deprotection was considered as a pseudo-first order rate reaction. The irreversibility of the reaction being a function of the reaction co-ordinate, we used an excess of acid for the reaction to go practically to completion. The apparent rate constant were calculated according to:

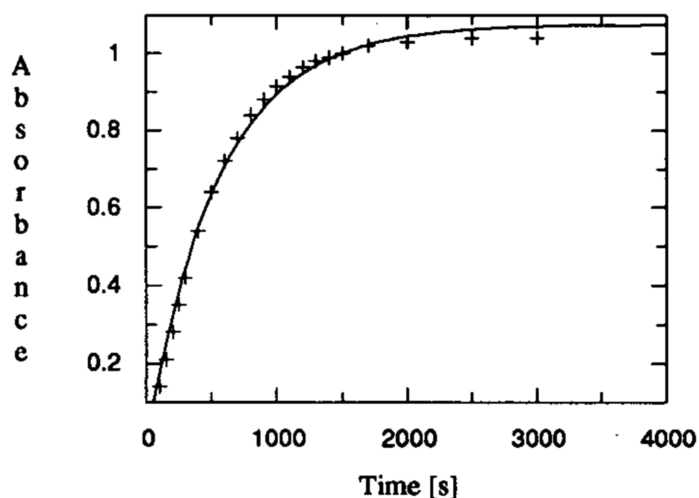
$$Ln((1-A_t)/A_\infty) = -k_x t$$

Where A_∞ is the absorbance at infinite time and A_t is the absorbance at time (t). The values for A_∞ and A_t were taken from the polynomial curvefit of *Fig. 2.9*. The reaction rate k was obtained from the gradient of $Ln((1-A_t)/A_\infty)$ against t. The results are summarised in *Table 2.4*.

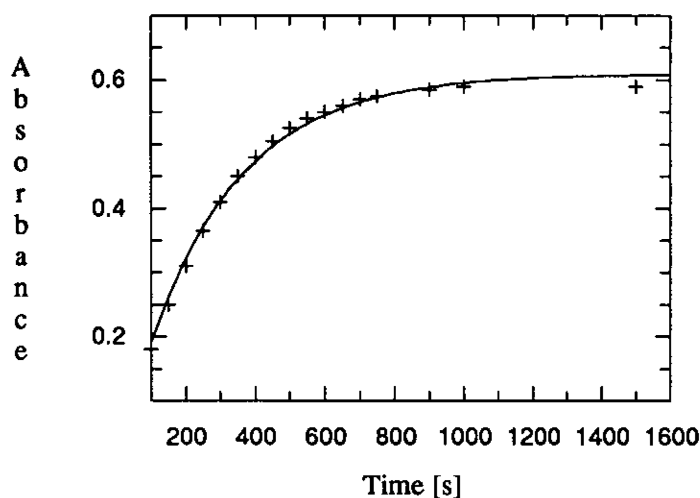
Compound	$10^{-3} \cdot k$ [s^{-1}]	τ [s]	A_∞	χ^2
5'-DMTr-T	1.8 ± 0.1	385	1.0752 ± 0.012	$57.88 \cdot 10^{-5}$
5'-Tbf-DMTr-T (90)	3.7 ± 0.1	187	0.6094 ± 0.005	$7.40 \cdot 10^{-5}$
5'-Tbf-Tr-T (89)	not detected	-	-	-

Table 2.4: Deprotection study results

Comparing the rate constant values k for the deprotection of thymidine in DMTr-T and Tbf-DMTr-T, protecting group Tbf-DMTr was found to be twice as acid labile as DMTr-T under the same conditions. The half-time life τ of a first order reaction is independent of the initial concentration of the reactant and are obtained from the equation $\tau = (Ln\ 2)/k$. From the results in *Table 2.4*, half the concentration of DMTr-T is deprotected after 385 s and half the concentration of Tbf-DMTr-T is deprotected after 187 s under the experimental conditions described above. This result was expected as the methylene group between Tbf and trityl has inductive electron-donating properties, making the compound more acid labile. On the other hand the rate of deprotection of compound (89) could not be determined under the same conditions as it is much lower than the rate of (90) and 5'-DMTr-T. The rate of acid cleavage was studied by Khorana;[121] he found that each p-methoxy substituent enhances the rate of cleavage by a factor of 10.



(a)



(b)

Fig. 2.9: UV deprotection study of 5'-DMTr-T (a) and 5'-Tbf-DMTr-T (b)

2.6 Synthesis of 5'-Tbf-DMTr-Oligodeoxyribonucleotides

2.6.1 Coupling of 5'-Tbf-DMTr-T-phosphoramidite

As previously reported in section 2.5.1, the 5'-Tbf-DMTr-protected thymidine was successfully activated for the solid phase phosphoramidite oligonucleotide

synthesis cycle. Subsequently a series of oligonucleotides were synthesized using the standard protocol for the β-cyanoethyl-phosphoramidite chemistry delivered by ABI for their Model 380 DNA synthesizer, see Appendix I. Phosphoramidite (91) was used as a 0.1 M solution in acetonitrile/DCM : 9/1 and was connected to the synthesizer. The synthesis of oligonucleotide was carried out on a 1 μmol scale on CPG supports. The reactivity of the phosphoramidite (91) was first determined qualitatively by synthesizing a polythymidine, 5'-Tbf-DMTr-d(T)$_{12}$ (92), using 5'-O-(4-(17"-tetrabenzo[*a,c,g,i*]fluorenylmethyl)-4''',4''''-dimethoxytrityl)-thymidine-3'-O-N,N-diisopropyl-β-cyanoethyl phosphoramidite (91) in the final monomer addition, thus only the desired sequence would bear the fluorescent protecting group. Later a randomly selected 6-mer (93) sequence was synthesized in a similar fashion. The sequences were as follows:

(92) 5'-Tbf-DMTr-TTT-TTT-TTT-TTT-3'

(93) 5'-Tbf-DMTr-TAG-CGT-3'

In order to increase the coupling efficiency, the coupling time for monomer (91) was prolonged to 5 min instead of the usual 30 s. In each case we obtained a crude oligonucleotide solution in ammonium hydroxide that was strongly fluorescent under UV light (254 nm). This qualitative observation was a clear indication that the modified thymidine monomer (91) had coupled to the solid supported oligonucleotide chain. As expected control sequences that were left with the standard DMTr group at the 5'-end did not fluoresce.

A more thorough investigation was undertaken when oligodeoxyribonucleotides (94), (95) and (96) were synthesized :

(94) 5'-**Tbf-DMTr**-TTC-GAG-CCA-TAT-3'

(95) 5'-**DMTr**-TTC-GAG-CCA-TAT-3'

(96) 5'-$^{*}T_{c}{}^{*}T_{b}{}^{*}T_{a}$-T-3' (*T represents the phosphoramidite thymidine monomer protected with Tbf-DMTr at the 5'-hydroxyl, see page 97)

5'-Tbf-DMTr substituted oligonucleotide (94) was compared to a control oligodeoxyribonucleotide (95) bearing DMTr at the 5'-end. Both products were deprotected in concentrated ammonia according to the standard protocol and analysed by RP-HPLC where the retention times were consistent with the hydrophobicity order of the two protecting groups. *Fig. 2.10 (a)* shows the crude control oligonucleotide (95) after ammonia evaporation and *Fig. 2.10 (b)* the Tbf-DMTr bound oligodeoxyribonucleotide (94). In both chromatograms the failure sequences elute at ~32% buffer B (see section 3.1) or after 9 to 11 min. The more hydrophobic products elute at 65% buffer B (see section 3.1) or 21 min in the case of (95) whereas the highly hydrophobic oligodeoxyribonucleotide (94) elutes after 10 min at 100% buffer B (see section 3.1). One other important advantage of Tbf-DMTr substituted oligonucleotides is that they can be detected at 365 nm as shown in *Fig. 2.11*. These observations demonstrate that the addition of the Tbf moiety to DMTr increases the lipophilic character of synthetic oligonucleotides drastically and enables detection at UV wavelengths which do not damage DNA. The use of this protecting group could therefore be useful in RP-HPLC chromatographic purification of crude oligonucleotide mixtures.

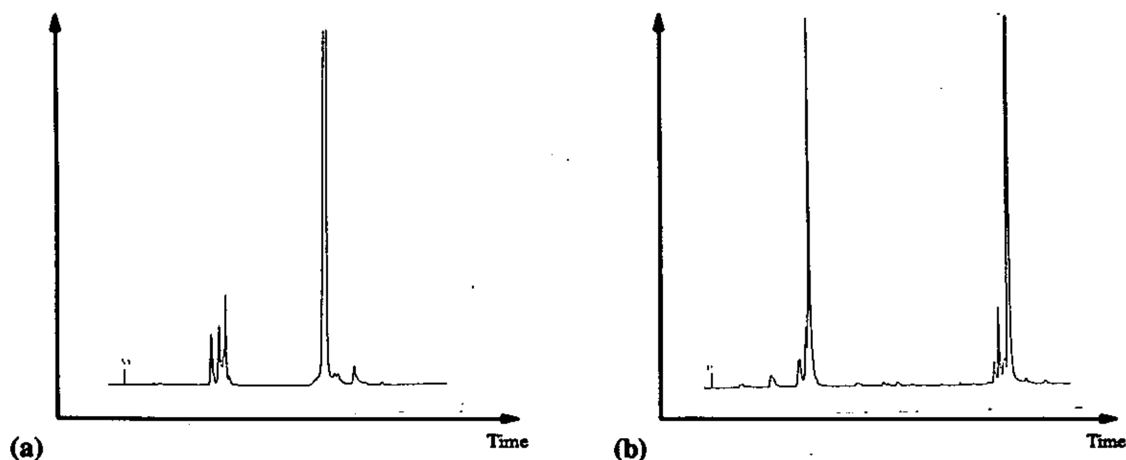


Fig. 2.10: (a) RP-HPLC spectra of (95) Gradient I,
(b) RP-HPLC spectra of (94) Gradient III at 280 nm

Fig. 2.11: RP-HPLC spectra of (94) at 365 nm, Gradient III

A quantitative measurement of overall yield was taken from the integration over the HPLC peaks. A yield of 85% is considered to be normal for the synthesis of a 12-mer oligonucleotide when an average coupling yield of 98% is achieved. For the DMTr bound oligonucleotide (95), 85% yield of the major peak was obtained whereas the product peak of Tbf-DMTr bound oligonucleotide (94) yielded only

60%. Similar RP-HPLC patterns were obtained for various other sequences and different monomer batches. These observations show conclusively that the coupling of the final Tbf-DMTr-monomer (91) proceeds with poor efficiency. The two sequences (95) and (94) were subsequently deprotected in 80% acetic acid/water for 30 min and an equal sample of both solutions was co-injected in the HPLC to give one single peak after 11 min with Gradient II (see section 3.1), thus indicating that the two sequences were identical.

In order to determine the exact coupling efficiency of monomer (91), sequence (96) was synthesized. $^{*}T_{a}$, $^{*}T_{b}$ and $^{*}T_{c}$ represent the phosphoramidite thymidine monomer protected with Tbf-DMTr at the 5'-hydroxyl (91); a,b or c is the order of addition. The coupling efficiencies of each coupling step could be determined by comparing the UV absorbance of the released Tbf-DMTrityl carbonium ion liberated after each 5'-deprotection in the synthesis process. The yellow detritylation solutions were diluted in 0.1 M p-toluene sulphonic acid in acetonitrile and the absorbance measured at 500 nm. The oligonucleotide required three additions of the monomer, resulting in two measurable coupling yield. The results of two experiments carried out with different coupling times are shown in *Table 2.5*.

Coupling time [min]	Absorbance of Tbf-Trityl cation	Coupling yield of $^{*}T_{b}$ [%]	Coupling yield of $^{*}T_{c}$ [%]
5	a: 0.317 b: 0.224 c: 0.197	71	88
15	a: 0.284 b: 0.232 c: 0.167	82	72

Table 2.5: Coupling efficiency of the 5'-Tbf-DMTr-T phosphoramidite (91)

The poor coupling efficiencies observed by RP-HPLC were again confirmed by this more accurate method. Even when trebling the reaction time from 5 to 15 min, coupling efficiencies ranging from 71 to 88% were obtained. The discrepancy in the yields compared to the yields normally obtained with DMTr-protected nucleosides, is difficult to explain but we suggest that the bulky structure of the monomer (91) is the cause for low coupling yields. Moreover with this approach, four additional ports for the four modified Tbf-monomers would have to be available on commercial DNA synthesizers, unfortunately complicating their machine design. Therefore in the general use of Tbf-DMTr-monomer, the problem of low coupling yields and additional engineering on synthesizers had to be resolved.

2.6.2 Reaction of Tbf-DMTr-Cl on Solid Phase

The preparation of phosphoramidite nucleotidic monomers requires a certain amount of care, as anhydrous and inert atmosphere conditions are required. These compounds are rather unstable and a fresh solution of a phosphoramidite monomer has to be used within 5 days of first usage otherwise unwanted by-products such as phosphonates and phosphate are formed. The synthesis of these compounds involves two steps: 5'-hydroxyl protection and phosphitylation. Tbf-DMTr-monomers are only used once at the end of the chain elongation cycle and are therefore used infrequently when compared to the DMTr-monomers. To overcome these obstacles, the Tbf-DMTritylation reaction was attempted on the 5'-end of the oligodeoxyribonucleotide while still attached to the support, usually CPG. As there was no reference in the literature of a solid phase tritylation reaction with oligonucleotides; we first investigated this reaction with the commercially available DMTr-Cl. The following two oligonucleotide sequences were first synthesized:

(97) : 5'-DMTr-TGC-AA-3' (Auto Tritylation)

(98) : 5'-DMTr-TGC-AA-3' (Manual Tritylation)

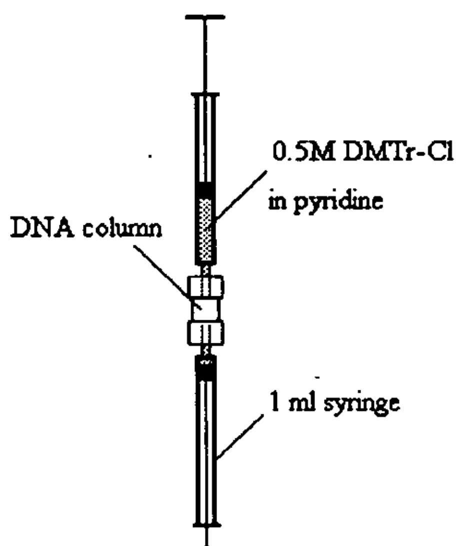


Fig. 2.12: Manual DMTritylation on a DNA column

Sequence (97) was synthesized according to the standard protocol in the "Trityl-on" mode where the 5'-DMTr group is left intact at the end of the synthesis, see Appendix I. The synthesis scale was 0.2 μmol with a CPG support. Oligodeoxyribonucleotide (98) was synthesized in the "Trityl-off" mode (removal of the 5'-DMTr group at the end of the synthesis) so that a tritylation reaction with DMTr-Cl and the free 5'-hydroxyls was carried out subsequently with the help of two syringes connected to each end of the column, see *Fig. 2.12*. The syringes contained a 0.5 M solution of DMTr-Cl in dry pyridine which was flushed through the column over 2 h, in order to perform the tritylation reaction (see section 3.4). After a thorough wash of the column with pyridine and acetonitrile, it was remounted onto the synthesizer and the product was cleaved off the support with concentrated ammonium hydroxide. The ammonia used for the cleavage and deprotection was then removed by evaporation or liophilisation. It is important to note that the DMTr group is unstable to heat and acid.

Therefore, evaporation should be done at room temperature, a drop of triethylamine should be added periodically throughout the evaporation process as a precaution. After the samples were dried, they were redissolved immediately in 1 or 2 ml of the HPLC buffer. Then, the oligonucleotides (97) and (98) were compared by RP-HPLC with Gradient I (see Section 3.1) and showed identical patterns as seen in *Fig. 2.13* and *2.14*. After peak integration similar yields were obtained for both syntheses.

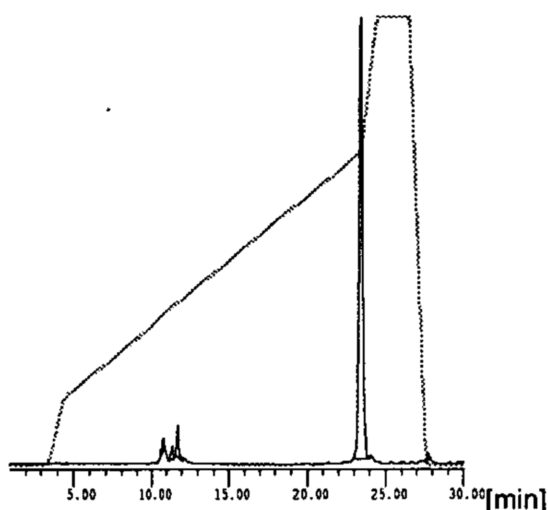


Figure 2.13: RP-HPLC of (97)

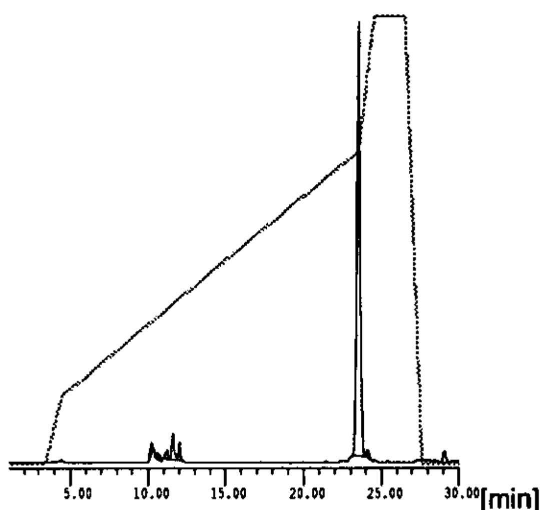


Figure 2.14: RP-HPLC of (98)

Following these results, longer oligodeoxyribonucleotides of 12 and 30 monomer units of random sequences were synthesized according to the same procedure. The yields obtained are presented in *Table 2.6*. AUTO tritylation is indicative for DMTr-oligonucleotide synthesized with the "Trityl-on" mode and MANUAL tritylation is indicative for DMTr-oligonucleotide synthesized with the "Trityl-off" mode and tritylated with the syringe method described in Section 3.4. Clearly, the solid phase DMTritylation of the 5'-hydroxyl of an oligonucleotide bound to CPG, is equally efficient when performed post-synthetically as when introduced by

a DMTr-monomer during the synthesis cycle. Also, the size of the oligonucleotide has little influence on the manual tritylation.

AUTO				
Protecting group	Length [units]	OD units of crude (a)	HPLC Yield [% area]	Theor. Yield [%]
DMTr	5	8	92	93
DMTr	12	34	84	83
DMTr	30	74	56	64

MANUAL				
Protecting group	Length [units]	OD units of crude (a)	HPLC Yield [% area]	Theor. Yield [%] (b)
DMTr	5	9	92	93
DMTr	12	17	84	83
DMTr	30	41	60	64

(a) OD units measured at 260 nm.
(b) Theoretical yield calculated with 98.5% yield/coupling step.

Table 2.6: Comparative study of the 5'-OH DMTritylation

In an identical manner, we synthesized compound (99) and (100) and compared the reactivity of Tbf-DMTr-Cl and DMTr-Cl on a support bound oligonucleotide.

(99) : 5'-DMTr-TGC-AA-3' (Manual Tritylation)

(100) : 5'-**Tbf-DMTr**-TGC-AA-3' (Manual Tritylation)

Both oligonucleotides were synthesized with the "Trityl-off" mode and were subsequently manually reacted with a 0.5 M pyridine solution of their respective 5'-hydroxyl protective group. The reaction time was set to 2 h. 0.5 M Solutions of Tbf-DMTr-Cl were close to saturation and happened to crystallize with fluctuations in ambient temperatures; 0.4 M solutions were later used giving the same results. Both

products were analysed by RP-HPLC and gave similar yields according to HPLC peak integration. Subsequently we repeated the experiment for longer oligonucleotides as summarised in *Table* 2.7. The results obtained demonstrate that Tbf-DMTr-Cl reacts as efficiently as DMTr-Cl on a support bound oligonucleotide in spite of its greater steric bulk; perhaps this is compensated for by a higher chemical reactivity. The substitution of the 5'-hydroxyl of oligonucleotides with G, C or A as the terminal unit, was also investigated and gave comparable product quality. This method of introduction of the Tbf-DMTr group onto an oligonucleotide proceeds to completion in all cases, possibly due to the vast excess in Tbf-DMTr-Cl used (~1000 equivalents) and therefore makes the solution phase approach using the monomer (91) much less attractive. Although Tbf-DMTr-oligonucleotide were successfully synthesized by automated addition of Tbf-DMTr-Cl, a stability test showed that Tbf-DMTr-Cl/pyridine solution turned dark brown and became inactive after 1 day. It is therefore necessary to prepare the Tbf-DMTr-Cl/pyridine solution just before applying it to the column.

MANUAL				
Protecting group	Length [units]	OD units of crude (a)	HPLC Yield [% area]	Theor. Yield [%] (b)
DMTr	5	9	92	93
DMTr	12	17	84	83
DMTr	30	41	60	64
Tbf-DMTr	5	9	89	93
Tbf-DMTr	12	44	82	83
Tbf-DMTr	30	69	57	64

(a) OD units measured at 260 nm.
(b) Theoretical yield calculated with 98.5% yield/coupling step.

Table 2.7: Comparative study of the Tritylation of 5'-OH with DMTr-Cl and Tbf-DMTr-Cl

In conclusion, an alternative method to introducing Tbf-DMTr as a 5'-terminal group has been developed. A single reagent: Tbf-DMTr-Cl is used for A, C, T or G terminated oligonucleotides of various lengths thus avoiding the complicated preparation of phosphoramidite monomers. Moreover the addition of this new protecting group can be automated. The fluorescent properties of this protecting group facilitates the detection, especially at wavelengths > 300 nm. The application of this methodology to DNA sequences longer than 50 units is described in section 2.9.

2.7 Purification of Short Oligonucleotides

In section 2.2, the Tbfmoc group developed by Ramage and Raphy[194] was described as an affinity based Nα-protecting group for peptides. This protecting group improved the purification of crude peptides by affinity chromatography on Porous Graphitised Carbon (PGC) columns. PGC acts as a very strong adsorbent for polycyclic aromatic structures, it has been studied in detail by Knox[214,215,216] for use in liquid chromatography. The Tbfmoc group is based on tetrabenzo[*a,c,g,i*]fluorene (53) and likewise is Tbf-DMTr, we therefore investigated the purification of oligonucleotides by PGC affinity chromatography. Preliminary experiments were designed to show the affinity of the Tbf-DMTr group to PGC. 5'-Tbf-DMTr-thymidine (90) and commercial 5'-DMTr-thymidine were dissolved in equimolar amounts in methanol and then PGC powder was added and stirred for 10 min. This experiment was monitored by RP-HPLC at 264 nm so that two peaks 1 and 2 were observed before the addition of carbon, the peak with the longer retention time 2 being the more lipophilic 5'-Tbf-DMTr-thymidine (90) as shown in *Fig. 2.15 (a)*. Tbf contributes greatly to the absorbance of (90) hence the greater intensity for peak 2. When a sample of the supernatant solution after PGC addition was injected, a single

peak was observed at a retention time corresponding to 5-DMTr-thymidine, see *Fig. 2.15 (b)*. A total retention of compound (90) was achieved clearly demonstrating the affinity of tetrabenzo[*a,c,g,i*]fluorene to PGC.

