Design and Synthesis of Novel Protecting Groups for the solid phase synthesis of Oligonucleotides

By

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November 2000
This thesis is dedicated to Chris Barker

International Diamond Thief, Secret Agent and Postgraduate Biologist, in his spare time, Chris Barker was an irrepressible and inspirational figure to everyone he met. To the casual observer it may have appeared he was mad. To those in the know, while some of his ideas were clearly insane, we knew he was most often on to something big.

Tragically Chris died on June 24, 2001 just a few months short of receiving his own doctorate in Biology.
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Abstract

Four different compounds have been investigated for their usefulness as protecting groups for the solid phase synthesis of RNA and DNA, covering both 5’ and 2’ protection.

First is a 5’ hydroxyl protecting group containing the highly hydrophobic Tbf (tetrabenz[a,c,g,i]fluorene) moiety based on the trityl protecting group. This new group has been successfully incorporated into the synthesis of oligonucleotides pre-attached to the final nucleotide. The high hydrophobicity of the Tbf moiety is utilised to purify the sequence and it is then cleaved using existing protocols intended for dimethoxy trityl deprotection. This group could been used for oligodeoxyribonucleotides, oligoribonucleotides, peptides and sugars.

Three separate compounds were tried for the 2’ position of ribose and would therefore be of general applicability in oligoribonucleotide synthesis. These protecting groups were planned to have a two stage deprotection protocol intended to be compatible with the synthesis of oligoribonucleotides while also meeting the strict steric restraints of the position.
# Table of Contents

## Part 1 Introduction

1.1 Introduction to RNA and DNA  
1.1.1 History of DNA  
1.1.2 Biological Role of DNA and RNA  
1.1.2.1 The RNA World  
1.1.3 Structure of DNA and RNA  
1.1.3.1 Primary Structure  
1.1.3.2 Secondary Structure  
1.1.4 DNA Triplex  

1.2 Applications of Synthetic Oligomers of DNA and RNA  
1.2.1 Antisense  

1.3 Chemical Synthesis of Oligodeoxyribonucleotides  
1.3.1 The Phosphodiester Method  
1.3.2 The Phosphotriester Method  
1.3.3 The Phosphite-triester / Phosphoramidite Methods  
1.3.4 The H-Phosphonate Method  

1.4 Solid Phase Synthesis of Oligodeoxyribonucleotides  
1.4.1 The Solid Support and Linker  
1.4.2 Solid Phase Synthesis Reaction Cycle  
1.4.3 Purification of Oligomers  
1.4.4 Protecting Groups for Solid Phase Synthesis  
1.4.4.1 Exocyclic Base Protection  
1.4.4.2 Protection of the 5’ Position  

1.5 Solid Phase Synthesis of Oligoribonucleotides
Part 2 Results and Discussion

2.1 Aims of this Research
   2.1.1 5' Protection 55
   2.1.2 2' Protection 57
      2.1.2.1 Propargyl System 59
      2.1.2.2 Butynol System 62
      2.1.2.3 O-Creosyl System 65

2.2 Protection of 5' position 66
   2.2.1 Synthesis of Tbf C_{10} Tr 66
   2.2.2 Shorter Linker Approach 72
   2.2.3 Synthesis of Tbf C_{10} DMTr 73
      2.2.3.1 Solid Phase Synthesis 78
   2.2.4 Structural Motif 82

2.3 Protection of 2' position of ribonucleotides 83
   2.3.1 Propargyl System 83
   2.3.2 Butynol System 85
   2.3.3 O-Creosyl System 87

2.4 Conclusion and Future work 92
   2.4.1 5' Protection 92
   2.4.2 2' Protection 94

Part 3 Experimental

3.1 Notes on Instrumentation and General Techniques 95

3.2 Synthetic Procedures 98
3.2.1 Deprotection Test 127

3.3 Oligodeoxyribonucleotide Synthesis 128
   3.3.1 Synthesiser Details and Procedure 128
   3.3.2 Purification 129
   3.3.3 Results 129

Part 4 References 131

Part 5 Appendices 139

   5.1 Abbreviations 139
   5.2 Two dimensional NMR 142
   5.3 Courses Attended 150
   5.4 Presentations 151
   5.5 DNA Synthesiser Synthesis Cycle 152
   5.6 Diagram list 154
1.1 Introduction to RNA and DNA

1.1.1 History of DNA

In 1869, thirty years after the cell had been identified as the basic unit of life, Friedrich Miescher isolated from cell nuclei a phosphorus-containing substance that was acidic in nature. Because of its acidity and origin, he named it Nucleinsaure (nucleic acid). In the 1920's Phoebus Levene identified the components of nucleic acids as four nitrogen-containing bases, a five-carbon sugar and a phosphate group. Levene also identified RNA as different from DNA in the sugar component and suggested how nucleotides might form long chains. However, he then put forward the tetranucleotide theory that has since been shown to be misguided. In this theory the bases repeated in regular order. This mistaken belief arose because it was thought nucleic acids were too simple to be the genetic information store and could as a consequence only play a structural role within the nucleus. Since the twenty amino acids offered much more variety than the four nucleic acid bases proteins were at the time thought to be the molecules of heredity. Erwin Chargaff first cast doubt on the tetranucleotide theory in the 1940's when he showed that nucleotide levels were not equal and that the chromosomal DNA of different species had different levels of each nucleotide. In the tetranucleotide theory all base levels should be equal and that should carry through to all life. One of Chargaff's major discoveries was that thymine and adenine levels were equal, as were cytosine and guanine, independent of the origin of the DNA.

The true significance of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) was to be realised in 1944 with the discovery by Avery et al$^1$ that DNA and not proteins from one strain of pneumoccus could transform another. This work was reinforced in 1952 when Hershey et al$^2$ used bacteriophages to show that viruses used their DNA to infect and take over other cells. Hershey was awarded the Nobel
Prize in 1969 for this work that finally determined that DNA was the molecule responsible for the transmission of inherited characteristics. The next big step forward came in 1953 with the publication in the journal Nature of the double helix structure of DNA by Watson and Crick. In their model two complementary antiparallel strands of oligonucleotides wind around each other with specific base pairs interacting across the strands. The implied mechanism for self-replication inherent in the structure was significant and subsequent discoveries that the base pair sequences directly translate into amino acid sequences for proteins have ensured that DNA