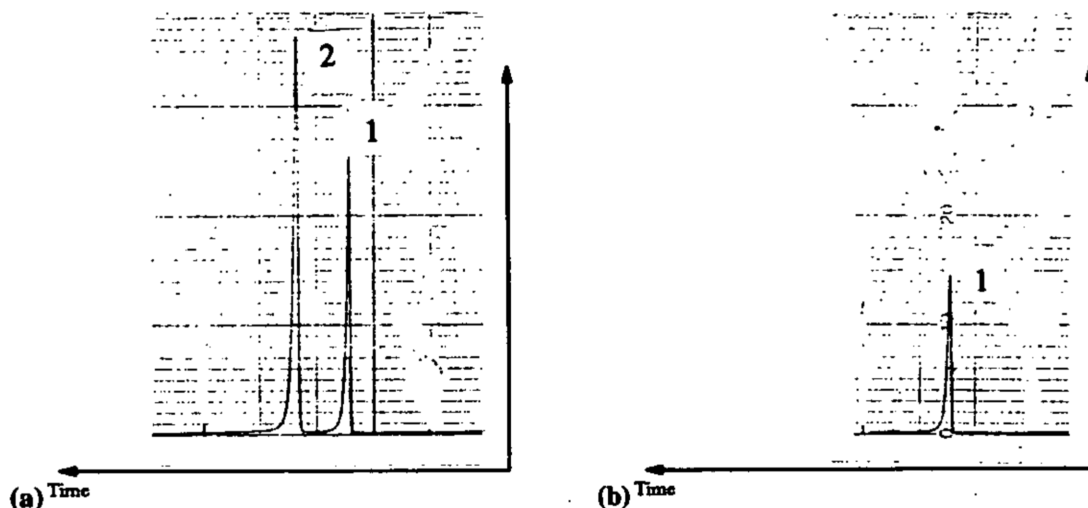


Fig. 2.15: Affinity of Tbf-DMTr to PGC; (a) before (b) after addition of PGC

Encouraged by the above results, we tried to purify a crude oligonucleotide bearing the Tbf-DMTr group at the 5'-end. For reasons of economy and simplicity, a 1 µmol scale batch of a 5'-Tbf-DMTr-$(dT)_{12}$ was synthesized and used in the PGC affinity experiments. PGC was kindly donated by Knox,[217] and had the following physical properties: particle size = 50-100 µm; specific surface area = 68 $m^2 \cdot g^{-1}$. An aliquot of an aqueous solution of the crude oligonucleotide was stirred with PGC powder. 10 to 40 % of dioxane was usually added in order to increase the homogeneity of the solution with PGC. Dioxane and tetrahydrofuran were described by Knox and Kaur[214] to have a greater eluotropic strength than acetonitrile or methanol in PGC chromatography. The experiments were monitored by HPLC, injecting a sample of the supernatant. We found that a weight ratio of oligonucleotide to PGC of 1:3, was necessary to have complete adsorption of the Tbf-DMTr bound thymidine. The absence of Tbf-DMTr material in solution was also confirmed by the

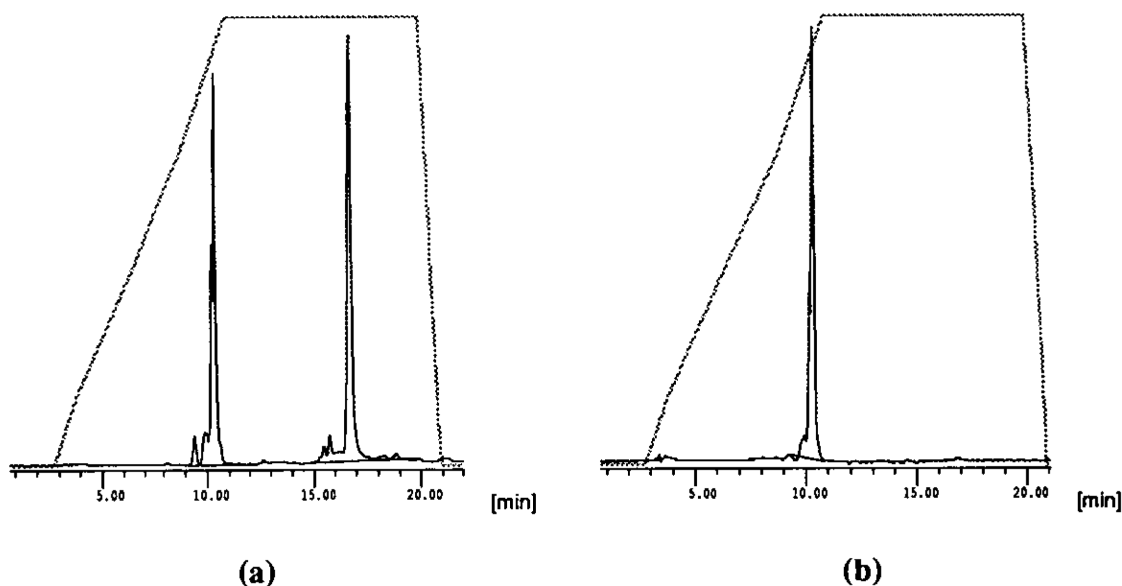
disappearance of fluorescence under UV light. Following this, the suspension was filtered to remove the by-products and the PGC was washed with dioxane/water. The PGC bound oligonucleotide was then treated with a 2% water/dioxane solution of TFA to be freed into solution. Deprotection times between 5 min and 2 h were investigated as well as acid concentrations from 2-100 %. The polarity of the solution was varied by using mixtures of water/dioxane, water/acetonitrile but unfortunately no free oligonucleotide could be detected in the supernatant. It appeared that oligonucleotides tend to be adsorbed irreversibly to PGC. At this stage we did not carry out a more thorough investigation but concentrated instead on the highly hydrophobic character of Tbf-DMTr in the scope of an alternative purification procedure for synthetic oligodeoxyribonucleotides.

Reverse phase silica chromatography for example separates a mixture of compounds according to their hydrophobicity. A simple purification method, based on the fact that Tbf-DMTr attached oligonucleotides are much more hydrophobic than free oligonucleotides, was developed. This method makes use of 1.5 cm long polypropylene cartridges, packed with 250 mg C18 silica gel, available commercially from Waters under the brand name SEP-PAK. These small and cheap columns are normally used for desalting peptide solutions. Each of them contain an inlet and an outlet which can be connected to a syringe. In a first instance we determined at which solvent polarity failure sequences (impurities) and Tbf-DMTr-bound oligonucleotides would elute. By using analytical HPLC, we carried out a series of isocratic runs from 5% to 45% acetonitrile (or 45% to 5% 0.1 M aqueous ammonium acetate), with the following crude oligonucleotide (*Fig. 2.16 (a)*):

5'-Tbf-DMTr-TTC-GAG-CCA-TAT-3'

The failure sequences started to elute with 5% acetonitrile and finished eluting with 10% of acetonitrile. The Tbf-DMTr-oligonucleotide did not elute until 40% of

acetonitrile. We were then able to use the correct solvent polarity for the isocratic purification of oligonucleotides on SEP-PAK cartridges. About 20 OD units of the crude oligonucleotide were loaded onto a cartridge and the elution was monitored by analytical HPLC, see *Fig. 2.16*. The best loading was obtained with a flow rate of about 1 drop every 2 s. Under UV light, at 254 nm, a clear narrow fluorescent band could be detected on the top layer of the silica cartridge. The deletion oligonucleotides were eluted with 10% acetonitrile/90% 0.1M aqueous ammonium acetate without any of the Tbf-DMTr-oligonucleotide, as shown on the HPLC trace in *Fig. 2.16 (b)*, Rt = 10.5 min. The pure oligonucleotide was finally eluted with 20% acetonitrile/80% 0.1M aqueous ammonium acetate, retention time = 10.5 min, after deprotection on the cartridge (*Fig. 2.16 (c)*).



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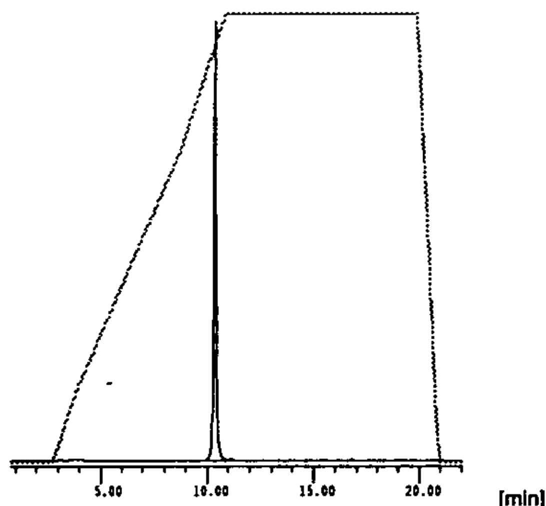


(c)

Fig. 2.16: Purification on a SEP-PAK cartridge

This purification method was repeated with various 0.2 μmol batches of 12-mer oligonucleotide as well as a 1 μmol batch 6-mer, the same efficiency was obtained in each case. Subsequently a 7 point procedure for the purification of short oligonucleotides on C18 silica gel SEP-PAK cartridges was drafted:

1. Flush the SEP-PAK cartridge with 5 ml acetonitrile, followed by 5 ml 0.1 M aqueous ammonium acetate with a properly adapted syringe.

2. Take the crude ammonia solution, evaporate it, and dissolve the crude oligonucleotide in 2 ml of 0.1M ammonium acetate. Place the solution in the syringe and slowly push it through the cartridge.

3. Slowly wash the cartridge with 2 x 5 ml of 10% acetonitrile/90% 0.1M ammonium acetate.

4. Wash the cartridge with 5 ml of deionized water.

5. Push in ca. 2 ml of a 2% solution of trifluoroacetic acid in water to deprotect the Tbf-DMTr bound oligonucleotide, and wait 10 min. A yellow coloration should appear.

6. Flush the cartridge with 5 ml of deionized water.

7. Elute the purified oligonucleotide with 2 ml of 20% acetonitrile/80% 0.1 M ammonium acetate

The purification method described above has also been carried out with DMTr bound oligonucleotide but gave inconsistent results. The fact that Tbf-DMTr is much more lipophilic than DMTr and consequently that failure sequences elute nearer to DMTr-oligonucleotides, allows to be more flexible in the solvent polarity choices when using Tbf-DMTr as the protecting group. The purification of an oligonucleotide is reduced to 15 min, instead of 2 h on preparative HPLC. Although the purification of longer and more valuable oligonucleotides (> 20-mer) could be envisaged, it is generally preferable to use preparative RP-HPLC in order to avoid any loss of material.

We purchased similar cartridges from Applied Biosystems but packed with polystyrene beads. Polystyrene has the advantage over C18 silica gel to be stable at pH > 10. Hence the loading of the ammonia solution (denaturing conditions) containing the crude oligonucleotide could be envisaged. The purification of the 12-mer oligonucleotide mentioned above was carried out using the same protocol as for SEP-PAK cartridges. *Fig. 2.17* shows the HPLC trace of the eluates after removal of the truncated sequences (a) and after deprotection with TFA (b). A small amount of Tbf-DMTr bound oligonucleotide broke through the polystyrene column but could be reloaded by passing the eluate through the column once again. This breakthrough is probably due to the poor packing of the cartridges. The free oligonucleotide could be eluted as a single peak, therefore demonstrating that this purification method is also applicable to a polystyrene stationary phase when Tbf-DMTr is used as a hydrophobic group.

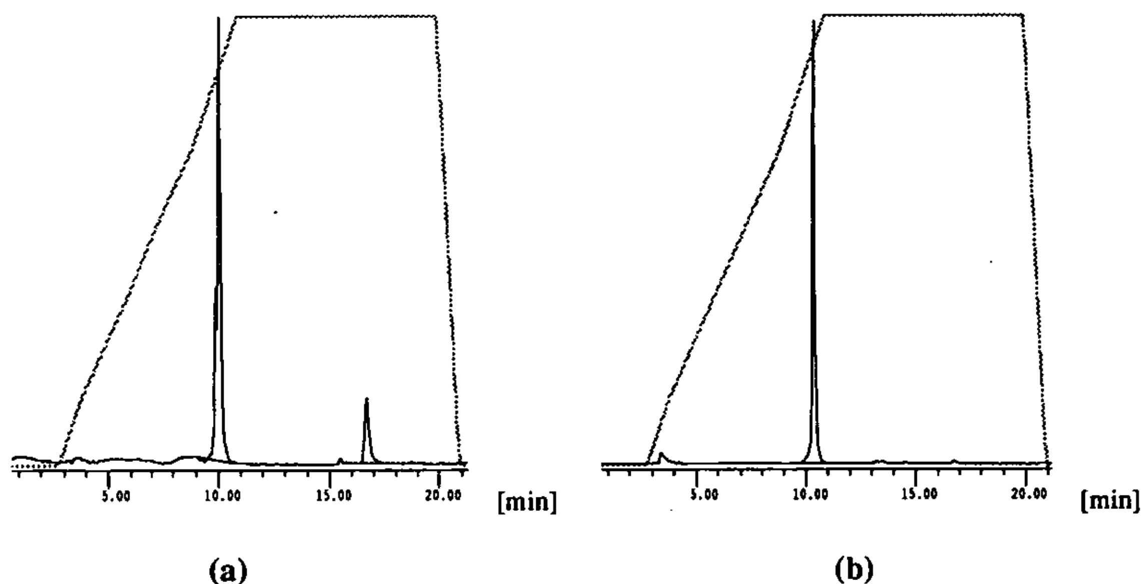


Fig. 2.17: Purification with a polystyrene cartridge

In summary, we have demonstrated that the purification of short oligonucleotides can be done by stepwise isocratic rather than the time consuming gradient elution and hence the overall DNA synthesis may be facilitated. This method enables column-capturing of the desired oligomer on small RP-C18 silica gel or polystyrene cartridges.

2.8 Purification of Long Oligonucleotides

The synthesis of oligonucleotides longer than 50 monomer units becomes a challenge as over 200 chemical steps are involved, each of them producing impurities which build up, complicating the purification. In a good synthesis the coupling yields after each step are normally not greater than 98.5%; this means that the overall yield of a 50-mer oligonucleotide would be < 47%, for a 100-mer it would be < 22% and for a 150-mer it would be < 10%. These theoretical calculations illustrate how

important the purification factor has become in a situation where the amount of impurities exceeds greatly that of product. Long oligonucleotides also become more hydrophobic and therefore the difference in retention time on RP-HPLC between failure sequences and the tritylated product is lowered. Often the product peak will also appear very broad due to several stable conformations, a phenomenon which can increase and overlap with the impurity peaks. In order to demonstrate the usefulness of the Tbf-DMTr group in the purification of long oligonucleotides, we synthesized various DNA sequences of different lengths and varying terminal nucleotide. A 51-mer oligonucleotide (101) was synthesized in 0.2 μmol scale on CPG support with the Tbf-DMTritylation being performed manually as described in section 2.6.2.

(101) 5'-**Tbf-DMTr**-GAT-CTG-TTG-ACA-ATT-AAT-CAT-CGG-CTC-GTA-TAA-TGT-GTG-GAA-TTG-GTC-GAC-3'

(102) 5'-**DMTr**-GAT-CTG-TTG-ACA-ATT-AAT-CAT-CGG-CTC-GTA-TAA-TGT-GTG-GAA-TTG-GTC-GAC-3'

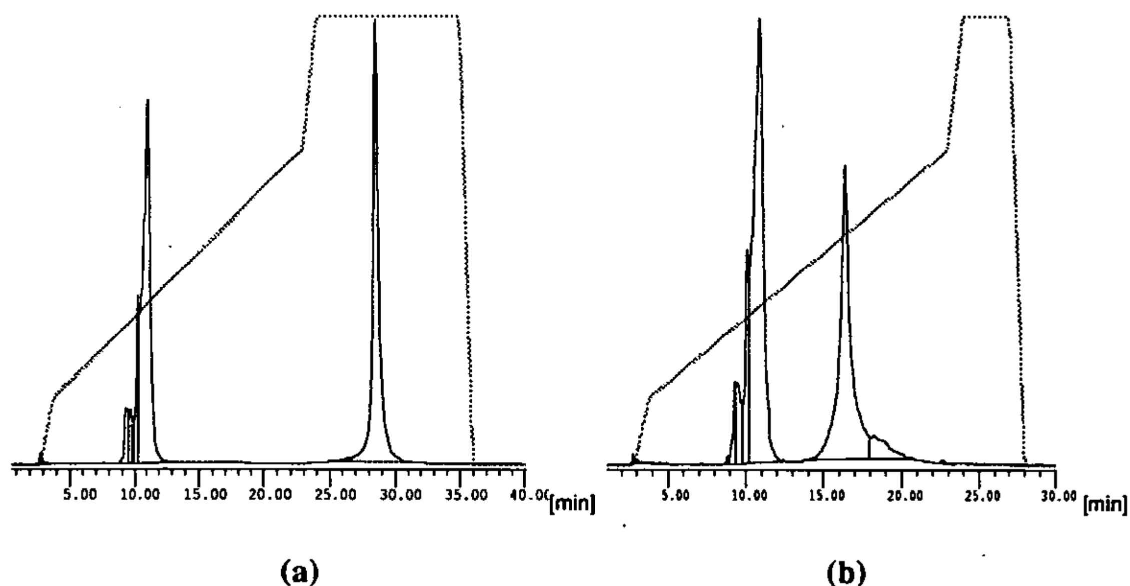


Fig. 2.18: HPLC profile of* (101) *((a), Gradient III) and* (102) *((b), Gradient I)

The same sequence bearing the DMTr group at the 5'-end (102) was synthesized for comparison. The HPLC profiles of the two crude products at 280 nm are shown in *Fig. 2.18.* No significant difference in yield was observed for both products; confirming on one side that the syntheses proceeded normally and that the Tbf-DMTritylation reaction was performed successfully. Subsequently a preparative HPLC was performed to collect the fluorescent Tbf-DMTr fraction, the latter was treated with 80% acetic acid to remove the protecting group and desalted on an Sephadex G-25 column. During the desalting step a number of contaminants are removed, such as benzamide, isobutyramide, Tbf-DMTr-OH, ammonium acetate and other organic and inorganic impurities, consequently the analyses and purification provide more accurate results. At this stage the product was analysed by capillary zone gel electrophoresis, see *Fig. 2.19.* As a result of the removal of the Tbf-DMTr group, two sequences of different size were revealed. The by-product could not have arisen from the acetic acid treatment because depurination may not occur at this stage as the bases are much more stable to acid after the base deprotection. The more likely explanation for this additional product, is that an incomplete capping step during the build up of the sequence left a reactive site for the final Tbf-DMTritylation. This observation emphasizes the importance of the capping steps during oligonucleotide synthesis which may have to be doubled in the synthesis of longer strands. The 51-mer was finally purified by a second preparative HPLC to give a product exhibiting a single peak on HPLC (Gradient II, see Section 3.1) yielding ~5 OD units. The purity was confirmed by capillary zone gel electrophoresis, see electropherogram *Fig. 2.20,* and PAGE, see *Fig. 2.21.*

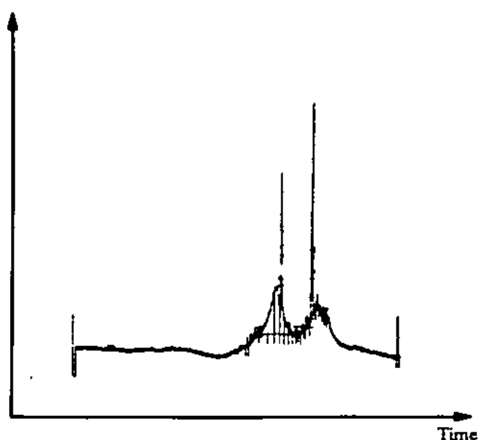


Fig. 2.19: CE trace after deprotection

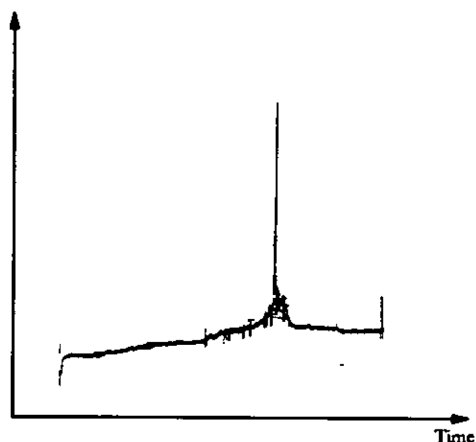


Fig. 2.20: CE trace of pure 51-mer

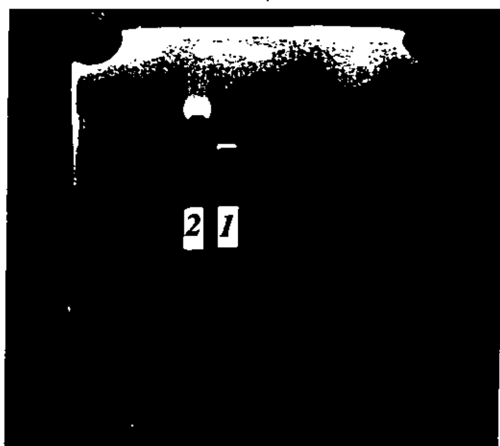


Fig. 2.21: PAGE of the purified 51-mer (lane 1) and a 45-mer reference sequence (lane 2)

Next a 102-mer oligonucleotide (102) was synthesized by the standard protocol on a 0.2 μmol scale with double capping steps. The sequence chosen consisted of twice the sequence of the above 51-mer (101) so that the occurrence of the uncapped truncated sequence observed in the 51-mer can be attributed to either insufficient capping time or to a sequence specific steric problem.

(102) 5'-**Tbf-DMTr**-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-GTG-GAA-TTG-GTC-GAC-3'

With a coupling yield of 98.5% on average, the expected overall yield ought to be 21.4%. From the integrated HPLC trace of the crude oligonucleotide (102) (*Fig. 2.22*) an overall yield of 11% was obtained. Although the deviation from the theoretical yield is great, it was considered as satisfactory because yields of long oligonucleotides can vary greatly according to the sequence and the freshness of the monomers. After preparative RP-HPLC and removal of the Tbf-DMTr group, two products were again obtained (*Fig. 2.23*), thus suggesting that the formation of this by-product due to an inefficient capping step, is sequence specific. The product was finally obtained as a single peak after a second preparative HPLC yielding ~1 OD unit. Attempts to analyse the oligodeoxyribonucleotide by PAGE or capillary zone gel electrophoresis (CE) was unsuccessful in this case as well as for the longer oligodeoxyribonucleotides because of small amounts of material available after purification. In PAGE, UV shadowing analysis or ethidium bromide staining analysis requires high amount of oligonucleotide, typically 0.5 to 1.5 OD units for sequences < 50 bases and 1.5 to 3 OD units for oligonucleotides > 50 bases. In capillary zone gel electrophoresis oligonucleotide concentrations of approximately 0.1-0.5 OD units/ml are appropriate.

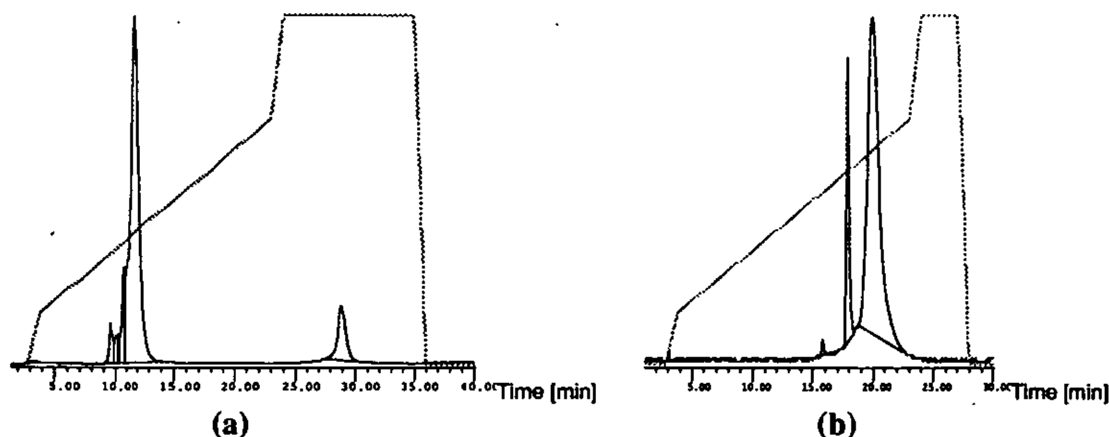


Figure 2.23: HPLC profile of crude* (102) *(a), gradient III and after deprotection (b), gradient II

The DNA synthesis efficiency of oligonucleotide > 100-mer can be increased when polystyrene support loadings are used.[218] Because polystyrene is hydrophobic, traces of water are less likely to be present as in CPG and moreover CPG beads may contain some uncapped silanol. The commercially available polystyrene supports have a loading capacity of 40 nmol. We used this support for the synthesis of a 153-mer oligonucleotide (103).

(103) 5'-**Tbf-DMTr**-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-GTG-GAA-TTG-GTC-GAC-GAT-CTG-TTG-ACA-ATT-AAT-CAT-CGG-CTC-GTA-TAA-TGT-GTG-GAA-TTG-GTC-GAC-3'

The synthesis was performed with double capping steps and fresh reagents. The HPLC profile of the crude 153-mer (103) is shown in *Fig. 2.24 (a)*. From the integration of the spectra, a product yield of 7% was obtained which compares to the calculated yield of 10% (with an average coupling step of 98.5%). Long oligonucleotides can form secondary structures with stable conformations which often results in a broadening of the peaks or in the appearance of several peaks. For these reasons the HPLC analysis of this 153-mer oligonucleotide was performed under a different gradient (Gradient IV, see Section 3.1) whereby the Tbf-DMTr-

oligonucleotide elutes under non-isocratic conditions. Fortunately this particular sequence eluted as a narrow peak. After collecting the more hydrophobic fraction and deprotecting it, the free oligonucleotide was analysed by HPLC and revealed a impurity peak with a shorter retention time, see *Fig. 2.24 (b)*. The sequence of (103) was designed to be three times the sequence of (101) where a similar HPLC pattern was obtained after removal of the Tbf-DMTr group. This observation again confirms that this recurrent side-product resulting from the Tbf-DMTritylation of an uncapped sequence is a sequence specific problem. With the final yields being so small this impurity could not be isolated and compared with those detected in (101) and (102). The purified oligonucleotide (103) was finally obtained as a single peak, see *Fig. 2.24 (c)*, with 0.1 OD unit.

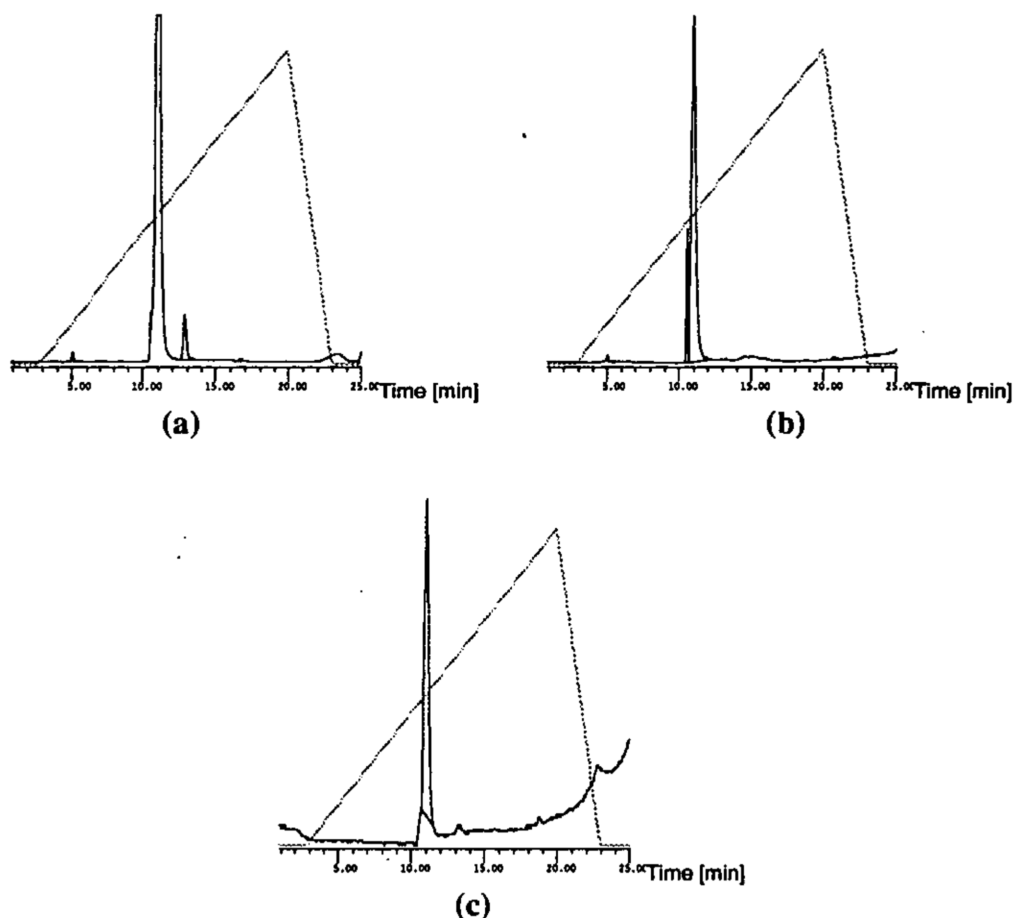


Figure 2.24: HPLC profile of (103) (a): crude, gradient IV; (b): after deprotection, gradient IV; (c): purified, gradient IV

The hydrophobic 5'-DMTr-oligonucleotides usually appear as separate peaks from the failure sequences but for longer sequences they can elute as shoulders of the main peak (failure sequences peak), due to the increased hydrophobic character of longer failure sequences. In this case it can be difficult to know when to start or to stop collecting. The following example illustrates the usefulness of the much more hydrophobic Tbf-DMTr group in the synthesis and purification of the two 130-mer oligonucleotide (104) and (105):

(104) 5'-**DMTr**-CCG-GAT-CCG-CGT-TAG-GGT-CAA-TGG-AGG-TGG-CTT-CAC-TTA-CGC-CAA-TGT-CGT-TAT-CCA-GCG-GTC-GTC-GGG-TGA-ACT-GAT-CGC-GCA-GCA-ATT-CGC-CCT-ATA-GTG-AGT-CGT-ATT-ACG-CGC-TGC-AGG-AAT-TCC-G-3'

(105) 5'-**Tbf-DMTr**-CCG-GAT-CCG-CGT-TAG-GGT-CAA-TGG-AGG-TGG-CTT-CAC-TTA-CGC-CAA-TGT-CGT-TAT-CCA-GCG-GTC-GTC-GGG-TGA-ACT-GAT-CGC-GCA-GCA-ATT-CGC-CCT-ATA-GTG-AGT-CGT-ATT-ACG-CGC-TGC-AGG-AAT-TCC-G-3'

The two syntheses were carried out on a 0.2 μmolar scale on CPG support. The RP-HPLC profile of the two crude oligonucleotides are shown in *Fig. 2.25*. The Tbf-DMTr bound oligonucleotide does clearly elute later than the failure sequences whereas the DMTr bound oligonucleotide (104) elutes closely to the truncated sequences and to what appear to be stable secondary structures of the product. The Tbf-DMTr-oligonucleotide was eluted under isocratic conditions with gradient III whereby no secondary structures of the product can be detected. Instead a sharp peak was obtained that enabled easy collection of the fraction. Oligonucleotide (105) was subsequently treated with acid to remove the Tbf-DMTr group, desalted over sephadex column and purified by HPLC to give a single peak product.

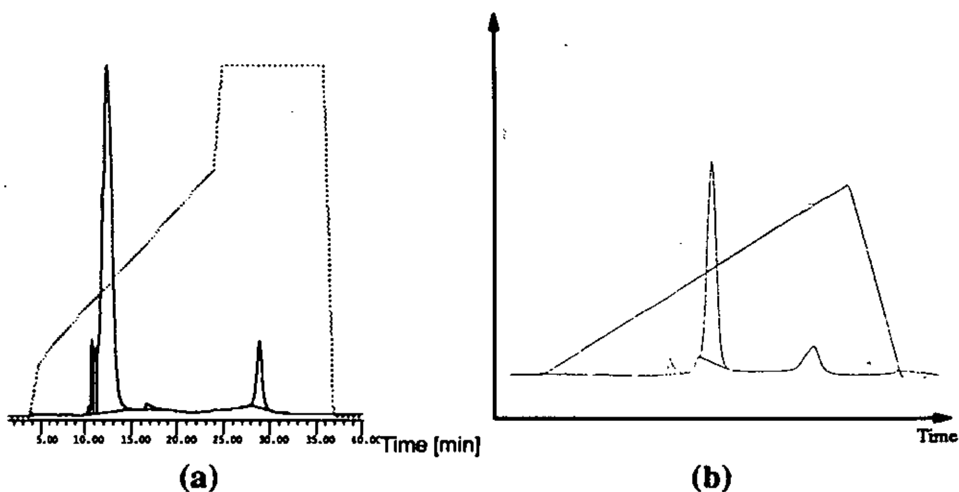


Figure 2.25: HPLC profiles; (a): crude (105), gradient I
(b): crude (105), gradient III

As an alternative to hydrocarbon-bonded silica columns, a polymeric adsorbent such as highly cross-linked, rigid polystyrene beads, can be considered. They offer stability a high temperature and pH and would probably have a very high capacity for "Tbf-DMTr-on" oligonucleotides, which would enable to run HPLC under denaturing conditions, therefore eliminating most secondary problems that one could encounter with long oligodeoxyribonucleotides; the resolution could be enhanced. RP-HPLC at alkaline pH would also eliminate the potential loss of Tbf-DMTr groups during the purification procedure.

2.9 Synthesis of a New Fluorescent Label for Oligonucleotides

The base-pairing property of oligomeric strands of DNA allows specific recognition of a particular base sequence in a target strand by a complementary base sequence in a probe strand. However since DNA probe strands do not naturally contain any convenient reporter groups, spectroscopic monitoring of hybridization is difficult.

~~contain any convenient reporter groups, spectroscopic monitoring of hybridization is difficult.~~

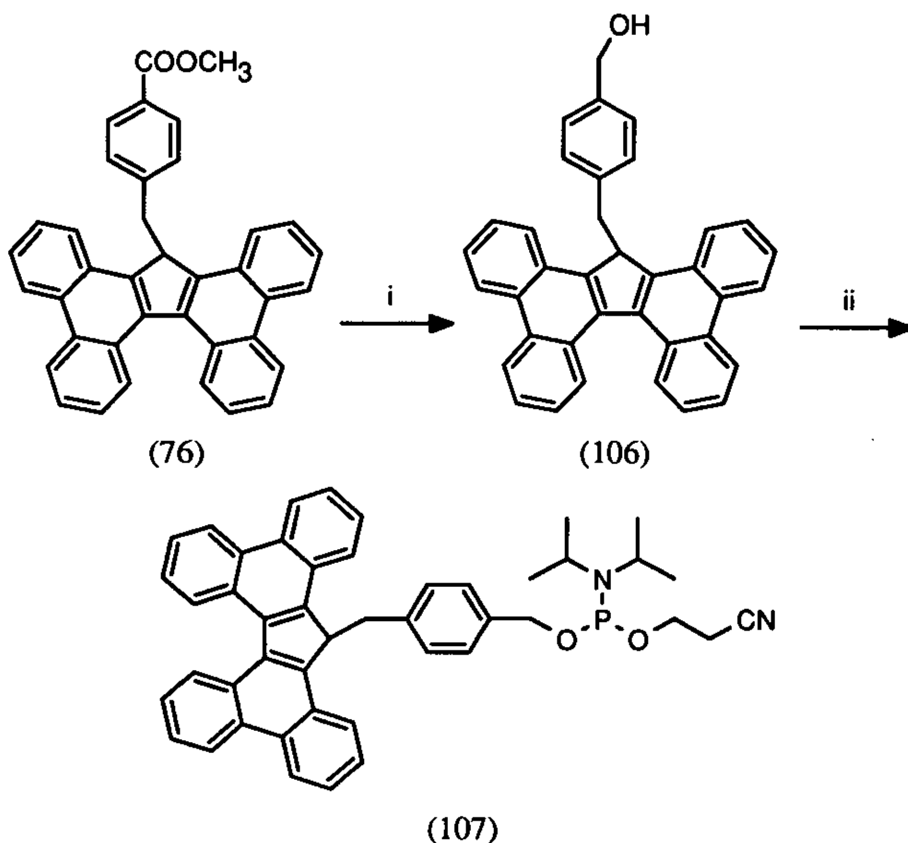
A method to overcome this problem is to prepare a synthetic oligonucleotide with the desired base sequence that also contains a covalently attached label (reporter group). In general, the functionalization of oligonucleotides for substituting reporter or reactive groups can be achieved using the following methods:

- Modification of nucleosides so that they contain a masked primary amino group on the heterocyclic base. These are incorporated into the oligonucleotide during synthesis.[219,220]
- Functionalization of deprotected oligonucleotides through their 5'-phosphate (which can be introduced by enzymatic reactions).[221,222]
- Coupling of suitably protected chemical moieties at the 5'- or 3'-terminus of the protected oligonucleotide.[223,224]

In our approach, we intended to introduce the reporter group during automated DNA synthesis at the 5'- or 3'-terminus for reasons of simplicity. Solid phase modifications of the oligonucleotide 3'-end are more difficult to develop and need the preparation of a modified support. Hence, we decided to introduce our labelling group at the 5'-end.

Earlier we mentioned the use of tetrabenzo[*a,c,g,i*]fluorene (53) for the purification of oligodeoxyribonucleotides. We have also investigated the fluorescent properties of this molecule and have used it as the basis for the development of a non-radioactive labelling group. We wished to design the labelling group so that it would be introduced onto the 5'-terminus of an oligonucleotide *via* an inert spacer. The synthetic fluorescent marker ought to be compatible with β-cyanoethyl phosphoramidite chemistry used on most DNA synthesizers.

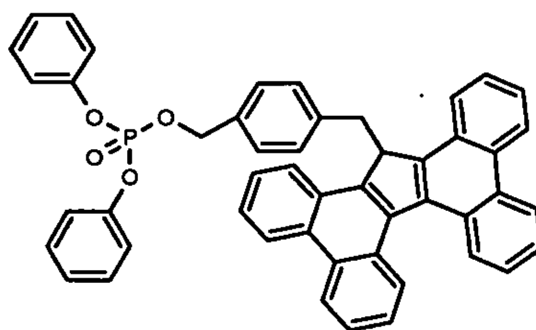
Methyl 4-(17'-tetrabenzo[*a,c,g,i*]fluorenylmethyl) benzoate (76) was already useful in the synthesis of Tbf-DMTr-OH (84) and its reduction to the corresponding benzyl alcohol derivative (106) would enable direct activation into a phosphoramidite species for introduction onto an oligonucleotide. The synthesis of 4-(17'-tetrabenzo[*a,c,g,i*]fluorenylmethyl) benzyl alcohol (106) was carried out under strong reductive conditions using diisobutylaluminium hydride (DIBAL) in THF yielding 85%, see *Scheme 2.16*. The first attempts using lithium aluminium hydride failed.



Scheme 2.16; i: DIBAL, THF; ii: bis(diisopropylammonium)tetrazolide, (2-cyanoethoxy)bis(diisopropylamino)phosphine

With this concept in mind, we successfully synthesized 4-(17'-tetrabenzo[*a,c,g,i*]fluorenylmethyl) benzyl-(N,N-diisopropyl-β-cyanoethyl) phosphoramidite (107) but when we tried to react it with the 5'-hydroxyl of a 20-mer oligonucleotide (5'-CCT-AAG-TTC-GAT-CCT-AGG-AT-3') bound to CPG no

fluorescent product was observed after treatment with ammonia. HPLC analysis of the crude also confirmed that the oligonucleotide was not coupled to compound (107). In order to test the stability of phosphate derivatives of (106) in concentrated ammonia, we reacted chlorodiphenyl phosphate with (106) in the presence of triethylamine in DCM to obtain the corresponding phosphate-triester (108), see *Fig. 2.26*. The phosphate produced was left in ammonia at 50°C for several hours whereby its stability was monitored by ^{31}P nmr. A single signal at -10.6 ppm was observed throughout the experiment indicating that the phosphate-triester (108) is stable under the cleavage conditions used in the oligonucleotide phosphoramidite chemistry. Consequently the absence of compound (107) on the 20-mer oligonucleotide is not due to a chemical problem but to the steric bulk of the structure.

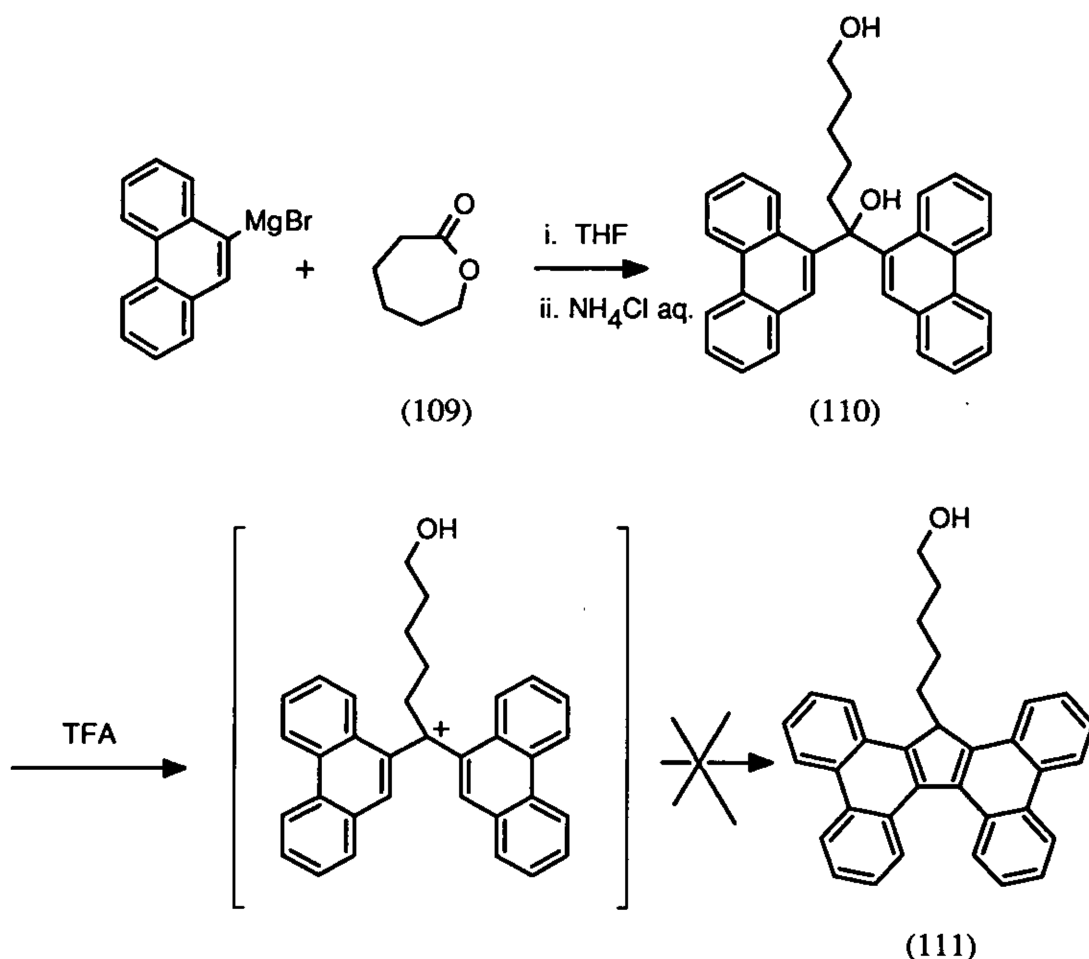


(108)

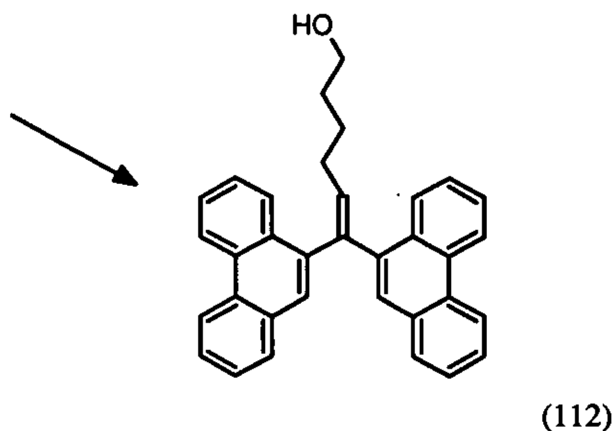
Fig. 2.26

Subsequently we investigated on the synthesis of a fluorescent compound with less steric hindrance. This may be achieved by increasing the length of the spacer arm between the fluorescent group and the oligonucleotide with an aliphatic chain of at least three carbon atoms. The bulk of the Tbf structure would therefore move further away from the reactive phosphorous atom thus minimising the possibility of interaction which may interfere with coupling. In section 2.3.3.1 we described the synthesis of di(phenanthren-9-yl)-α-hydroxy alkyls by Grignard reactions involving

esters and anhydrides. By a similar approach we synthesized 1-(diphenanthren-9'-yl)-1,6-hexanediol (110) from phenanthrenemagnesium bromide and ε-caprolactone (109) in 26 % yield. Subsequently we attempted the cyclisation of (110) in the presence of TFA to give 6-(17'-tetrabenzo[*a,c,g,i*]fluorenyl)hexanol (111) but instead the elimination product 1-(diphenanthren-9'-yl)-1-hexene-6-ol (112) was obtained in 81 % yield after purification by dry flash chromatography (*Scheme 2.17*). Neither did the [2+2] cyclisation occur when using milder acids such as TCA, acetic acid, sulphuric acid nor with heat.

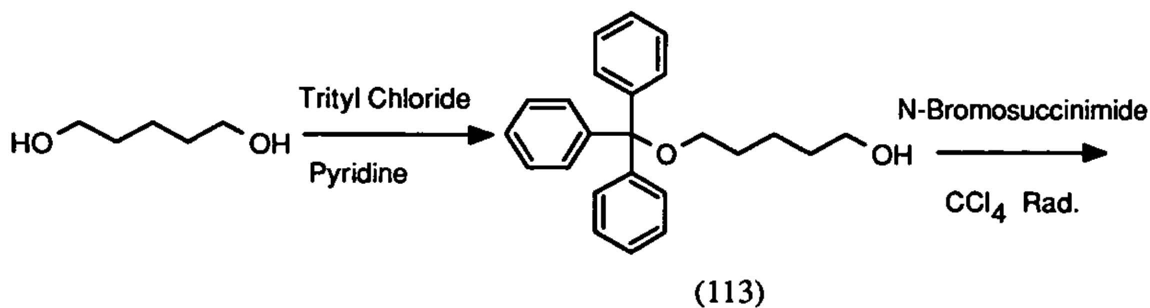


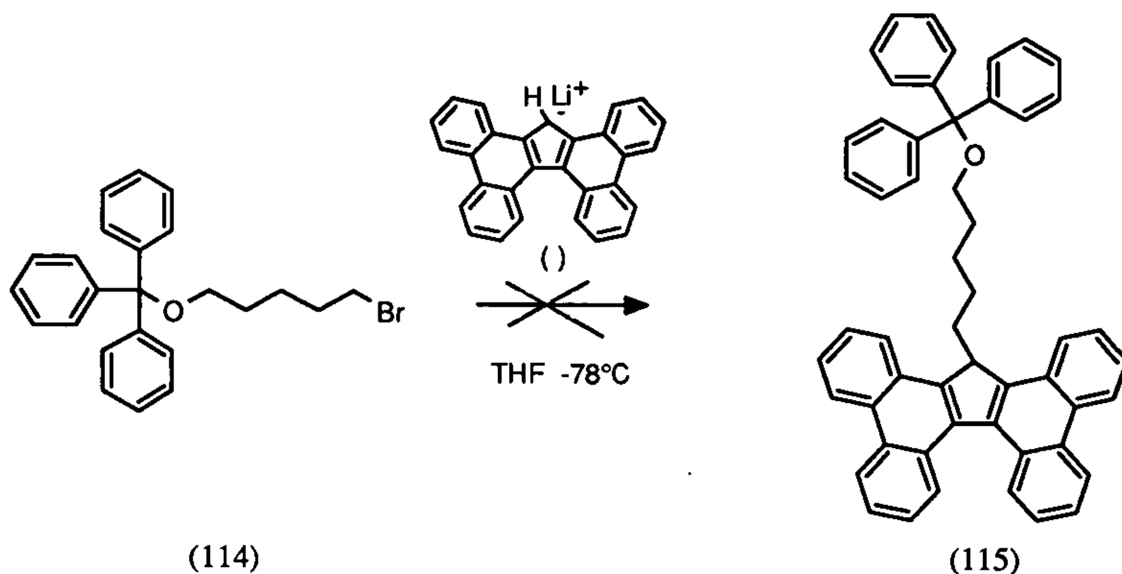
Cont. ↳



Scheme 2.17

Next we investigated the alkylation of Tbf with a protected alcohol alkyl halide which after deprotection would release a hydroxyl function which in turn can be phosphitylated. Using an excess of 1,5-pentanediol and a low concentration in the reaction with trityl chloride, we could selectively monotritylate the diol to obtain 5-hydroxy-1-triphenylmethoxy-pentane (113) in 51 % yield as a white solid. The less polar ditritylated derivative was only detected in traces by t.l.c. The free hydroxyl function was subsequently brominated with N-bromosuccinimide in carbon tetrachloride to give the crystalline 1-triphenylmethoxy-5-bromopentane (114) in 77% yield. The lithium derivative (73) of Tbf was prepared in a similar fashion (see section 2.3.3.2) and then reacted with the alkyl bromide (114) at -78°C and separatly at room temperature in THF but neither reaction gave any of the product (115) (*Scheme 2.18*). We considered the use of iodo derivatives, but this is unlikely to improve upon the reaction. Because allyl halides are more reactive to direct nucleophilic substitution than primary alkyl halides, we abandonned the route and used the more reactive allyl bromide.

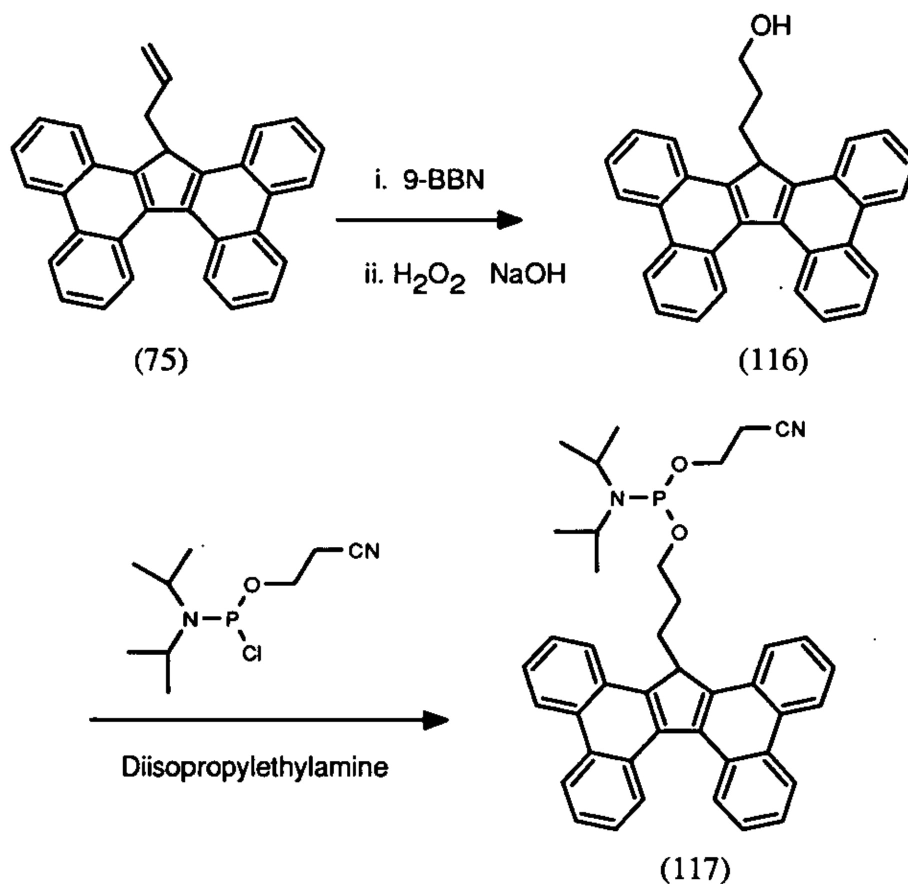




Scheme 2.18

The synthetic route finally chosen to produce the new fluorescent label is shown in *Scheme 2.19*. The anion of Tbf (73) was prepared by addition of butyllithium at low temperature and this highly reactive species readily reacted with allyl bromide to give the alkene (75) in 63 % yield, this reaction was previously described in section 2.3.3.2. This alkene was successfully hydroborated using 9-BBN (9-borabicyclo[3.3.1]nonane) in THF and subsequently reacted with sodium hydroxide and hydrogen peroxide to generate the corresponding primary alcohol 3-(17'-tetrabenzo[*a,c,g,i*]fluorenyl)propanol (116) in 57% yield. Lower yields were obtained when sodium borohydride was used instead of 9-BBN. The phosphitylation of (116) was carried out in anhydrous THF using chloro-N,N,diisopropylamino-

cyanoethoxyphosphine.[68] The reaction gave one product on t.l.c. and after rapid aqueous work-up 3-(17'-tetrabenzo[*a,c,g,i*]fluorenyl) propyl-N,N-diisopropyl-β-cyanoethyl phosphoramidite (117) was obtained as a yellow foam in 86% yield. The overall yield starting from tetrabenzofluorene was 38%. For reasons of simplicity we abbreviate compound (116) with Tbf-Prop (Tetrabenzo[*a,c,g,i*]fluorenylpropyl) and Tbf-Prop-P (Tetrabenzo[*a,c,g,i*]fluorenylpropyl phosphate) when tagged to an oligonucleotide.



Scheme 2.19

2.10 Tbf-Prop-P Labelled Oligonucleotides

Compound (117) was found to be poorly soluble in anhydrous acetonitrile, the standard solvent for DNA synthesis monomers. Hence the resulting amidite (117) was dissolved in anhydrous acetonitrile/DCM : 9/1 at a concentration of 0.1 M. A series of short test sequences were synthesized using a 380B DNA synthesizer from Applied Biosystems with the standard cycle for 0.2 μmol scale DNA synthesis, with a normal 30 s coupling time. The first sequence was a polythymidine oligonucleotide $(dT)_6$ whereby the coupling efficiency of the fluorescent label was determined. The Tbf-Prop-P (17-tetrabenzofluorenylpropyl phosphate) tagged oligonucleotide was obtained in 82% yield according to the integrated HPLC spectrum. This result is comparable to the expected theoretical yield with coupling efficiencies of 98.5% per step. Subsequently we synthesized Tbf-Prop-P labelled oligonucleotides on a 0.2 μmol and 1 μmol scale, the results of which are exemplified with the following 20-mer oligonucleotide (118):

(118) 5'-**Tbf-Prop-P**-TTG-AGT-GCG-TCG-TGG-TCT-CA-3'

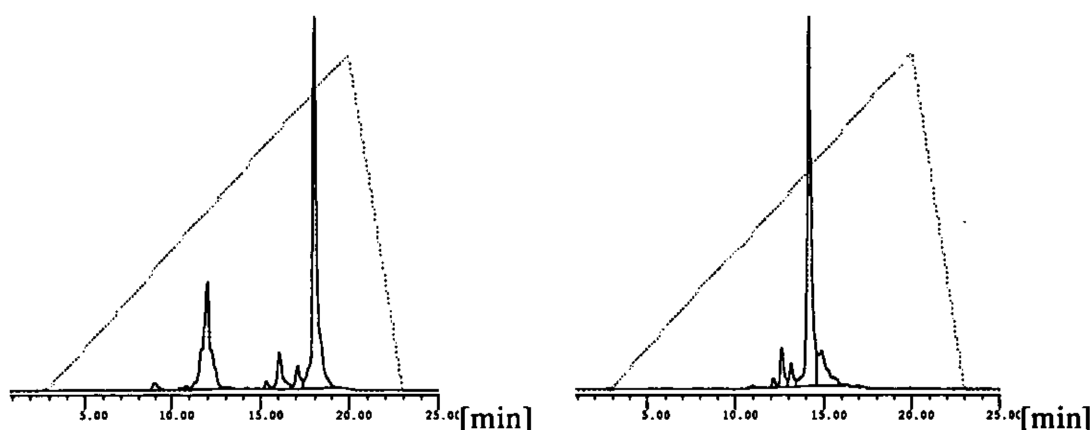


Fig. 2.27: HPLC Spectra of crude oligonucleotide (118), (a) at 280 nm, (b) at 365 nm

After deprotection in ammonia at 50°C the oligonucleotide was analysed by analytical RP-C_{18} HPLC at 280 nm, see *Fig. 2.27 (a).* The HPLC characteristics of oligonucleotides containing the hydrophobic Tbf-Prop label were very different to those of unlabelled failure sequences. The Tbf-Prop oligonucleotides had retention times between 5 and 10 min longer than unlabelled oligonucleotides. Clearly the hydrophobicity of the reporter group increases the retention time of the product drastically and therefore makes the purification easier. In *Fig. 2.27 (b)* the detector was set at 365 nm where only the fluorescent label absorbs. We are therefore able to monitor the labelled oligonucleotide outside 254-260 nm and thus avoid the DNA damaging wavelengths. The labelled oligonucleotide was finally purified by HPLC and desalted using a NAP-10 cartridge to give 53 OD units of pure fluorescent product as demonstrated by capillary gel electrophoresis with a single peak at 17 min, *Fig.2.28.*

Fig. 2.28: Capillary Gel Electrophoresis of purified oligonucleotide (118)

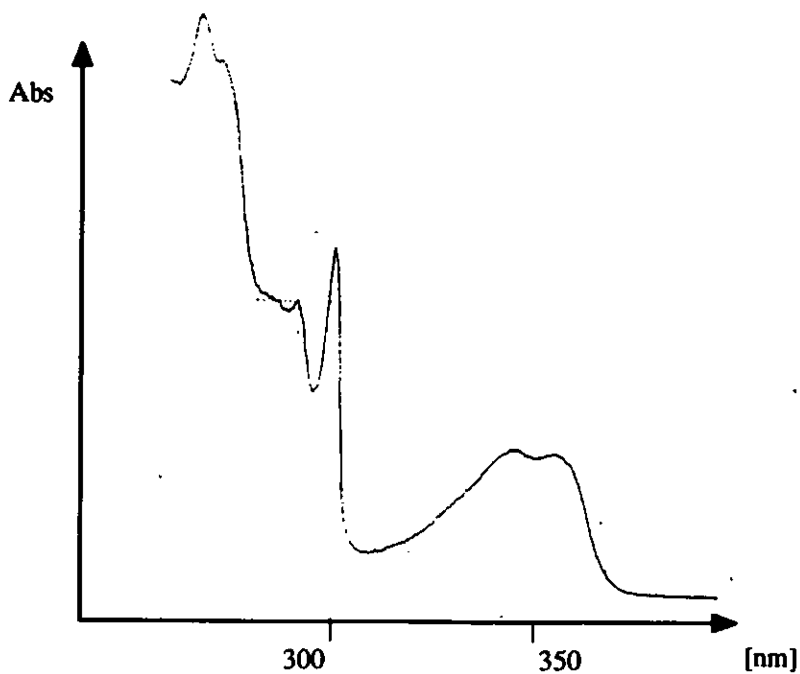


Fig. 2.29: UV absorption spectra of (116) in DCM

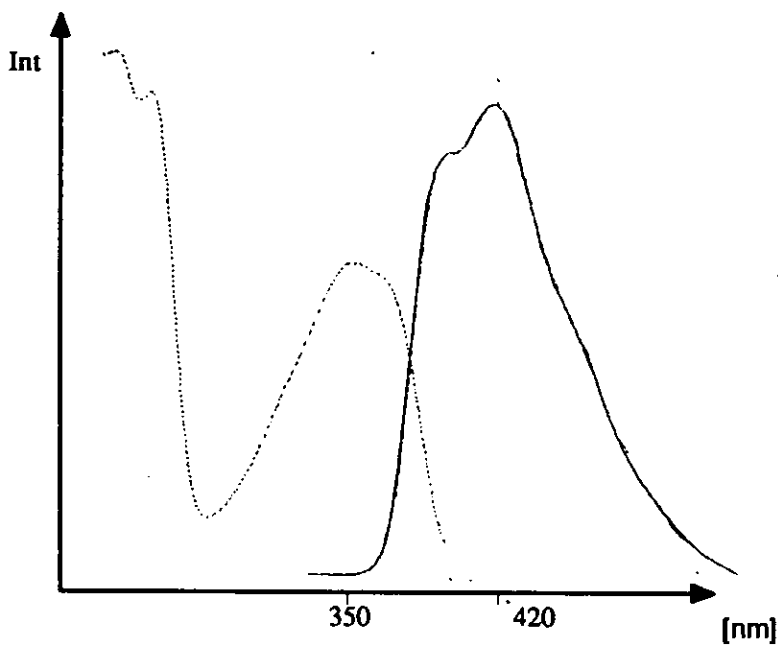


Fig. 2.30: Fluorescence spectra of (116) in DCM (Excitation spectra in dotted line, Emission spectra in plain line).

Fig. 2.29 shows the UV absorption spectrum of the Tbf-Prop label in DCM. The absorption band between 330 and 400 nm is characteristic of the tetrabenzo[*a,c,g,i*]fluorene structure and can be used for monitoring the processes undergone by a labelled primer at wavelengths where most molecules do not absorb. Moreover when excited at 300 or 375 nm, the fluorescent label emits light with a maximum at 416 nm, see *Fig. 2.30*. In order to illustrate this important characteristic *Fig. 2.31* shows a PAGE of the pure oligonucleotide (118) in lane 2 and a reference 40-mer sequence unlabelled in lane 1. When the gel slab is irradiated by UV light (254 nm) only the Tbf-Prop-P-oligonucleotide is visualised thanks to its fluorescent tag. Both oligonucleotides can be revealed when stained with ethidium bromide. The detection limit was determined with an aqueous solution of (118) by successive dilution and measured on an Perkin-Elmer LS-50 luminescence spectrophotometer. By subtraction of the water background the labelled oligonucleotide could be detected at a concentration of 10^{-11} M, see *Fig. 2.32*.

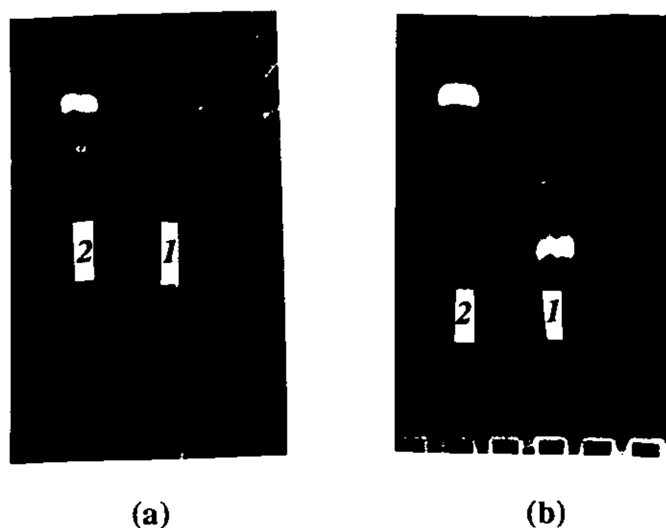


Fig. 2.31: PAGE 12% of oligonucleotide (118) (Lane 2) and a reference 40-mer sequence (Lane 1), (a) before and (b) after ethidium bromide staining

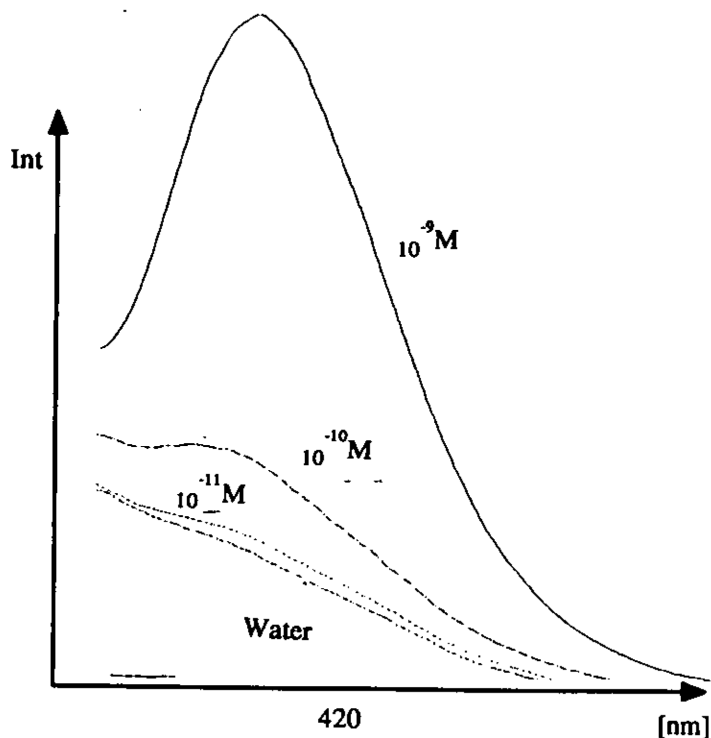


Fig. 2.32: Fluorescence emission spectra of oligonucleotide (118) at various concentrations

Given the ability to synthesize these internally labelled oligonucleotides, it was necessary to confirm that covalent attachment of the label does not adversely affect either the label itself or the modified oligonucleotide. The first effect can be determined by measuring the UV spectrum of the Tbf-Prop-labelled oligonucleotide over a period of time. The fluorescent oligonucleotide remained fluorescent indefinitely and the UV absorption spectrum was identical to the one of Tbf-Prop on its own. The second effect was determined by making melting temperature measurements when hybridized with its complementary strand. Two identical duplexes, but one containing the Tbf-Prop-labelled strand were made. The buffer system used for the melting curves determination was 100 mM sodium chloride, 10 mM sodium phosphate, 1 mM sodium EDTA adjusted to pH 7 by using NaOH. The

complementary strands were annealed by heating equimolar solutions to 80°C and cooling to room temperature for 1 h. The melting curves were obtained by slowly heating the samples to the high end of the run (80°C) and allowing equilibration for 20 min. The samples were then cooled to the low end of the run (20°C) at a rate of 10°C/h. The absorbance readings were made at 264 nm. The result of this experiment shows that the melting temperature of the Tbf-Prop-labelled duplex is 2.7±1°C lower than for the unlabelled duplex. Although this value is almost within the error margin one could still speculate that the intercalation of Tbf would be expected to lead to stabilization of the duplex (increase of the melting temperature), but the opposite phenomenon was observed. This may be caused by the steric interaction of the somewhat bulky Tbf-Prop group with the duplex formation. This interaction could be reduced if the spacer chain between the Tbf group and the phosphorous atom was extended.

2.11 Conclusions

The synthesis of tetrabenzo[*a,c,g,i*]fluorene and some of its derivatives has been investigated. Some of these derivatives present interesting characteristics, they represented the basis for the development of a new hydrophobic 5'-protecting group Tbf-DMTr for oligonucleotide synthesis and purification. We have shown that Tbf-DMTr-Cl can be used to introduce the Tbf-DMTr group directly onto the support bound oligonucleotides, without lowering the synthesis yield. Synthetic oligonucleotides of more than 150 monomer units can equally be substituted with the Tbf-DMTr group. The removal of this new protecting group in acid is twice as fast as DMTr and its fluorescent properties enable easy detection at DNA non-damaging wavelengths. Due to the high hydrophobicity of Tbf-DMTr, the purification of long

oligonucleotides > 100 units is easily achieved by RP-HPLC. Purification is usually carried out in two steps. In the first step, 5'-Tbf-DMTrityl terminated oligonucleotides are separated from the crude reaction mixture by HPLC. In the second step, detritylation of the material in the separated fraction from the first step is performed, and the desired oligomers are separated from this reaction mixture by HPLC. Tbf-DMTr has a great potential as a useful 5'-protecting group for RNA synthesis and in carbohydrate chemistry.

We have shown that tetrabenzo[*a,c,g,i*]fluorene can be used as a fluorophore in the labelling of oligonucleotide by a technique involving the standard phosphoramidite chemistry used in oligonucleotide synthesis. This fluorescent label offers significant advantages over common fluorescent labels. It is chemically inert and therefore will not adversely affect the oligonucleotide and its environment. It is entirely compatible with the established β-cyanoethyl phosphoramidite chemistry and does not require any derivatisation of the heterocyclic bases or the use of an aminohexyl linker. The hydroxyl group is at the opposite end of the molecule to the Tbf moiety, hence minimising any possibility of steric interaction between the bulky Tbf group and the phosphoramidite group which may interfere with coupling. It is achiral. Its strongly fluorescent property enables the detection of oligonucleotides at very low concentrations. The synthesis of this reporter group involves few steps and low cost. It is unlike any other molecule previously used to label oligonucleotides, and is thus unlikely to infringe existing patents. Tbf-Prop should also be investigated for its intercalating effects on DNA double strands a phenomenon which might be expected to occur should the spacer arm between the Tbf group and the phosphorous atom be extended. Polycyclic aromatic hydrocarbons in general are known to associate with natural DNA duplexes under certain conditions.[225]

Chapter 3: Experimental

3.1 Instrumentation and General Techniques

NMR Spectroscopy

^{1}H NMR

Routine proton nuclear magnetic resonance, ^{1}H NMR, spectra were obtained using a Jeol PMX-60 MHz spectrometer. Higher field spectra were obtained on a Bruker WP-80 (80 MHz), WP-200 (200 MHz) or a WH-360 (360 MHz) spectrometer. Measurements were carried out in the solvent indicated and chemical shifts (δ) reported in parts per million using tetramethylsilane (δ = 0.00) as an external reference.

^{13}C NMR

Carbon nuclear magnetic resonance, ^{13}C NMR, spectra were recorded at 50.3 MHz on a Bruker WP-200 spectrometer or at 90.6 MHz on a Bruker WH-360 spectrometer. Measurements were carried out in the solvent indicated and chemical shifts (δ) reported in parts per million using tetramethylsilane (δ = 0.00) as an external reference.

^{31}P NMR

Phosphorus ^{31}P NMR spectra were recorded at 36 MHz on a Jeol FX 90 or a Bruker WP-200 operating at 81 MHz. Measurements were carried out in the solvent indicated and chemical shifts (δ) reported in parts per million using phosphoric acid (δ = 0.00) as an external reference.

Infra-red Spectroscopy

Infra-red spectra were recorded on a Perkin-Elmer 781 spectrophotometer or a Biorad SPC 3200. Samples were examined as bromoform mulls or methylene chloride solutions, both on potassium bromide plates. Calibration was achieved by reference to the characteristic polystyrene peak at 1603 cm^{-1}.

Mass Spectrometry

Mass spectra, both low and high resolution were recorded on a Kratos MS-50 TC spectrometer operated by Mr. A. Taylor or on a AEI MS-902 spectrometer operated by Miss E. Stevenson. Fast Atom Bombardment (FAB) techniques were carried out with the Kratos spectrometer using 3-nitrobenzyl alcohol as the matrix, accurate mass spectra were calibrated with CsI. Electron Impact (EI) techniques were carried out on the AEI spectrometer.

Elemental Analysis

Elemental analysis for carbon, hydrogen and nitrogen were carried out on a Carlo-Erba elemental analyser, model 1106, operated by Mrs E. McDougall or Ms L. Eades. The compounds that bear a polycyclic aromatic structure such as Tbf, were generally measured with low carbon values. A study with extended combustion time

and the use of a catalyst such as vanadium oxide did not improve the results obtained for the carbon values.[227]

X-Ray Crystallography

Single crystal X-ray structure determination was determined on a Stoe Stadi-4, four circle diffractometer, graphite monochromated (Cu-Ka radiation; l = 1.54184 Å) by Dr. S. Blake and Dr. R. Gould.

Melting Points and Boiling Points

Melting points were measured in open capillaries on an electrically heated Buechi 510 melting point apparatus, or on an electrically heated Reichert 7905 hot stage and are quoted without correction. Boiling points were measured using a Buechi Kugelrohr distillation apparatus; the temperatures were recorded by placing a thermometer inside the glass oven.

HPLC

High performance liquid chromatography (HPLC) was performed using either a Gilson system i.e. 2 x 306 pumps, a 811B automatic gradient controller and a model 115 ultraviolet detector; or an Applied Biosystems system, i.e. 1783A Absorbance Detector-Controller, a 1406A Solvent Delivery System and a 491 Dynamic Mixer/Injector. Analytical separations by HPLC were carried out on ABI (RP-C_{18}) Aquapore OD-300 columns, 300 Å pore size, 7 mm spherical silica (4.6 mm x 220 mm), loop volume = 1 ml, with a flow rate of 1 ml/min. Preparative separations were performed with Brownlee Aquapore C_8 columns, 25 cm x 10 mm, loop volume = 1 ml and a flow rate of 3 ml/min. The following gradients were used :

Gradient I								
Time (min)	0	3	4	23	24	27	28	30
%B	0	0	15	70	100	100	0	0

Buffer A = 100% 0.1M aqueous ammonium acetate
Buffer B = 50% acetonitrile / 50% 0.1M aqueous ammonium acetate

Gradient II								
Time (min)	0	3	4	23	24	27	28	30
%B'	0	0	15	70	100	100	0	0

Buffer A = 100% 0.1M aqueous ammonium acetate
Buffer B' = 20% acetonitrile / 80% 0.1M aqueous ammonium acetate

Gradient III								
Time (min)	0	3	4	23	24	35	36	40
%B	0	0	15	70	100	100	0	0

Buffer A = 100% 0.1M aqueous ammonium acetate
Buffer B = 50% acetonitrile / 50% 0.1M aqueous ammonium acetate

Gradient IV				
Time (min)	0	3	20	23
%B"	0	0	90	0

Buffer A = 100% 0.1M aqueous ammonium acetate
Buffer B" = 100% acetonitrile

Capillary zone gel electrophoresis

Analyses of oligonucleotides by capillary zone gel electrophoresis (CE), were conducted on a Applied Biosystems Model 270A with Microgel capillaries. The following conditions were used: Buffer 75 mM Tris-phosphate/10 % methanol, pH 7.5; detector 260 nm; samples ~5 μM; loading voltage = -5 kV; temperature = 30°C; running voltage = -15 kV.

Polyacrylamide gel electrophoresis

Polyacrylamide Gel Electrophoresis (PAGE) was performed with a slab gel apparatus of 1 mm width and 20 cm length. The sample wells were loaded with ~3 μl of 6 OD unit oligonucleotide solutions. 12% Acrylamide gel were used for the separation of 40 to 200 bp fragments, their preparation was made according to the ABI method[226]. The runs were carried out at 30 mA for ~2 h. The analyses were carried out in the department of Biochemistry with the assistance of Dr. L. Powell.

U.V. Spectroscopy

Ultraviolet spectra were run on a Varian Cary 210 spectrophotometer using dichloromethane as the solvent and matched 1 cm silica cells. Fluorescence spectra were recorded a Perkin Elmer LS-50 luminescence spectrophotometer.

Thin Layer Chromatography

For analytical purposes, aluminium backed plates, coated with a 0.2 mm layer of silica gel 60 GF_{254} (Merck 5735), and containing a fluorescent indicator were used. The solvents systems used were:

(A) chloroform

(B) chloroform/methanol/triethylamine (90:10:1)

(C) chloroform/methanol (9:1)

(D) benzene

(E) diethyl ether/n-hexane (3:7)

(F) dichloromethane/acetonitrile (8:2)

(G) ethylacetate

(H) diethyl ether/dichloromethane/triethylamine (45:45:1)

(I) ethyl acetate/cyclohexane (4:1)

The component spots were visualised by ultra-violet light at 254 nm or 352 nm or iodine spray. A sulfuric acid (in methanol) spray was used to visualise the position of trityl containing compounds and an anisaldehyde spray (in acidic ethanol) to reveal sugar containing compounds.

Drying and Purification of Solvents

All solvents were distilled before use and the following were dried using the drying reagents indicated when required: benzene (sodium wire), dichloromethane (calcium hydride), diethyl ether (sodium wire), n-hexane (molecular sieves type 4 Å), N,N-dimethylformamide (calcium hydride), pyridine (calcium hydride), tetrahydrofuran (sodium wire).

Purchase of Reagents and Chemicals

Nucleosides and nucleotides were purchased from Applied Biosystems, Cruachem or Raylo Chemicals. Other organic compounds were purchased from Aldrich or Fluka.

3.2 Synthetic Procedures

Di(phenanthren-9-yl) methanol (69)

9-Bromophenanthrene (5 g, 19.5 mmol) was dissolved in dry THF (10 ml) and slowly added to magnesium turnings (0.5 g, 2.1 mmol) together with a crystal of iodine under nitrogen. The exothermic reaction was controlled with the addition of 9-bromophenanthrene. After 1 h stirring, a solution of methyl formate (0.43 g, 9.7 mmol) in dry THF (5 ml) was slowly added. After 2 h, ice (5 g) then aqueous ammonium chloride (5 g in 10 ml water) was added and a white solid precipitated. The precipitate was filtered off and washed with water. The white solid was dissolved in DCM and combined with the DCM extract of the aqueous filtrate. The solution was dried over magnesium sulphate and the solvent removed *in vacuo*. The residue was finally triturated with diethyl ether to give a white solid (2.3 g, 60%); **m.p.** 238-239°C; **C,H,N** found: C, 91.03; H, 5.69; $C_{29}H_{20}O$ requires C, 90.59; H, 5.25 %; **t.l.c.** $Rf_{(A)}$ 0.27; ν_{max} (bromoform mull) 3420 (OH), 3080 (aromatic CH), 1610, 1500 (aromatic rings) cm^{-1}; λ_{max} 298 (ε 20110 $dm^3mol^{-1}cm^{-1}$), 278 (25010), 255 (111000) nm; δ_H ($CDCl_3$, 200 MHz), 8.77 (2H, d, ^3J = 7.7 Hz, aromatic), 8.69 (2H, d, ^3J = 8.3 Hz, aromatic), 8.09 (2H, d, ^3J = 8.6 Hz, aromatic), 7.79-7.49 (12H, m, aromatic), 7.26 (1H, d, ^3J = 4.6 Hz, CH); **m/z** (FAB) 384 (M+), 367, 178; HRMS (FAB) found 384.15141, $C_{29}H_{20}O$ requires 384.15140, (< 1ppm).

8bH-Tetrabenzo[*a,c,g,i*]fluorene (70)

Di(phenanthren-9-yl-methanol (48.0 g, 0.13 mol) was suspended in dichloromethane (200 ml) and trifluoroacetic acid (ca. 50 ml) was carefully added at this stage the reaction can be monitored by the brief appearance of a blue colour. The

yellow suspension was left stirring for 10 min after which it was evaporated to dryness. Trifluoroacetic acid was extracted by successive evaporation of freshly added dichloromethane. Finally the yellow residue was triturated with diethyl ether and filtered off to afford the title compound as a yellow solid, (41.0 g, 86%); **m.p.** 280-282°C; **C,H,N** found: C, 95.25; H, 5.15; $C_{29}H_{18}$ requires C, 95.05; H, 4.95 %; **t.l.c.** $Rf_{(A)}$ 0.69, $Rf_{(E)}$ 0.39; $\mathbf{v_{max}}$ (bromoform mull) 3054 (CH stretching, aromatic), 1610, 1505 (aromatic rings) cm^{-1}; λ_{max} 374 (ε 8947 $dm^3mol^{-1}cm^{-1}$), 300 (21553), 288 (20943), 254 (52867) nm; δ_H ($CDCl_3$, 200 MHz), 8.84-8.69 (2H, m, aromatic), 8.29-7.07 (15H, m, aromatic), 5.40 (1H, s, CH); **m/z** (FAB) 366 (M+); HRMS (FAB) found 366.14087 $C_{29}H_{18}$ requires 366.14085, (< 1ppm).

Tetrabenzo[*a,c,g,i*]fluorene (53)

8bH-tetrabenzo[*a,c,g,i*]fluorene (0.50 g, 1.37 mmol) was suspended in THF (50 ml) and a few drops of triethylamine were added. The suspension was stirred for 5 min after which the solvent was removed *in vacuo*. The yellow solid was then triturated in diethyl ether and filtered off to give the title compound as a yellow solid (0.42 g, 89%); **m.p.** 280-282°C (lit[195] 275-276°C); **C,H,N** found: C, 95.11; H, 5.03; $C_{29}H_{18}$ requires C, 95.05; H, 4.95 %; **t.l.c.** $Rf_{(A)}$ 0.69; $\mathbf{v_{max}}$ (bromoform mull) 3045 (CH stretching, aromatic), 1610, 1505 (aromatic rings) cm^{-1}; λ_{max} 374 (ε 10450 $dm^3mol^{-1}cm^{-1}$), 365 (11220), 300 (21470), 288 (19143), 254 (50365) nm; δ_H ($CDCl_3$, 200 MHz), 8.82-8.67 (6H, m, aromatic), 8.20-8.14 (2H, m, aromatic), 7.79-7.57 (8H, m, aromatic), 4.59 (2H, s, CH_2); **m/z** (FAB) 366 (M+); HRMS (FAB) found 366.14082 $C_{29}H_{18}$ requires 366.14085, (< 1ppm); **X-Ray analysis**, see section 2.3.2.

Tetrabenzo[*a,c,g,i*]fluorenone (58)

8bH-Tetrabenzo[*a,c,g,i*]fluorene (0.50 g, 1.37 mmol) was suspended in THF (60 ml) in an open flask when sodium methoxide (0.22 g, 3 equiv.) was added. The reaction mixture turned dark red, it was stirred at room temperature ovenight. The solution was then extracted in DCM, washed with 2 M aq. HCl and water twice, and dried over magnesium sulphate. After removal of the solvents, the residue was triturated with diethylether to give a brown red solid, (0.27, 52%); **m.p.** > 295°C; **C,H,N** found: C, 90.87; H, 4.60; $C_{29}H_{16}O$ requires C, 91.56; H, 4.24 %; **t.l.c.** $Rf_{(A)}$ 0.70; $\mathbf{v}_{max}$ (bromoform mull) 3080 (aromatic CH), 1700 (CO, ketone), 1610, 1500 (aromatic rings) cm^{-1}; λ_{max} 337 (ε 20064 $dm^3mol^{-1}cm^{-1}$), 256 (50160) nm; δ_H ($CDCl_3$, 200 MHz), 9.23-9.18 (2H, dd, aromatic), 8.78-8.74 (4H, dd, aromatic), 8.31-8.27 (2H, d, aromatic), 7.83-7.61 (8H, m, aromatic); δ_C ($CDCl_3$, 50 MHz), 134.6-122.7 (aromatic C's), weak sample; **m/z** (FAB) 381 (M+), 366; HRMS (FAB) found 381.12796, $C_{29}H_{17}O$ requires 381.12793, (< 1ppm).

17-Hydroxytetrabenzo[*a,c,g,i*]fluorene (79)

Sodium borohydride (1.0 g, 26 mmol) was added to a solution of tetrabenzo[*a,c,g,i*]fluorenone (0.52 g, 1.37 mmol) in THF (100 ml) and stirred at room temperature for 2 h. The deep red solution turned yellow after 15-25 min. After cooling with an ice-bath, the solution is acidified to pH ~ 1.5 by slow addition of HCl aq. 10%. The product was finally extracted with DCM, washed with $NaHCO_3$ aq. and water, and dried over magnesium sulphate. The solvents were evaporated to leave a yellow foam which was recrystallised from DCM/Petrol 60-80 to give a yellow solid, (0.37 g, 71%); **m.p.** (decomp.) 270-272°C; **C,H,N** found: C, 89.39; H, 4.90; $C_{29}H_{18}O$ requires C, 91.07; H, 4.74 %; **t.l.c.** $Rf_{(A)}$ 0.47; $Rf_{(C)}$ 0.90; $\mathbf{v}_{max}$ (bromoform mull)

3550, 3520 (OH stretching), 3080 (aromatic CH), 1610, 1500 (aromatic rings) cm^{-1}; λ_{max} 378 (ε 8404 $dm^3mol^{-1}cm^{-1}$), 303 (25518), 293 (23684) nm; δ_H ($CDCl_3$, 200 MHz), 9.05-8.91 (4H, m, aromatic), 8.65-8.47 (4H, dd, aromatic), 7.81-7.70 (8H, m, aromatic), 6.39 (1H, s, CH); δ_C ($CDCl_3$, 50 MHz), 141.9, 139.4, 134.8, 131.4-122.4 (aromatic C's), 74.4 (CH); **m/z** (EI) 381 (M+), 380, 365; HRMS (EI) found 382.1359, $C_{29}H_{18}O$ requires 382.13576, (< 1ppm).

17-Bromotetrabenzo[*a,c,g,i*]fluorene (81)

N-Bromosuccinimide (1.60 g, 9.0 mmol) was added to a solution of 8bH-tetrabenzo[*a,c,g,i*]fluorene (3.0 g, 8.2 mmol) in carbon tetrachloride (50 ml). Traces of dibenzoylperoxide were subsequently added to the yellow suspension which was heated to reflux for 1 h, whilst being irradiated by a 500W halogen lamp. After cooling to room temperature the solvents were evaporated, the orange residue taken up in DCM (1.3 l) and extracted with water (2 x 70 ml) and finally dried over magnesium sulphate. The solvent was evaporated and the product crystallised in diethylether to give an orange solid, (2.70 g, 75%); **m.p.** (decomp.) 265°C; **C,H,N** found: C, 77.80; H, 3.97; $C_{29}H_{17}Br$ requires C, 78.37; H, 3.86 %; **t.l.c.** $Rf_{(A)}$ 0.68; ν_{max} (bromoform mull) 3060 (aromatic CH), 2970 (alkyl CH), 1610, 1500 (aromatic rings) cm^{-1}; λ_{max} 308 (ε 32040 $dm^3mol^{-1}cm^{-1}$), 262 (64080), 255 (65860) nm; δ_H ($CDCl_3$, 200 MHz), 8.83-8.38 (8H, m, aromatic), 7.81-7.60 (8H, m, aromatic), 6.59 (1H, s, CH); δ_C ($CDCl_3$, 50 MHz), 132.4, 130.9, 128.0, 126.9, 126.7, 125.4, 124.7, 123.7, 123.3, (aromatic C's), 44.3 (CH); **m/z** (FAB) 444 (M+), 365; HRMS (FAB) found 444.05146, $C_{29}H_{17}Br$ requires 444.051410, (< 1ppm).

Methyl 4-(17'-tetrabenzo[*a,c,g,i*]fluorenyl)oxybenzoate (82)

Sodium pieces (0.03 g, 1.23 mmol) were added to a solution of methyl-4-hydroxybenzoate (0.19 g, 1.23 mmol) in THF (10 ml) at room temperature under nitrogen. The suspension was stirred for 2.5 h until no more hydrogen evolution could be observed. Then 17-bromotetrabenzo[*a,c,g,i*]fluorene (0.50 g, 1.12 mmol) was added to the white suspension and was heated to reflux for 8 h. The reaction mixture was then evaporated *in vacuo* to give a yellow residue which was redissolved in DCM (20 ml). The resulting solution was washed twice with water (2 x 40 ml) and dried over magnesium sulphate. The volatiles were removed *in vacuo* and the product was purified by flash chromatography, eluting with DCM. The title compound was obtained as a bright yellow solid, (0.15 g, 26%); **m.p.** (decomp.) 250-255°C; **C,H,N** found: C, 86.10; H, 5.00; $C_{37}H_{24}O_3$ requires C, 86.03; H, 4.68 %; **t.l.c.** $Rf_{(A)}$ 0.38, $Rf_{(D)}$ 0.68; $\mathbf{v_{max}}$ (bromoform mull) 3080, 3060 (aromatic CH), 2950 (alkyl CH), 1725 (ester CO), 1605, 1505 (aromatic rings) cm^{-1}; λ_{max} 384 (ε 3922 $dm^3mol^{-1}cm^{-1}$), 255 (31373) nm; δ_H (DMSO-d6, 200 MHz), 8.9-8.2 (m, broad, aromatic), 7.7-7.2 (m, broad, aromatic), 7.28, 7.23 (2H, d, CH aromatic), 6.60, 6.55 (2H, d, CH aromatic), 6.81 (1H, s, CH), 3.51 (3H, s, CH_3); **m/z** (FAB) 515 (M+), 365; HRMS (FAB) found 515.16476, $C_{37}H_{23}O_3$ requires 515.16476, (< 1ppm).

1-(17'-Tetrabenzo[*a,c,g,i*]fluorenyl)-prop-2-ene (75)

A solution of n-butyllithium in cyclohexane (1.1 ml, 1.51 mmol; 1.1 equiv.; 1.26 M titrated) was added dropwise to a suspension of 8bH-tetrabenzo[*a,c,g,i*]fluorene (0.50 g, 1.37 mmol) in dry THF (10 ml) at -78°C under nitrogen. The orange suspension was stirred for 1 h at -78°C . Then allyl bromide (0.17 g, 1.37 mmol; 1 equiv.) was added giving an orange solution which was stirred for 30 min at -78°C

and a further 3 h at room temperature. The reaction mixture was then evaporated *in vacuo* to give a brown oil which was redissolved in DCM (20 ml). The resulting solution was washed twice with water and dried over magnesium sulphate. The volatiles were removed *in vacuo* to give a brown oil which was crystallized from DCM/n-hexane and subsequently recrystallised from diethylether. The title compound was obtained as a beige solid which was filtered and dried, (0.35 g, 63%); **m.p.** 118-120°C; **C,H,N** found: C, 89.62; H, 5.57; $C_{32}H_{22}$ requires C, 94.55; H, 5.45 %; **t.l.c.** $Rf_{(A)}$ 0.70, $Rf_{(E)}$ 0.33; ν_{max} (bromoform mull) 3070 (CH stretching, aromatic), 2970, 2910, 2860 (CH stretching, alkyl), 1640 (C=C alkene) 1605, 1500 (aromatic rings) cm^{-1}; λ_{max} 380 (ε 16565 $dm^3mol^{-1}cm^{-1}$), 365 (17215), 300 (39626), 288 (31506), 253 (64310) nm; δ_H ($CDCl_3$, 200 MHz), 8.83-8.66 (6H, m, aromatic), 8.26-8.19 (2H, m, aromatic), 7.77-7.58 (8H, m, aromatic), 5.00 (1H, s, CH Tbf), 4.79-4.68 (1H, m, CH), 4.42-4.23 (2H, m, CH_2 3-propene), 3.36-3.30 (2H, dd, CH_2 1-propene); δ_C ($CDCl_3$, 50 MHz), 143.6, 136.8, 131.2, 130.3, 128.6, 127.9, (quaternary aromatic C's), 131.9, 126.7, 125.8, 125.6, 125.1, 124.9, 124.4, 123.4 (aromatic CH's), 116.6 (=CH_2), 46.8 (Tbf CH), 37.6 (CH_2); **m/z** (FAB) 406 (M+), 365, HRMS (FAB) found 406.17217, $C_{32}H_{22}$ requires 406.17215, (< 1ppm).

Methyl-4-(bromomethyl)benzoate (78)

N-Bromosuccinimide (97.9 g, 0.55 mol) and a catalytic amount of dibenzoylperoxide were added to a solution of methyl-2-methylbenzoate (75.1 g, 0.50 mol) in carbon tetrachloride (300 ml). The mixture was then heated under reflux whilst irradiating with a 500W halogen lamp. The reaction became vigorous for the first 20 min. After 2h, the mixture was allowed to cool to room temperature, then filtered and the filtrate concentrated *in vacuo* to give the product as a yellow oil which crystallized overnight. It was purified by distillation under reduced pressure to give a

colourless solid, (89.0 g, 77%); **b.p.** 124°C/1.5 Torr; **C,H,N** Found: C, 46.91; H, 4.41; $C_9H_9BrO_2$ requires: C, 47.19; H, 3.96 %; **t.l.c.** $Rf_{(A)}$ 0.50; ν_{max} (bromoform mull) 3005 (CH stretching, aromatic), 2900, 2843 (CH stretching, alkyl), 1724 (C=O), 1612, 1508 (aromatic rings) cm^{-1}; λ_{max} 246 (ε 14313 $dm^3mol^{-1}cm^{-1}$) nm; δ_H ($CDCl_3$, 80 MHz), 8.02, 7.91 (2H, d, J = 8.5 Hz, CH aromatic), 7.46, 7.35 (2H, d, J = 8.5 Hz, CH aromatic), 4.45 (2H, s, CH_2), 3.87 (3H, s, CH_3); δ_C ($CDCl_3$, 50 MHz), 165.8 (C=O), 142.2 (quaternary aromatic C), 129.5, 128.5 (aromatic CH's), 51.7 (CH_3), 31.9 (CH_2); **m/z** (EI) 229 (M+), 149.

Ethyl-17-ethoxycabonyl-(17-tetrabenzo[*a,c,g,i*]fluorenyl)-3'-propionate (72)

A solution of ethylacrylate (0.11 g, 1.14 mmol), piperidine (0.097 g, 1.14 mmol) in dioxane (5 ml) was stirred for 15 min at room temperature. Then, 17-ethoxycarbonyltetrabenzo[*a,c,g,i*]fluorene (0.50 g, 1.14 mmol) was added and stirred over 4 days at room temperature to give a yellow solution. The reaction mixture was then evaporated *in vacuo* to give a brown oil which was redissolved in DCM (20 ml). The resulting solution was washed twice with water (2 x 40 ml) and dried over magnesium sulphate. The volatiles were removed *in vacuo* to give a brown oil which was purified by flash chromatography, eluting with dichloromethane. Finally the product was recrystallised from ethanol to give yellow crystals, (0.12 g, 20%); **m.p.** 127-129°C; **C,H,N** found: C, 81.83; H, 5.83; $C_{37}H_{30}O_4$ requires C, 82.50; H, 5.62 %; **t.l.c.** $Rf_{(A)}$ 0.17, $Rf_{(D)}$ 0.36; ν_{max} (bromoform mull) 2975 (CH stretching, alkyl), 1722 (CO, ester), 1612, 1501(aromatic rings) cm^{-1}; λ_{max} 380 (ε 16140 $dm^3mol^{-1}cm^{-1}$), 305 (52972), 293 (44695), 253 (189541) nm; δ_H ($CDCl_3$, 80 MHz), 8.86-8.54 (6H, m, aromatic), 8.36-8.19 (2H, m, aromatic), 7.84-7.50 (8H, m, aromatic), 3.98 (2H, q, ^3J = 7.2 Hz, CH_2-O-CO), 3.68 (2H, q, ^3J = 7.2 Hz, CH_2-O-CO), 2.62 (4H, broad, CH_2), 1.20 (3H, t, CH_3), 0.74 (3H, t, CH_3); δ_C ($CDCl_3$, 50 MHz), 175.3

(carbonyl C), 140.4, 137.6, 132.3, 131.0, (quaternary aromatic C's), 128.0, 127.4, 126.6, 126.4, 125.2, 123.5, 123.3 (aromatic CH's), 84.4 (quaternary 17-C), 62.8 (CH_2-O-CO), 58.3 (CH_2), 18.2, 13.6 (CH_3); **m/z** (FAB) 538 (M+), 437; HRMS (FAB) found 538.21442, $C_{37}H_{30}O_4$ requires 538.21439, (< 1ppm).

Ethyl 3-(17'-tetrabenzo[*a,c,g,i*]fluorenyl) propanoate (74)

A solution of n-butyllithium in cyclohexane (0.94 ml, 1.51 mmol, 1.1 equiv.; 1.6M titrated) was added dropwise to a suspension of 8bH-tetrabenzo[*a,c,g,i*]fluorene (0.50 g, 1.37 mmol) in dry THF (5 ml) at -78°C under nitrogen. The orange suspension was stirred for 30 min at room temperature. Then, ethylacrylate (0.15 g, 1.51 mmol, 1.1 equiv.) was added dropwise and stirred at room temperature for 2 h. The reaction was quenched by adding water and the product was extracted with diethylether. The resulting solution was washed twice with water (2 x 20 ml) and dried over magnesium sulphate. The volatiles were removed *in vacuo* to give a brown oil. This was purified by flash chromatography, eluting with dichloromethane to give the title compound as a pale brown solid, (0.10 g, 16%); **m.p.** 175-177°C; **C,H,N** found: C, 87.2; H 5.84; $C_{34}H_{26}O_2$ requires C, 87.53; H, 5.62 %; **t.l.c.** $Rf_{(A)}$ 0.40, $Rf_{(D)}$ 0.65; $\mathbf{v_{max}}$ (bromoform mull) 2983, 2941 (CH stretching, alkyl), 1725 (CO, ester), 1608, 1500 (aromatic rings) cm^{-1}; λ_{max} 380 (ε 19064 $dm^3mol^{-1}cm^{-1}$), 365 (20546), 301 (46600), 289 (38550), 254 (76255) nm; δ_H ($CDCl_3$, 60 MHz), 9.0-8.6 (6H, m, aromatic), 8.4-8.1 (2H, m, aromatic), 7.9-7.4 (8H, m, aromatic), 5.1 (1H, t, ^3J = 4 Hz, CH), 3.6 (2H, q, ^3J = 7 Hz, CH_2-ethyl), 2.9 (2H, m, CH_2), 1.3 (2H, t, CH_2-CO), 0.8 (3H, t, ^3J = 7 Hz, CH_3); δ_C ($CDCl_3$, 50 MHz), 172.9 (carbonyl C), 142.8, 136.9, 131.3, 130.3, 128.4, 127.7 (quaternary aromatic C's), 127.4, 126.9, 126.0 125.7, 125.5, 125.0, 124.2, 123.4, (aromatic CH's), 59.8

(CH_2-ethyl), 45.7 (CH), 28.2, 27.2 (CH_2), 13.6 (CH_3); **m/z** (FAB) 466 (M+), 421, 365, HRMS (FAB) found 466.19348, $C_{34}H_{26}O_2$ requires 466.19327, (< 1ppm).

Methyl 4-(17'-tetrabenzo[*a,c,g,i*]fluorenylmethyl) benzoate (76)

A solution of n-butyllithium in cyclohexane (1.8 ml, 2.27 mmol; 1.1 equiv.; 1.26 M titrated) was added dropwise to a suspension of 8bH-tetrabenzo[*a,c,g,i*]fluorene (0.75 g, 2.0 mmol) in dry THF (10 ml) at -78°C under nitrogen. The suspension was stirred for 1 h at -78°C . Then a solution of methyl-4-(bromomethyl)-benzoate (0.52 g, 2.27 mmol; 1.1 equiv.) in dry THF (1 ml) was added giving an orange solution which was stirred a further 3.5 h at room temperature. The reaction mixture was then evaporated *in vacuo* to give a brown oil which was redissolved in DCM (20 ml). The resulting solution was washed twice with water (2 x 40 ml) and dried over magnesium sulphate. The volatiles were removed *in vacuo* to give a brown oil which was crystallized from DCM/n-hexane. The title compound was obtained as a pale yellow solid which was filtered and dried, (0.75 g, 71%); **m.p.** 241-243°C; **t.l.c.** $Rf_{(A)}$ 0.37, $Rf_{(F)}$ 0.85; **C,H,N** found: C, 87.23; H, 5.35; $C_{38}H_{26}O_2$ requires C, 88.68; H, 5.10 %; ν_{max} (bromoform mull) 3063 (CH stretching, aromatic), 2936, 2900 (CH stretching, alkyl), 1714 (CO, ester), 1608, 1500 (aromatic rings) cm^{-1}; λ_{max} 367 (ε 17362 $dm^3mol^{-1}cm^{-1}$), 302 (41577), 290 (33810), 254 (110110) nm; δ_H ($CDCl_3$, 80 MHz), 8.84-8.67 (4H, m, aromatic), 8.36-8.19 (4H, m, aromatic), 7.81-7.37 (8H, m, aromatic), 6.98 (2H, d, ^3J = 8.49 Hz, aromatic), 5.84 (2H, d, ^3J = 8.49 Hz, aromatic), 5.17 (1H, t, ^3J = 4.50 Hz, CH), 3.79 (2H, d, ^3J = 4.55 Hz, CH_2), 3.61 (3H, s, O-CH_3); δ_C ($CDCl_3$, 50 MHz), 166.6 (carbonyl C), 142.6, 141.0, 137.1, 131.0, 130.3, 128.6, 127.7, 127.3 (quaternary aromatic C's), 128.3, 127.5, 127.1, 126.8 125.6, 124.9, 124.3, 123.6, 123.3 (aromatic CH's), 51.4 (CH), 47.6 (CH_3), 39.7 (CH_2); **m/z** (FAB)

514 (M+), 365, HRMS (FAB) found 514.19325, $C_{38}H_{26}O_2$ requires 514.19327 , (< 1ppm).

4-(17'-Tetrabenzo[*a,c,g,i*]fluorenylmethyl)-trityl alcohol (83)

Tbf-Tr-OH

A solution of bromobenzene (4.27 g, 27.2 mmol) in dry THF (20 ml) was added to magnesium turnings (0.66 g, 27.2 mmol) and a crystal of iodine. After the reaction commenced the temperature was maintained at 20-25°C with an ice bath until the end of the addition. The reaction mixture was then heated to reflux for 45 min and then cooled to room temperature. A solution of methyl 4-(17'-tetrabenzo[*a,c,g,i*]fluorenylmethyl) benzoate (1.40 g, 2.72 mmol) in dry THF (30 ml) was added dropwise. The greenish reaction mixture was then heated to reflux for 2 h. After cooling to room temperature the solution was poured onto a saturated solution of aqueous ammonium chloride (100 ml) and the product extracted with DCM (100 ml). The organic layer was separated and washed with $NaHCO_3$ aq. (25 ml), then water (25 ml) and finally dried over magnesium sulphate. The volatiles were evaporated *in vacuo* and a brown oil was obtained. The product was purified by silica gel dry flash chromatography in DCM and crystallized in DCM/n-hexane, filtered and dried, to give the title compound as a beige crystalline solid, (1.1 g, 63%); **m.p.** 236-238°C; **t.l.c.** $Rf_{(A)}$ 0.44; **C,H,N** found: C, 91.82; H, 5.30; $C_{49}H_{34}O$ requires C, 92.13; H, 5.36 %; $\mathbf{v_{max}}$ (bromoform mull) 3572, 3433 (OH stretching), 3062 (CH stretching, aromatic), 2935, 2865 (CH stretching, alkyl), 1603, 1500 (aromatic rings) cm^{-1}; $\boldsymbol{\lambda_{max}}$ 378 (ε 13400 $dm^3mol^{-1}cm^{-1}$), 366 (14036), 303 (33814), 290 (26800), 254 (56144) nm; $\boldsymbol{\delta_H}$ ($CDCl_3$, 80 MHz), 8.83-8.71 (4H, m, aromatic), 8.41-8.22 (4H, m, aromatic), 7.75-7.47 (8H, m, aromatic), 7.09-6.69 (10H, m, aromatic), 6.17 (2H, d, ^3J = 8.55 Hz, aromatic), 5.72 (2H, d, ^3J = 8.52 Hz, aromatic), 5.23 (1H, t, ^3J = 4.53

Hz, CH), 3.76 (2H, d, ^3J = 4.46 Hz, CH_2), 2.16 (1H, s, OH); δ_C ($CDCl_3$, 50 MHz), 146.4, 144.1, 143.2, 137.1, 134.5, 131.1, 130.2, 128.7, 127.8, 127.1 (quaternary aromatic C's), 127.4, 126.8, 126.6, 125.9, 125.8, 125.5, 124.8, 124.4, 123.5, 123.4 (aromatic CH's), 81.2 (quaternary trityl C), 47.9 (CH), 39.5 (CH_2), 39.7 (CH_2); **m/z** (FAB) 640 (M+), 623, 366, HRMS (FAB) found 638.26106, $C_{49}H_{34}O$ requires 638.26096, (< 1ppm).

4-(17'-tetrabenzo[*a,c,g,i*]fluorenylmethyl)-4'',4'''-dimethoxytrityl alcohol (84) Tbf-DMTr-OH

A solution of 4-bromoanisole (4.36 g, 23.3 mmol) in dry THF (15 ml) was added to magnesium turnings (0.57 g, 23.3 mmol) and a crystal of iodine. After the reaction commenced, the temperature was maintained at 20-25°C with an ice bath until the end of the addition. The reaction mixture was heated at reflux for 1 h after which it was cooled to room temperature. A solution of methyl 4-(17'-tetrabenzo[*a,c,g,i*]fluorenylmethyl) benzoate (4.0 g, 7.78 mmol) in dry THF (50 ml) was added dropwise whilst the temperature was maintained below 20°C with an ice bath. The greenish reaction mixture was then stirred at room temperature for 3 h. The solution was poured into a saturated aqueous solution of ammonium chloride (100 ml) and the product extracted with DCM (100 ml). The organic layer was separated and washed with $NaHCO_3$ aq. (25 ml), then water (25 ml) and finally dried over magnesium sulphate. The volatiles were evaporated *in vacuo* and a brown oil was obtained. The product was purified by silica gel dry flash chromatography in DCM and crystallized in DCM/n-hexane, filtered and dried, to give the title compound as a beige crystalline solid, (3.0 g, 55%); **m.p.** 209-211°C; **t.l.c.** $Rf_{(A)}$ 0.26, $Rf_{(G)}$ 0.72; **C,H,N** found: C, 87.57; H, 5.69; $C_{51}H_{38}O_3$ requires C, 87.64; H, 5.48 %; $\mathbf{V_{max}}$ (bromoform mull) 3583, 3550 (OH stretching), 3070 (CH stretching, aromatic), 2955,

2929, 2835 (CH stretching, alkyl), 1608, 1508 (aromatic rings), 1249, 1032 (C-O ether stretching) cm^{-1}; λ_{max} 380 (ε 17013 $dm^3mol^{-1}cm^{-1}$), 366 (17450), 302 (41880), 290 (34900), 254 (70236), 237 (68928) nm; δ_H ($CDCl_3$, 80 MHz), 8.82-8.71 (4H, m, aromatic), 8.41-8.22 (4H, m, aromatic), 7.75-7.47 (8H, m, aromatic), 6.59-6.56 (8H, d, aromatic), 6.15 (2H, d, ^3J = 8.39 Hz, aromatic), 5.68 (2H, d, ^3J = 8.40 Hz, aromatic), 5.22 (1H, t, ^3J = 4.17 Hz, CH), 3.75 (2H, d, ^3J = 4.20 Hz, CH_2), 3.72 (6H, s, CH_3O-), 2.14 (1H, s, OH); δ_C ($CDCl_3$, 50 MHz), 158.0 (quaternary aromatic C, C-O) 144.4, 143.2, 139.1, 136.9, 134.2, 131.0, 130.1 (quaternary aromatic C's), 128.6, 127.6, 127.0, 126.7, 125.7, 125.6, 125.3, 124.6, 124.4, 123.4, 112.5 (aromatic CH's), 80.6 (quaternary trityl C), 55.0 (CH_3O), 47.8 (CH), 39.5 (CH_2), 39.4 (CH_2); **m/z** (FAB) 698 (M+), 681, 365, HRMS (FAB) found 698.28213, $C_{51}H_{38}O_3$ requires 698.28208, (< 1ppm); **X-Ray analysis**, see section 2.4.

4-(17'-Tetrabenzo[*a,c,g,i*]fluorenylmethyl)-trityl chloride (85)
Tbf-Tr-Cl

Acetyl chloride (0.61 g, 7.84 mmol), previously distilled over phosphorus pentachloride, was added to a suspension of 4-(17'-tetrabenzo[*a,c,g,i*]fluorenylmethyl)-trityl alcohol (0.50 g, 0.784 mmol) in dry benzene (20 ml) at room temperature. It was heated to reflux for 3 h and then cooled to room temperature. The volatiles were evaporated *in vacuo* to give an orange solid. Finally the product was recrystallised from fresh benzene (10 ml) to give a pale yellow solid (0.45 g, 88%); **m.p.** 214-215°C; **t.l.c.** $Rf_{(A)}$ 0.67; **C,H,N** found: C, 88.70; H, 6.43; $C_{49}H_{33}Cl$ requires C, 89.55; H, 5.06 %; ν_{max} (bromoform mull) 3061 (CH stretching, aromatic), 2914, 2846 (CH stretching, alkyl), 1605, 1495 (aromatic rings); λ_{max} 380 (ε 16851 $dm^3mol^{-1}cm^{-1}$), 367 (17250), 303 (40336), 290 (33702), 254 (140115) nm; δ_H ($CDCl_3$, 200 MHz), 8.79-8.77 (4H, m, aromatic), 8.39-8.31 (4H, m, aromatic),

7.75-7.51 (8H, m, aromatic), 7.13-6.72 (10H, m, aromatic), 6.17 (2H, d, ^3J = 8.5 Hz, aromatic), 5.71 (2H, d, ^3J = 8.5 Hz, aromatic), 5.26 (1H, t, ^3J = 4.5 Hz, CH), 3.80 (2H, d, ^3J = 4.5 Hz, CH_2); δ_C ($CDCl_3$, 50 MHz), 144.9, 143.1, 142.3, 137.1, 135.1, 131.1, 130.2, 128.7 (quaternary aromatic C's), 129.2, 127.6, 127.2, 126.8, 125.8, 125.5, 124.9. 124.4, 123.6, 123.4 (aromatic CH's), 80.8 (quaternary trityl C), 47.8 (CH), 39.4 (CH_2); **m/z** (FAB) 773 (M + matrix), 621, 365.

4-(17'-tetrabenzo[*a,c,g,i*]fluorenylmethyl)-4",4"'-dimethoxytrityl chloride (86) Tbf-DMTr-Cl

Acetyl chloride (1.12 g, 14.3 mmol), previously distilled over phosphorus pentachloride, was added to a suspension of 4-(17'-tetrabenzo[*a,c,g,i*]fluorenylmethyl)-4",4"'-dimethoxytrityl alcohol (1.0 g, 1.43 mmol) in benzene (10 ml) at room temperature. The mixture was heated to reflux for 4 h and then cooled to room temperature. The volatiles were evaporated *in vacuo* to give an orange solid. Finally the product was recrystallised in fresh benzene (10 ml) to give a pale yellow solid (0.95 g, 93%); **m.p.** 176-179°C; **t.l.c.** $Rf_{(E)}$ 0.70; ν_{max} (bromoform mull) 3068 (CH stretching, aromatic), 2955, 2929, 2836 (CH stretching, alkyl), 1605, 1508 (aromatic rings), 1250, 1032 (C-O ether stretching) cm^{-1}; λ_{max} 498 (ε 6900 $dm^3mol^{-1}cm^{-1}$), 438 (3450), 382 (16851), 368 (17250), 304 (40336), 290 (33702), 254 (140115) nm; δ_H ($CDCl_3$, 200 MHz), 8.78-8.74 (4H, m, aromatic), 8.38-8.24 (4H, m, aromatic), 7.78-7.45 (8H, m, aromatic), 6.64-6.48 (8H, d, aromatic), 6.15 (2H, d, ^3J = 8.39 Hz, aromatic), 5.67 (2H, d, ^3J = 8.40 Hz, aromatic), 5.14 (1H, t, ^3J = 4.17 Hz, CH), 3.75 (2H, d, ^3J = 4.20 Hz, CH_2), 3.76 (6H, s, CH_3O-); **m/z** (FAB) 835 (M + matrix), 681, 365.

5'-O-(4-(17"-Tetrabenzo[*a,c,g,i*]fluorenylmethyl)-trityl)-thymidine (89)

5'-O-Tbf-Tr-T

Thymidine (0.17 g, 0.70 mmol) was twice co-evaporated with dry pyridine then dissolved in 10 ml of pyridine, distilled triethylamine (0.06 g, 0.70 mmol) and a trace of N,N-dimethylaminopyridine. Tbf-Tr-Cl (0.52 g, 0.78 mmol) was added at room temperature under argon and stirred for 18 h. It was then evaporated to dryness *in vacuo* and dissolved in dichloromethane (20 ml). The solution was washed successively with saturated sodium carbonate solution and water, dried over magnesium sulphate, and the solvent removed *in vacuo* to give a brown oil. This was purified by flash chromatography, eluting with 0-10% methanol in dichloromethane, the product was crystallised from dichloromethane/n-hexane to produce the title compound as a white solid, (0.21 g, 35%); **m.p.** (softening point) 156-158°C; **t.l.c.** $Rf_{(C)}$ 0.30, $Rf_{(A)}$ 0.05; **C,H,N** found: C, 81.40; H, 5.89; N, 3.19; $C_{59}H_{46}N_2O_5$ requires C, 82.10; H, 5.38; N, 3.25 %; ν_{max} (bromoform mull) 3387, 3193 (OH, NH stretching), 2931, 2869 (CH stretching, alkyl), 1682, (C=O ketone), 1610, (aromatic rings), 1276 (C-O ether stretching) cm^{-1}; λ_{max} 380 (ε 20257 $dm^3mol^{-1}cm^{-1}$), 366 (20688), 303 (50858), 290 (43962), 254 (137920), 237 (136196) nm; δ_H ($CDCl_3$, 80 MHz), 8.83-8.72 (4H, m, aromatic), 8.41-8.25 (4H, m, aromatic), 8.10 (1H, s, 6-H), 7.75-7.45 (8H, m, aromatic), 7.09-6.76 (8H, m, aromatic), 6.27 (2H, d, ^3J = 4.4 Hz, aromatic), 6.22 (1H, t, 1'-H), 5.73 (2H, d, ^3J = 8.4 Hz, aromatic), 5.27 (1H, t, Tbf-CH), 4.12 (1H, m, 4'-H), 3.77 (2H, d, Tbf-CH_2), 2.75 (2H, dd, 5'-CH_2), 2.13 (2H, m, 2'-CH_2); δ_C ($CDCl_3$, 50 MHz), 163.7 (2-C=O), 150.4 (4-C=O), 143.9, 140.9, 136.2, 135.6, 134.4, 130.8 (quaternary aromatic C's), 129.4, 129.1, 128.5, 127.2, 126.5, 126.2, 126.0, 125.3, 125.0, 124.0, 112.9 (aromatic CH's), 109.5 (5-C), 85.8 (quaternary trityl C), 85.3 (3'-C), 83.7 (1'-C), 70.5 (4'-C), 63.5 (5'-C), 47.6 (Tbf-CH), 40.0 (Tbf-CH_2), 40.0 (2'-CH_2), 11.6 (5-CH_3); **m/z** (FAB) 864 (M+), 775, 639, 366, HRMS (FAB) found 862.34070, $C_{59}H_{46}N_2O_5$ requires 862.34065, (< 1ppm).

5'-O-(4-(17''-Tetrabenzo[*a,c,g,i*]fluorenylmethyl)-4''',4''''-dimethoxytrityl)-thymidine (90)

5'-O-Tbf-DMTr-T

Thymidine (0.25 g, 1.03 mmol) was twice co-evaporated with dry pyridine then dissolved in 3 ml of pyridine, distilled triethylamine (0.11 g, 1.13 mmol) and a trace of N,N-dimethylaminopyridine. Tbf-DMTr-Cl (0.96 g, 1.34 mmol, 1.3 equiv.) was added at room temperature under argon and stirred for 18 h. Methanol (3 ml) was added to quench the reaction. Then, it was evaporated to dryness *in vacuo* and dissolved in dichloromethane (20 ml). The solution was washed with saturated sodium carbonate and water, dried over magnesium sulphate, and the solvent removed *in vacuo* to give a brown oil. This was purified by flash chromatography, eluting with 0-10% methanol in dichloromethane, the product was crystallised from dichloromethane/n-hexane to produce the title compound as a pale solid, (0.43 g, 45%); **m.p.** 175-177°C; **t.l.c.** $Rf_{(C)}$ 0.38, $Rf_{(A)}$ 0.05; ν_{max} (bromoform mull) 3401, 3314, 3181 (OH, NH stretching), 2926, 2833 (CH stretching, alkyl), 1700, 1666 (C=O ketone), 1606, 1505 (aromatic rings), 1253, 1037 (C-O ether stretching) cm^{-1}; λ_{max} 380 (ε 14137 $dm^3mol^{-1}cm^{-1}$), 366 (14137), 303 (35036), 290 (31348), 254 (62696), 238 (61467) nm; δ_H ($CDCl_3$, 200 MHz), 11.29 (1H, s, NH) 8.97-8.93 (4H, d, aromatic), 8.47-8.29 (4H, dd, aromatic), 7.83-7.56 (8H, m, aromatic), 7.20 (1H, s, 6-H), 6.72-6.58 (8H, m, aromatic), 6.19 (2H, d, ^3J = 8.2 Hz, aromatic), 6.07 (1H, t, 1'-H), 5.59 (2H, d, ^3J = 8.2 Hz, aromatic), 5.59 (1H, t, Tbf-CH), 5.20 (1H, d, 3'-OH), 4.02 (1H, m, 4'-H), 3.66 (2H, d, Tbf-CH_2), 3.36 (6H, s, CH_3O-), 2.76 (2H, m, 5'-CH_2), 1.97 (2H, m, 2'-CH_2), 1.17 (3H, s, CH_3); δ_C ($CDCl_3$, 50 MHz), 163.7 (2- C=O), 150.4 (4- C=O), 143.9, 140.9, 136.2, 135.6, 134.4, 130.8 (quaternary aromatic C's), 129.4, 129.1, 128.5, 127.2, 126.5, 126.2, 126.0, 125.3, 125.0, 124.0, 112.9 (aromatic CH's), 112.9 (5-C), 85.3 (quaternary trityl C), 85.3 (3'-C), 83.7 (1'-C), 70.6 (4'-C), 63.4 (5'-C), 55.1 (CH_3O), 47.6 (Tbf-CH), 40.0 (Tbf-CH_2), 40.0 (2'-CH_2), 12.5 (5-CH_3); **m/z**

(FAB) 922 (M+), 681, 365, HRMS (FAB) found 922.36181, $C_{61}H_{50}N_2O_7$ requires 922.36178, (< 1ppm).

5'-O-(4-(17"-tetrabenzo[*a,c,g,i*]fluorenylmethyl)-4''',4''''-dimethoxytrityl)-thymidine-3'-O-N,N-diisopropyl-β-cyanoethyl phosphoramidite (91)

5'-O-(4-(17"-Tetrabenzo[*a,c,g,i*]fluorenylmethyl)-4''',4''''-dimethoxytrityl)-thymidine (0.25 g, 0.271 mmol) was co-evaporated twice in methylene chloride (10 ml) and then the flask was closed with a septum pierced with a needle and filled with argon and the needle removed. An argon filled balloon was attached to the flask and the nucleoside was dissolved in dry methylene chloride (10 ml). N,N-Diisopropylamine (0.070 g, 0.54 mmol) and then N,N-diisopropyl-β-cyanoethyl phosphoramidyl chloride (0.077 g, 0.32 mmol) were added and the reaction was stirred for 30 min before being quenched with methanol (0.8 equiv.) and poured into 10 ml each of DCM and sat. aqueous sodium carbonate and extracted. The organic layer was washed with water (10 ml) and dried over magnesium sulphate and the solvent removed *in vacuo* to give a brown oil. The residue was dissolved in DCM (5 ml) and precipitated with n-hexane (250 ml) at -20°C. The solid was filtered off and dried in an evacuated dessicator to give the title compound as a white powder (0.20, 66%); **m.p.** (softening point) 71-75°C; **t.l.c.** $Rf_{(F)}$ 0.71; $\mathbf{V_{max}}$ (bromoform mull) 2930, 2830 (CH stretching, alkyl), 1700, 1682 (C=O ketone), 1608, 1508 (aromatic rings), 1253, 1034 (C-O ether stretching) cm^{-1}; δ_H ($CDCl_3$, 200 MHz), 11.34 (1H, s, NH) 8.98-8.95 (4H, d, aromatic), 8.50-8.26 (4H, dd, aromatic), 7.84-7.61 (8H, m, aromatic), 7.22 (1H, s, 6-H), 6.74-6.48 (8H, m, aromatic), 6.20 (2H, d, ^3J = 8.1 Hz, aromatic), 6.18 (1H, t, 1'-H), 5.59 (2H, d, ^3J = 8.2 Hz, aromatic), 4.26 (1H, m, 4'-H), 3.71-3.28 (Tbf-CH_2, CH_3O-, 3'-H, β-cyanoethyl CH_2), 2.76 (2H, m, 5'-CH_2), 1.97

(2H, m, 2'-CH_2), 1.22-0.76 (5-CH_3, ^{i}pr CH_3); δ_P ($CDCl_3$, 36 MHz), 148.51, 148.03; **m/z** (FAB) 1124 (M+), 682, 365.

4-(17'-Tetrabenzo[*a,c,g,i*]fluorenylmethyl) benzyl alcohol (106)

Methyl 4-(17'-tetrabenzo[*a,c,g,i*]fluorenylmethyl) benzoate (2.06 g, 4.0 mmol) was dissolved in dry THF (40 ml) under nitrogen whereupon diisobutylaluminium hydride DIBAL (8.8 ml, 4.4 mmol, 1.0 M in DCM) was carefully added at room temperature. After 2 h stirring the solution had faded from orange to yellow and was poured onto aqueous HCl 2 M (60 ml). The product was extracted with ethyl acetate (70 ml), washed with aqueous sodium hydrogen carbonate (40 ml) and water (50 ml), and dried over magnesium sulphate. After evaporation of the solvents, the crude beige solid was recrystallized from ethyl acetate / methanol to give a beige solid, (1.65 g, 85%); **m.p.** 230-232°C; **C,H,N** found: C, 91.44; H, 5.89; $C_{37}H_{26}O$ requires C, 91.33; H, 5.39 %; **t.l.c.** $Rf_{(A)}$ 0.20, $Rf_{(C)}$ 0.82; ν_{max} (bromoform mull) 3584, 3555, 3473 (OH stretching), 2927, 2858 (CH stretching, alkyl), 1607, 1500 (aromatic rings) cm^{-1}; λ_{max} 380 (ε 11340 $dm^3mol^{-1}cm^{-1}$), 366 (11664), 303 (28188), 291 (23004), 254 (46332) nm; **Fluorescence** Ex 385 nm, Em_{max} 427 nm; δ_H ($CDCl_3$, 80 MHz), 8.84-8.68 (4H, m, aromatic), 8.37-8.16 (4H, m, aromatic), 7.81-7.35 (8H, m, aromatic), 6.24 (2H, d, ^3J = 8.0 Hz, aromatic), 5.75 (2H, d, ^3J = 8.0 Hz, aromatic), 5.15 (1H, t, ^3J = 4.4 Hz, CH), 4.05 (2H, s, CH_2), 3.73 (2H, d, ^3J = 4.5 Hz, CH_2); δ_C ($CDCl_3$, 50 MHz), 143.1, 137.9, 137.0, 134.8, 131.0, 130.2, 128.4 (quaternary aromatic C's), 128.7, 127.0, 126.8, 125.8, 125.4, 124.9, 124.7, 124.4, 123.5, 123.3, (aromatic CH's), 64.7 (CH_2OH), 47.9 (CH), 39.5 (CH_2); **m/z** (FAB) 487 (M+), 366, HRMS (FAB) found 486.19836, $C_{37}H_{26}O$ requires 486.19835, (< 1ppm).

4-(17'-Tetrabenzo[*a,c,g,i*]fluorenylmethyl) benzyl-(N,N-diisopropyl-β-cyanoethyl) phosphoramidite (107)

4-(17'-Tetrabenzo[*a,c,g,i*]fluorenylmethyl) benzyl alcohol (0.70 g, 1.44 mmol) was co-evaporated twice in methylene chloride (10 ml) and then the flask was closed with a septum pierced with a needle and filled with argon and the needle removed. An argon filled balloon was attached to the flask and the solid was dissolved in dry methylene chloride (30 ml). Bis(diisopropylammonium) tetrazolide (0.18 g, 0.75 equiv.), the catalyst, and then (β-cyanoethoxy)bis(diisopropylamino) phosphine (0.87 g, 2 equiv.) were added under argon and the reaction was stirred for 1 h before being poured into DCM (15 ml and saturated aqueous sodium hydrogen carbonate (15 ml) and extracted. The aqueous layer was washed with DCM (10 ml) and the collected organic fractions were washed with brine (30 ml). Finally they were dried over magnesium sulphate and evaporated *in vacuo* to give a brown oil. The residue was purified by short column chromatography on silica gel with ethyl acetate / DCM / triethylamine : 9 / 9 / 2. The collected fractions were evaporated to give a pale yellow solid (0.85, 86%); **m.p.** (softening point) 75-78°C; **t.l.c.** $Rf_{(H)}$ 0.77; ν_{max} (bromoform mull) 2940, 2850 (CH stretching, alkyl), 1604, 1508 (aromatic rings), cm^{-1}; δ_H ($CDCl_3$, 80 MHz), 8.84-8.69 (4H, m, aromatic), 8.39-8.16 (4H, m, aromatic), 7.81-7.37 (8H, m, aromatic), 6.31 (2H, d, ^3J = 8.1 Hz, aromatic), 5.80 (2H, d, ^3J = 8.1 Hz, aromatic), 5.16 (1H, t, Tbf-CH), 4.23-3.39 (8H, m, Tbf-CH_2, CH_2O, ^iPrCH), 2.35 (2H, t, CH_2CN), 1.31-0.82 (12H, m, ^{i}pr CH_3); δ_C ($CDCl_3$, 50 MHz), 143.2, 137.0, 136.3, 136.1, 134.8, 131.0, 130.2, (quaternary aromatic C's), 128.7, 128.2, 127.8, 126.8, 126.2, 125.8, 125.1, 124.8, 124.5, 123.5, (aromatic CH's), 117.5 (CN), 64.9 (CH_2O), 58.3 (CH_2CN), 48.0 (Tbf-CH), 42.8 (^iPrCH), 39.6 (Tbf-CH_2), 24.3 (^{i}pr CH_3), 20.1 (OCH_2); δ_P ($CDCl_3$, 36 MHz), 148.48; **m/z** (FAB) 686 (M+), 633, 469, 365; HRMS (FAB) found 687.31405, $C_{46}H_{44}N_2O_2P$ requires 687.31405, (< 1ppm).

1-(Diphenanthren-9'-yl)-1,6-hexanediol (110)

9-Bromophenanthrene (2.83 g, 11 mmol) was dissolved in dry THF (40 ml) and slowly added to magnesium turnings (0.28 g, 11.5 mmol) together with a crystal of iodine under nitrogen. The exothermic reaction was controlled with the addition of 9-bromophenanthrene. After 30 min stirring, a solution of ε-caprolactone (0.57 g, 5 mmol) in dry THF (30 ml) was slowly added. After 2 h at reflux, ice then aqueous ammonium chloride was added until pH 3. The product was extracted into ethyl acetate, washed with aqueous sodium hydrogen carbonate (40 ml) and brine (50 ml), and dried over magnesium sulphate. After evaporation of the solvents, the residue was crystallised in DCM to give a white solid, (0.6 g, 26%); **m.p.** 238-240°C; **C,H,N** found: C, 86.16; H, 6.74; $C_{34}H_{30}O_2$ requires C, 86.78; H, 6.43 %; **t.l.c.** $Rf_{(C)}$ 0.58; $\mathbf{v_{max}}$ (bromoform mull) 3559, 3470 (OH), 3059 (aliphatic CH), 2947, 2848 (CH, aromatic) 1597, 1493 (aromatic rings) cm^{-1}; λ_{max} 278 (ε 22410 $dm^3mol^{-1}cm^{-1}$), 254 (122600) nm; δ_H ($CDCl_3$, 80 MHz), 8.79-7.03 (18H, m, aromatic), 3.30 (4H, s, CH_2), 1.49-1.16 (6H, m, CH_2), **m/z** (FAB) 470 (M^+), 452, 383; HRMS (FAB) found 470.22458, $C_{34}H_{30}O_2$ requires 470.22457, (< 1ppm).

Tetrabenzo[*a,c,g,i*]fluorene/Picric acid Complex

In order to grow crystals of the charge transfer complex between tetrabenzo[*a,c,g,i*]fluorene and picric acid for X-ray analysis, equimolar amounts of both compounds were first dissolved separately in a minimum of hot benzene. The two solutions were subsequently mixed together to give a dark red solution. Dark red crystals grew upon cooling to room temperature and after partial evaporation of the solvent overnight. After filtration and washing with cold benzene, the crystals were dried in a vacuum oven at 40°C. **m.p.** 231-233°C; **t.l.c.** $Rf_{(A)}$ 0.10 and 0.70 (two

spots); $\mathbf{V_{max}}$ (bromoform mull) 3400 (OH), 3094 (CH stretching, aromatic), 2925 (CH stretching, alkyl), 1699 (C=O, ketone), 1631, 1604, (aromatic rings), 1544, 1342 (N=O, nitro) cm^{-1}; **m/z** (EI) 381, 366 (M+), 229 (M+); **X-Ray analysis**, see section 2.3.2.

1-(Diphenanthren-9'-yl)-1-hexene-6-ol (112)

1-(Diphenanthren-9'-yl)-1,6-hexanediol (13·0 g, 0·028 mol) was suspended in DCM (100 ml) and TFA (~2·5 ml) was added dropwise, a blue coloration appearing after each drop. The brown solution was neutralized with triethylamine whilst being cooled with an ice bath. It was then washed with water, aqueous sodium hydrogen carbonate and brine, and finally dried over magnesium sulphate. The product was purified by dry flash cromatography with DCM / methanol to give a white solid (10.25 g, 81%); **m.p.** 67-70°C; **C,H,N** found: C, 89.56; H, 6.75; $C_{34}H_{28}O$ requires C, 90.23; H, 6.24 %; **t.l.c.** $Rf_{(C)}$ 0.52; $\mathbf{V_{max}}$ (bromoform mull) 3600, 3405 (OH), 3019 (CH stretching, aromatic), 2935, 2857 (CH stretching, alkyl), 1680 (C=C, alkene), 1594, 1492 (aromatic rings) cm^{-1}; $\boldsymbol{\lambda_{max}}$ 300 (ε 19675 $dm^3mol^{-1}cm^{-1}$), 254 (100110) nm; $\boldsymbol{\delta_H}$ ($CDCl_3$, 200 MHz), 8.83-7.47 (18H, m, aromatic), 6.36 (1H, t, ^3J = 7.3 Hz, CH alkene), 3.52 (2H, t, ^3J = 6.2 Hz, OCH_2), 2.20-2.17 (2H, m, CH_2), 1.72 (1H, s, OH), 1.56 (4H, m, CH_2); $\boldsymbol{\delta_C}$ ($CDCl_3$, 50 MHz), 139.6, 137.3, 137.0, 131.3, 131.1, 130.9, 130.7 (quaternary aromatic C's), 136.7 (CH alkene), 128.7, 128.4, 128.1, 127.8, 127.3, 126.8, 126.6, 126.4, 126.4, 126.1, 125.7, 123.4, 122.9, 122.3, 122.1 (aromatic CH's), 62.3 ($HOCH_2$), 32.1, 30.1, 25.5 (CH_2), 39.7 (CH_2); **m/z** (FAB) 452 (M+), 363, HRMS (FAB) found 452.21404, $C_{34}H_{28}O$ requires 452.21399 , (< 1ppm).

5-Hydroxy-1-triphenylmethoxy-pentane (113)

Triphenylmethyl chloride (11.15 g, 0.04 mol) was added to a solution of 1,5-pentandiol (4.17 g, 0.04 mol) in dry pyridine (170 ml) at 0°C under nitrogen. The clear solution was stirred at room temperature for 24 h. The reaction was quenched by adding methanol (20 ml) and evaporated to dryness *in vacuo*. The white residue was taken up in ethyl acetate and washed with water, aqueous sodium hydrogen carbonate and water and finally dried over magnesium sulphate. After evaporation of the solvent, the product was purified by flash chromatography with DCM to give a white solid, (7.0 g, 51%); **m.p.** 65-69°C; **C,H,N** found: C, 84.40; H, 8.00; $C_{24}H_{26}O_2$ requires C, 83.19; H, 7.57 %; **t.l.c.** $Rf_{(A)}$ 0.10, $Rf_{(C)}$ 0.53; ν_{max} (bromoform mull) 3601, 3390 (OH), 3055 (CH stretching, aromatic), 2938, 2867 (CH stretching, alkyl), 1597, 1489 (aromatic rings), 1076 (ether C-O stretching) cm^{-1}; λ_{max} 280 (ε 19850 $dm^3mol^{-1}cm^{-1}$), 254 (110150) nm; δ_H ($CDCl_3$, 200 MHz), 7.57-7.24 (15H, m, aromatic), 3.61 (2H, t, ^3J = 6.3 Hz, OCH_2), 3.16 (2H, t, ^3J = 6.5 Hz, OCH_2), 2.18 (1H, s, OH), 1.73-1.53 (6H, m, CH_2); δ_C ($CDCl_3$, 50 MHz), 144.3 (quaternary aromatic C), 128.5, 127.5, 126.7 (aromatic CH's), 86.1 (quaternary trityl C), 63.3, 62.6 (OCH_2), 32.4, 29.6, 22.3 (CH_2); **m/z** (EI) 347 (M+), 243, HRMS (EI) found 346.1931, $C_{24}H_{26}O_2$ requires 346.19327, (< 1ppm).

1-Triphenylmethoxy-5-bromopentane (114)

Carbon tetrabromide (9.5 g, 28 mmol) was added to a solution of 5-hydroxy-1-triphenylmethoxy-pentane (5.0 g, 14 mmol) and triphenylphosphine (4.5 g, 15.4 mmol) in dry DMF (75 ml) at 0°C. A few drops of diisopropylamine were added and after 15 min the reaction was quenched with methanol (20 ml). The yellow brown solution was evaporated to dryness and the residue taken up in ethyl acetate (150 ml)

and washed with aqueous sodium hydrogen carbonate and water, then dried over magneium sulphate. After evaporation of the solvent, the crude product was purified by flash chromatography with DCM. The product was finally crystallised in n-hexane / diethyl ether to give a white solid, (4.51 g, 77 %); **m.p.** 86-87°C; **C,H,N** found: C, 71.53; H, 6.48; $C_{24}H_{25}BrO$ requires C, 70.57; H, 6.17 %; **t.l.c.** $Rf_{(A)}$ 0.60; $\mathbf{v_{max}}$ (bromoform mull) 3045 (CH stretching, aromatic), 2940, 2866 (CH stretching, alkyl), 1596, 1492 (aromatic rings), 1073 (ether C-O stretching) cm^{-1}; λ_{max} 281 (ε 20653 $dm^3mol^{-1}cm^{-1}$), 254 (105110) nm; δ_H ($CDCl_3$, 200 MHz), 7.50-7.20 (15H, m, aromatic), 3.40 (2H, t, ^3J = 6.8 Hz, $BrCH_2$), 3.10 (2H, t, ^3J = 6.3 Hz, OCH_2), 1.85-1.49 (6H, m, CH_2); δ_C ($CDCl_3$, 50 MHz), 144.2 (quaternary aromatic C), 128.5, 127.6, 126.7 (aromatic CH's), 86.2 (quaternary trityl C), 63.0 (OCH_2), 33.7 ($BrCH_2$), 32.4, 29.0, 24.8 (CH_2); **m/z** (EI) 410 (M+), 332, 240; HRMS (EI) found 410.1080, $C_{24}H_{25}OBr$ requires 410.10695.

3-(17'-tetrabenzo[*a,c,g,i*]fluorenyl) propanol (116)

A solution of 9-BBN (17.7 ml, 0.5M, 8.9 mmol) in THF was added to a solution of 1-(17'-tetrabenzo[*a,c,g,i*]fluorenyl)-prop-2-ene (3.0 g, 7.4 mmol) in dry THF (20 ml) under nitrogen. The tempearture was maintained below 20°C. After 3 h stirring the solution became lighter, aqueous sodium hydroxide (0.35 g in 2 ml water, 8.9 mmol) was first added then hydrogen peroxide (1 ml, 30% in water, 8.9 mmol) the temperature rose to 45 °C. The reaction mixture was stirred at 60°C for 1 h and then cooled to room temperature. Fresh hydrogen peroxide (1 equiv) was added and left to stir overnight at room temperature. The product was extracted with DCM / methanol (9/1, 100 ml), washed with water, aqueous sodium hydrogen carbonate and dried over magnesium sulphate. After evaporation of the solvents, the residue was recrystallised in THF / DCM to give a white solid, (1.80 g, 57 %); **m.p.** 188-190°C;

C,H,N found: C, 90.58; H, 6.04; $C_{32}H_{24}O$ requires C, 90.53; H, 5.70 %; **t.l.c.** $Rf_{(A)}$ 0.13; $\mathbf{V_{max}}$ (bromoform mull) 3326 (OH), 3021 (CH stretching, aromatic), 2950, 2859 (CH stretching, alkyl), 1604, 1497 (aromatic rings) cm^{-1}; λ_{max} 380 (ε 17366 $dm^3mol^{-1}cm^{-1}$), 365 (18326), 300 (37548), 288 (32548), 254 (86966) nm; δ_H ($CDCl_3$, 200 MHz), 8.97-8.87 (4H, m, aromatic), 8.57 (2H, d, ^3J = 8.2 Hz, aromatic), 8.34 (2H, d, ^3J = 8.3 Hz, aromatic), 7.81-7.64 (8H, m, aromatic), 5.31 (1H, s, CH Tbf), 3.98 (1H, t, ^3J = 5.2 Hz, OH), 2.87-2.78 (2H, m, CH_2), 2.60-2.49 (2H, m, CH_2), 0.35-0.23 (2H, m, CH_2); δ_C ($CDCl_3$, 50 MHz), 144.6, 135.9, 130.9, 130.5, 129.9, (quaternary aromatic C's), 128.3, 127.8, 127.3, 126.7, 126.5, 126.1, 125.5, 124.7, 124.1, 123.8 (aromatic CH's), 60.7 ($HOCH_2$), 46.4 (Tbf CH), 30.1, 25.8 (CH_2); **m/z** (FAB) 425 (M+), 408, 366, HRMS (FAB) found 424.18273, $C_{32}H_{24}O$ requires 424.18270, (< 1ppm).

3-(17'-tetrabenzo[*a,c,g,i*]fluorenyl) propyl-N,N-diisopropyl-β-cyanoethyl phosphoramidite (117)

3-(17'-tetrabenzo[*a,c,g,i*]fluorenyl)propanol (0.64 g, 1.5 mmol) was co-evaporated twice with dry methylene chloride (10 ml) and then the flask was closed with a septum pierced with a needle and filled with nitrogen and the needle removed. An nitrogen filled balloon was attached to the flask and the solid was dissolved in dry methylene chloride (15 ml). Diisopropylethylamine (0.59 g, 4.5 mmol) and N,N-diisopropyl-β-cyanoethyl phosphoramidyl chloride (0.36 g, 1.5 mmol) were added under nitrogen and the reaction was stirred for 1 h before being poured into saturated aqueous sodium hydrogen carbonate (30 ml). The organic fraction was washed with water and dried over magnesium sulphate. After evaporation *in vacuo* the brown oil was purified by short column chromatography on silica gel with ethyl acetate/cyclohexane : 4/1. The collected fractions were evaporated to give a yellow

foam like solid (0.81, 86 %); **m.p.** 62-65°C; **C,H,N** found: C, 78.28; H, 7.18; N, 4.74; $C_{41}H_{41}O_2PN_2$ requires C, 78.82; H, 6.61; N, 4.48 %; **t.l.c.** $Rf_{(I)}$ 0.73; $\mathbf{V_{max}}$ (bromoform mull) 3072, 3023 (CH stretching aromatic), 2929, 2870 (CH stretching, alkyl), 2253 (CN), 1608, 1500 (aromatic rings), cm^{-1}; δ_H ($CDCl_3$, 200 MHz), 8.82-8.76 (4H, m, aromatic), 8.67 (2H, d, ^3J = 8.0 Hz, aromatic), 8.24 (2H, d, ^3J = 8.0 Hz, aromatic), 7.73-7.55 (8H, m, aromatic), 5.06 (1H, t, Tbf-CH), 3.41-3.04 (6H, m, Tbf-CH_2, CH_2O, ^iPrCH), 2.78-2.67 (2H, m, Tbf-CH_2), 2.21 (2H, t, ^3J = 6.6 Hz, CH_2CN), 1.30-1.18 (2H, m, CH_2), 1.00-0.79 (12H, dd, ^{i}pr CH_3), 0.67-0.52 (2H, m, Tbf-CH_2); δ_C ($CDCl_3$, 50 MHz), 143.7, 136.8, 131.2, 130.3, 128.6, 127.4 (quaternary aromatic C's), 126.8, 125.8, 125.6, 125.0, 124.3, 123.4 (aromatic CH's), 117.5 (CN), 63.1 (CH_2O), 58.0 (CH_2CN), 46.4 (Tbf-CH), 42.6 (^iPrCH), 30.0 (Tbf-CH_2), 24.2 (^{i}pr CH_3), 19.9 (OCH_2); δ_P ($CDCl_3$, 36 MHz), 147.47; **m/z** (FAB) 625 (M+), 633, 407, 364; HRMS (FAB) found 624.29063, $C_{41}H_{41}N_2O_2P$ requires 624.29055, (< 1ppm).

3.3 Oligodeoxyribonucleotide Synthesis

The synthesis of oligodeoxyribonucleotides was performed on an Applied Biosystems 380B DNA Synthesizer. A,C,G,T phosphoramidite monomers, tetrazole coupling catalyst, acetic anhydride and N-methyl imidazole capping reagents, trichloroacetic acid deprotection solution, iodine oxidation mixture, acetonitrile wash solvent, aqueous ammonia cleavage solution and the solid support were all purchased from Applied Biosystems or Cruachem Ltd. The monomers were dissolved in anhydrous acetonitrile. Synthesis scale varied from 0.2 μmol to 1 μmol using controlled pore glass (CPG) as the solid support and 40 nmol for polystyrene supports. In each case the first nucleoside was already attached to the support *via* a succinyl linker by the time of purchase. At the end of the synthesis the

oligonucleotides were cleaved from the solid support by treatment with concentrated aqueous ammonia and the exocyclic amine protecting groups were removed by heating the ammonium hydroxide solution to 50°C for 4 h. The water and ammonia were removed *in vacuo* and the residue was dissolved in 2 ml of water and analysed by RP-HPLC.

The chemical steps for one synthesis cycle are presented in appendix I. Multiple washes with the same solvent involve filtration between wash steps. Each step volume is 1 ml. For each μmol of nucleoside attached covalently to the support, 0.4M tetrazole and 0.1M monomer phosphoramidite are premixed in acetonitrile. I_2 solution is THF/lutidine/water : 2/2/1 containing 0.2M iodine.

3.4 5'-Tbf-DMTr-oligodeoxyribonucleotide Synthesis

The preparation of 5'-Tbf-DMTr-oligodeoxyribonucleotides, as presented in chapter 2.6.2, is generally performed manually directly with the synthesis columns. The oligodeoxyribonucleotide is synthesised according to the standard β-cyanoethyl phosphoramidite protocol in the "Trityl-off" and manual deprotection mode. The 3'-end of the oligonucleotide is therefore bound to the CPG-support while the 5'-end is free to react with the Tbf-DMTr-Cl protecting group. Prior to the reaction, a 0.4-0.3 M solution of Tbf-DMTr-Cl in dry pyridine is prepared from freshly synthesized Tbf-DMTr-Cl. Typically for 0.2 and 1 μmol syntheses, 1 ml of the orange solution is flushed through the column with two 1 ml syringes, see *Fig. 2.12*. For 10 μmol syntheses, 3 ml of Tbf-DMTr-Cl solution is necessary. Air bubbles can be expelled by keeping the column-syringe system upright. The protecting group is then left to react with the free 5'-hydroxyl groups for 1.5 to 2 h at room temperature. The nearly

saturated solution of Tbf-DMTr-Cl should not precipitate in the column. If it does it should be diluted with fresh pyridine. The column is then flushed slowly, so as not to break the fragile filters, with pyridine (5 ml) and acetonitrile (5 ml). The column can be put back onto the DNA synthesizer and the deprotection programmed in the "Trityl-On" and automatic deprotection mode. The 5'-Tbf-DMTr-oligonucleotide with the exocyclic amines still protected, is subsequently obtained in solution in ammonia. The base deprotection and subsequent work-up rejoins the standard protocol.

3.5 Oligodeoxyribonucleotide Deprotection and Purification

For phosphoramidite synthesized oligodeoxyribonucleotides, a final reaction step involving warming in the presence of concentrated ammonia is required for deprotection of the base protecting group. This is usually achieved by inserting the vial containing the crude product in a heating block at 50°C for 4 h. Afterwhat the only remaining protecting group is Tbf-DMTr at the 5'-end. The crude 5'-Tbf-DMTr-oligonucleotide is evaporated to dryness *in vacuo* or liophilized and dissolved in 0.7 ml of distilled water. For short sequences the purification is effected as outlined in section 2.8, whereas for longer sequences (> 30-mer) the purification is carried out by preparative RP-HPLC with a binary solvent system to give a gradient of acetonitrile in aqueous ammonium acetate buffer (section 3.1). The fluorescent hydrophobic Tbf-DMTr containing fractions are collected and evaporated or liophilized. The 5'-deprotection is then carried out by dissolving the residue in 1 ml of water and 4 ml of acetic acid. The solution is left to react for 45 min, after which it is evaporated or liophilized three times using 2 ml of water for each redissolution. The residue is subsequently dissolved in exactly 1 ml of water and loaded onto a Sephadex G-25

column for desalting. The pure oligonucleotide is finally obtained in an aqueous solution following elution of the Sephadex column with 1.5 ml of water.

Chapter 4: References

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Abbreviations

9-BBN	9-borabicyclo[3.3.1]nonane
A	Adenine
AP	Alkaline phosphatase
bp	Base pair
C	Cytosine
CE	Capillary gel electrophoresis
CNE	β-Cyanoethyl
CPG	Controlled pore glass
DBU	1,5-Diazabicyclo[5,4,0]undec-5-ene
DCCI	Dicyclohexylcarbodiimide
DCM	Dichloromethane
DIBAL	Diisobutylaluminium hydride
DMAP	N,N-Dimethylaminopyridine
DMTr	4,4'-Dimethoxytrityl
DNA	Deoxyribonucleic acid
DNP	2,4-Dinitrophenyl
EDTA	Ethylene diamine tetra-acetic acid
Fmoc	Fluoren-9-ylmethoxycarbonyl
G	Guanine
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
MMTr	4-monomethoxytrityl
mRNA	Messenger RNA
MSNT	1-Mesitylenesulphonyl-3-nitro-1,2,4-triazole
nmr	Nuclear magnetic resonance
NPE	2-(*p*-Nitrophenyl)ethyl
PAGE	Polyacrylamide gel electrophoresis
PGC	Porous graphitised carbon
RNA	Ribonucleic acid
RP-HPLC	Reverse-phase HPLC
SPS	Solid phase synthesis
T	Thymine

t.l.c.	Thin layer chromatography
TBAF	Tetra-*n*-butylammonium fluoride
Tbf	Tetrabenzo[*a*,*c*,*g*,*i*]fluorene
Tbf-DMTr	4-(17'-Tetrabenzo[*a*,*c*,*g*,*i*]fluorenylmethyl)-4",4'"-dimethoxytrityl
Tbf-DMTr-Cl	4-(17'-Tetrabenzo[*a*,*c*,*g*,*i*]fluorenylmethyl)-4",4'"-dimethoxytrityl chloride
Tbf-DMTr-OH	4-(17'-Tetrabenzo[*a*,*c*,*g*,*i*]fluorenylmethyl)-4",4'"-dimethoxytrityl alcohol
Tbf-Prop	Tetrabenzo[*a*,*c*,*g*,*i*]fluorenylpropyl
Tbf-Prop-Ph	Tetrabenzo[*a*,*c*,*g*,*i*]fluorenylpropyl phosphate
Tbf-Tr	4-(17'-Tetrabenzo[*a*,*c*,*g*,*i*]fluorenylmethyl)-trityl
Tbf-Tr-Cl	4-(17'-Tetrabenzo[*a*,*c*,*g*,*i*]fluorenylmethyl)-trityl chloride
Tbf-Tr-OH	4-(17'-Tetrabenzo[*a*,*c*,*g*,*i*]fluorenylmethyl)-trityl alcohol
Tbf-Trityl	4-(17'-Tetrabenzo[*a*,*c*,*g*,*i*]fluorenylmethyl)-trityl
Tbfmoc	Tetrabenzo[*a*,*c*,*g*,*i*]fluorenyl-17-methyloxycarbonyl
TCA	Trichloroacetic acid
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
tRNA	Transfer RNA
UV	Ultra-violet

Courses Attended

The following is a statement of the courses attended during the period of research:

Organic Research Seminars, various speakers, Department of Chemistry, University of Edinburgh, 1990-1993.

Merck, Sharp & Dohme, Medicinal Chemistry lectures, Prof. R. Baker and colleagues, Department of Chemistry, University of Edinburgh, 1991,1992.

Smith Kline and French, Medicinal Chemistry lectures, Department of Chemistry, University of Edinburgh, 1991.

2D Nuclear Magnetic Resonance, Dr. D. Reed, Dr. I.H. Sadler and Dr. J. Parkinson, Department of Chemistry, University of Edinburgh, 5 lectures.

Current Topics in Organic Chemistry, various speakers, Department of Chemistry, University of Edinburgh, 1990-1993.

Peptide Chemistry, Prof. R. Ramage, Department of Chemistry, University of Edinburgh, 1991.

Royal Society of Chemistry, Perkin Division, Scottish Post-graduate Symposia, 1991-1992.

Thirteenth American Peptide Symposium, Edmonton, Alberta, Canada, 1993.

Third Symposium in Solid Phase Synthesis, Biological & Biomedical Applications, Oxford University, 1993.

APPENDIX

Standard synthesis cycle on a ABI 380 DNA synthesiser, 2 μmol scale synthesis by the phosphoramidite method.

STEP NUMBER	FUNCTION £	NAME	STEP TIME	STEP ACTIVE FOR BASES A	G	C	T	5	6	7
1	10	£18 To Waste	4	Yes	Yes	Yes	Yes	Yes	Yes	Yes
2	9	£18 To Column	60	Yes	Yes	Yes	Yes	Yes	Yes	Yes
3	2	Reverse Flush	20	Yes	Yes	Yes	Yes	Yes	Yes	Yes
4	1	Block Flush	4	Yes	Yes	Yes	Yes	Yes	Yes	Yes
5	28	Phos Prep	3	Yes	Yes	Yes	Yes	Yes	Yes	Yes
6	+45	Group 1 On	1	Yes	Yes	Yes	Yes	Yes	Yes	Yes
7	90	TET to column	2	Yes	Yes	Yes	Yes	Yes	Yes	Yes
8	19	B+TET To Col 1	3	Yes	Yes	Yes	Yes	Yes	Yes	Yes
9	90	TET to column	2	Yes	Yes	Yes	Yes	Yes	Yes	Yes
10	19	B+TET To Col 1	2	Yes	Yes	Yes	Yes	Yes	Yes	Yes
11	9	£18 To Column	2	Yes	Yes	Yes	Yes	Yes	Yes	Yes
12	-46	Group 1 Off	1	Yes	Yes	Yes	Yes	Yes	[illegible]	[illegible]
13	+47	Group 2 On	1	Yes	Yes	Yes	Yes	Yes	[illegible]	[illegible]
14	10	£18 To Waste	7	Yes	Yes	Yes	Yes	Yes	[illegible]	[illegible]
15	1	Block Flush	4	Yes	Yes	Yes	Yes	Yes	[illegible]	[illegible]
16	90	TET to column	2	Yes	Yes	Yes	Yes	Yes	Yes	Yes
17	20	B+TET To Col 2	3	Yes	Yes	Yes	Yes	Yes	Yes	Yes
18	90	TET to column	2	Yes	Yes	Yes	Yes	Yes	[illegible]	[illegible]
19	20	B+TET To Col 2	2	Yes	Yes	Yes	Yes	[illegible]	[illegible]	[illegible]
20	9	£18 To Column	2	[illegible]	Yes	Yes	Yes	Yes	Yes	Yes
21	-48	Group 2 Off	1	[illegible]	Yes	Yes	Yes	Yes	Yes	Yes
22	+49	Group [illegible] On	1	Yes	Yes	Yes	Yes	Yes	Yes	Yes
23	10	£18 To Waste	7	Yes	Yes	Yes	Yes	Yes	Yes	Yes
24	1	Block Flush	4	Yes	Yes	Yes	Yes	Yes	Yes	Yes
25	90	TET to column	2	Yes	Yes	Yes	Yes	Yes	Yes	Yes
26	21	B+TET To Col 3	3	Yes	Yes	Yes	Yes	Yes	Yes	Yes
27	90	TET to column	2	Yes	Yes	Yes	Yes	Yes	Yes	Yes
28	21	B+TET To Col 3	2	Yes	Yes	Yes	Yes	Yes	Yes	Yes
29	9	£18 To Column	2	Yes	Yes	Yes	Yes	Yes	Yes	Yes
30	-50	Group 3 Off	1	Yes	Yes	Yes	Yes	Yes	Yes	Yes
31	4	Wait	30	Yes	Yes	Yes	Yes	Yes	Yes	Yes
32	16	Cap Prep	10	Yes	Yes	Yes	Yes	Yes	Yes	Yes
33	10	£18 To Waste	5	Yes	Yes	Yes	Yes	Yes	Yes	Yes
34	2	Reverse Flush	5	Yes	Yes	Yes	Yes	Yes	Yes	Yes
35	1	Block Flush	4	Yes	Yes	Yes	Yes	Yes	Yes	Yes
36	+45	Group 1 On	1	Yes	Yes	Yes	Yes	Yes	Yes	Yes
37	22	Cap To Col 1	15	Yes	Yes	Yes	Yes	Yes	Yes	Yes
38	-46	Group 1 Off	1	Yes	Yes	Yes	Yes	Yes	Yes	Yes
39	+47	Group 2 On	1	Yes	Yes	Yes	Yes	Yes	Yes	Yes
40	23	Cap To Col 2	12	Yes	Yes	Yes	Yes	Yes	Yes	Yes
41	-48	Group 2 Off	1	Yes	Yes	Yes	Yes	Yes	Yes	Yes
42	+49	Group 3 On	1	Yes	Yes	Yes	Yes	Yes	Yes	Yes
43	24	Cap To Col 3	12	Yes	Yes	Yes	Yes	Yes	Yes	Yes

STEP NUMBER	FUNCTION £	NAME	STEP TIME	A	G	C	T	5	6	7
44	-50	Group 3 Off	1	Yes	Yes	Yes	Yes	Yes	Yes	Ye
45	4	Wait	1	Yes	Yes	Yes	Yes	Yes	Yes	Ye
46	10	£18 To Waste	7	Yes	Yes	Yes	Yes	Yes	Yes	Ye
47	2	Reverse Flush	5	Yes	Yes	Yes	Yes	Yes	Yes	Ye
48	1	Block Flush	4	Yes	Yes	Yes	Yes	Yes	Yes	Ye
49	81	£15 To Waste	7	Yes	Yes	Yes	Yes	Yes	Yes	Ye
50	13	£15 To Column	15	Yes	Yes	Yes	Yes	Yes	Yes	Ye
51	4	Wait	30	Yes	Yes	Yes	Yes	Yes	Yes	Ye
52	10	£18 To Waste	10	Yes	Yes	Yes	Yes	Yes	Yes	Ye
53	2	Reverse Flush	20	Yes	Yes	Yes	Yes	Yes	Yes	Ye
54	1	Block Flush	4	Yes	Yes	Yes	Yes	Yes	Yes	Ye
55	9	£18 To Column	20	Yes	Yes	Yes	Yes	Yes	Yes	Yes
56	2	Reverse Flush	5	Yes	Yes	Yes	Yes	Yes	Yes	Yes
57	9	£18 To Column	10	Yes	Yes	Yes	Yes	Yes	Yes	Yes
58	2	Reverse Flush	5	Yes	Yes	Yes	Yes	Yes	Yes	Yes
59	9	£18 To Column	10	Yes	Yes	Yes	Yes	Yes	Yes	Yes
60	2	Reverse Flush	5	Yes	Yes	Yes	Yes	Yes	Yes	Yes
61	9	£18 To Column	10	Yes	Yes	Yes	Yes	Yes	Yes	Yes
62	2	Reverse Flush	5	Yes	Yes	Yes	Yes	Yes	Yes	Yes
63	1	Block Flush	4	Yes	Yes	Yes	Yes	Yes	Yes	Yes
64	**33**	**Cycle Entry**	**1**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**
65	10	£18 To Waste	3	Yes	Yes	Yes	Yes	Yes	Yes	Yes
66	9	£18 To Column	20	Yes	Yes	Yes	Yes	Yes	Yes	Yes
67	2	Reverse Flush	5	Yes	Yes	Yes	Yes	Yes	Yes	Yes
68	1	Block Flush	6	Yes	Yes	Yes	Yes	Yes	Yes	Yes
69	6	Waste-Port	1	Yes	Yes	Yes	Yes	Yes	Yes	Yes
70	5	Advance FC	1	Yes	Yes	Yes	Yes	Yes	Yes	Yes
71	82	£14 To Waste	5	Yes	Yes	Yes	Yes	Yes	Yes	Yes
72	14	£14 To Column	10	Yes	Yes	Yes	Yes	Yes	Yes	Yes
73	2	Reverse Flush	1	Yes	Yes	Yes	Yes	Yes	Yes	Yes
74	14	£14 To Column	10	Yes	Yes	Yes	Yes	Yes	Yes	Yes
75	2	Reverse Flush	1	Yes	Yes	Yes	Yes	Yes	Yes	Yes
76	14	£14 To Column	10	Yes	Yes	Yes	Yes	Yes	Yes	Yes
77	2	Reverse Flush	1	Yes	Yes	Yes	Yes	Yes	Yes	Yes
78	14	£14 To Column	10	Yes	Yes	Yes	Yes	Yes	Yes	Yes
79	2	Reverse Flush	1	Yes	Yes	Yes	Yes	Yes	Yes	Yes
80	14	£14 To Column	10	Yes	Yes	Yes	Yes	Yes	Yes	Yes
81	1	Block Flush	4	Yes	Yes	Yes	Yes	Yes	Yes	Yes
82	10	£18 To Waste	5	Yes	Yes	Yes	Yes	Yes	Yes	Yes
83	9	£18 To Column	30	Yes	Yes	Yes	Yes	Yes	Yes	Yes
84	2	Reverse Flush	5	Yes	Yes	Yes	Yes	Yes	Yes	Yes
85	10	£18 To Waste	5	Yes	Yes	Yes	Yes	Yes	Yes	Yes
86	9	£18 To Column	30	Yes	Yes	Yes	Yes	Yes	Yes	Yes
87	2	Reverse Flush	5	Yes	Yes	Yes	Yes	Yes	Yes	Yes
88	1	Block Flush	4	Yes	Yes	Yes	Yes	Yes	Yes	Yes
89	7	Waste-Bottle	1	Yes	Yes	Yes	Yes	Yes	Yes	Yes

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.

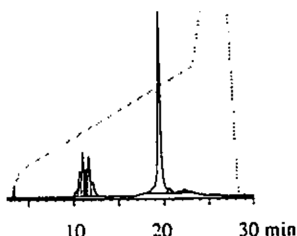

Figure 1

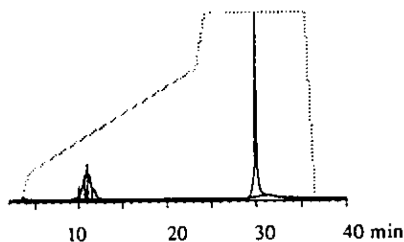

Figure 2

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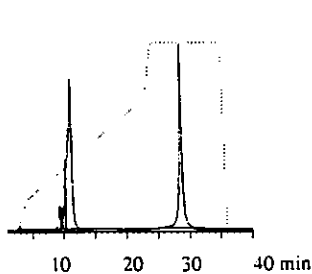

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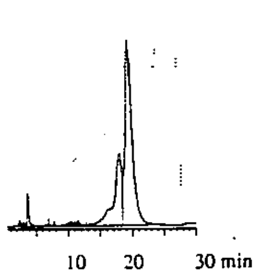

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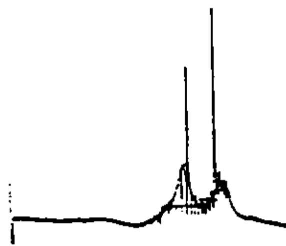
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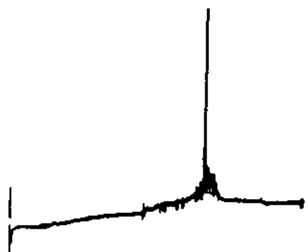

Figure 6

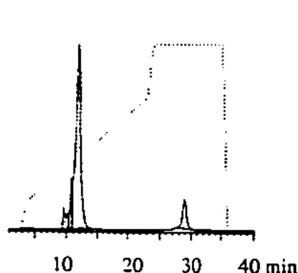

Figure 7

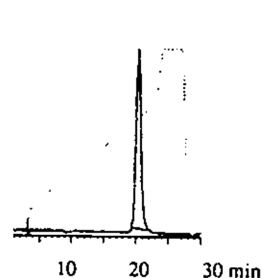

Figure 8

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.

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References

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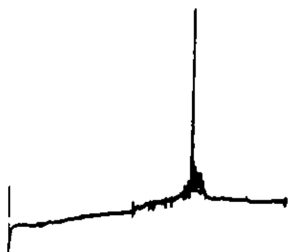

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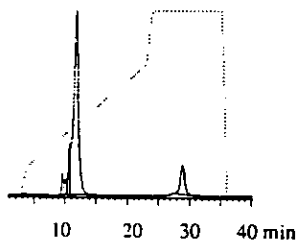
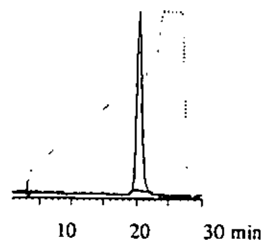


Figure 7



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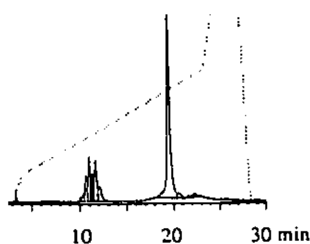
by the synthesizer without altering the synthesis cycle. Figure 1 shows the RP-HPLC profile of a crude 12-mer oligonucleotide (TTC-GAG-CCA-TAT) at 280 nm bearing DMTr at the 5'-terminus and Figure 2 shows the same crude oligonucleotide bearing a 5'-Tbf-DMTr terminus. The difference in retention time 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.



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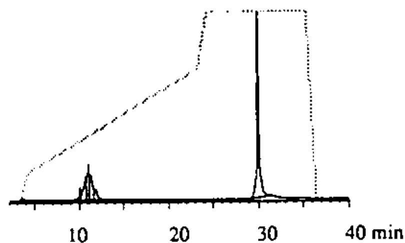

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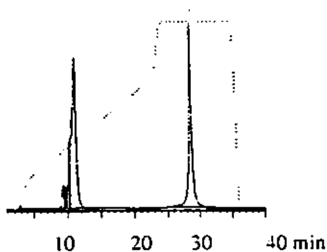

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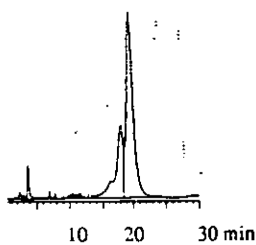


Figure 4

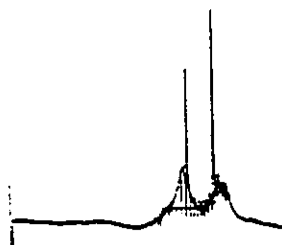


Figure 5

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

Robert Ramage*, Franck Olivier Wahl

Department of Chemistry, University of Edinburgh, West Mains Road, Edinburgh EH9 3JJ, Scotland

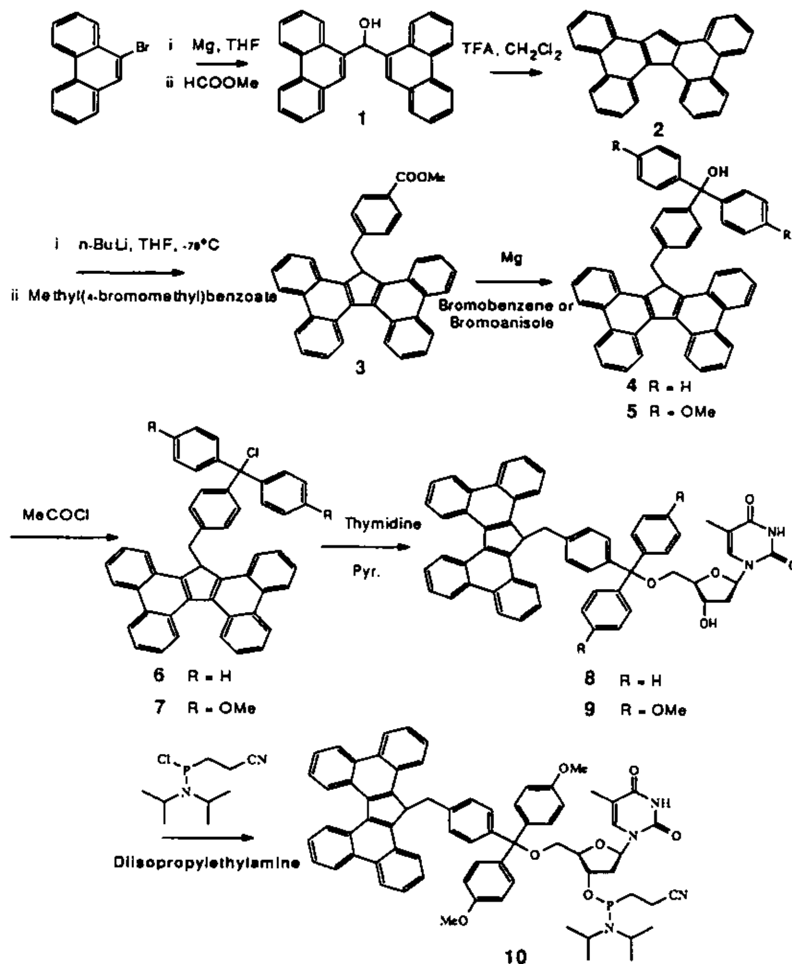
Keywords: RP-HPLC; 4-(17-Tetrabenzo[a,c,g,i]fluorenylmethyl)-4',4''-Dimethoxytrityl Chloride; Tbf-DMTr; DMTr; Protecting group; Oligonucleotide Synthesis.

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

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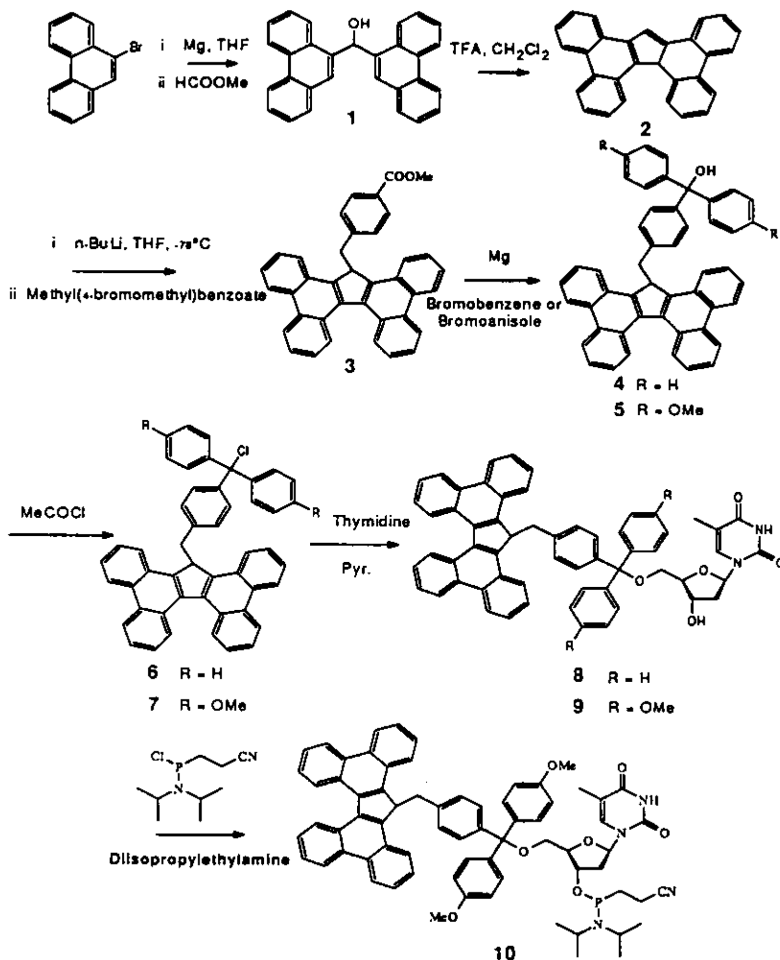

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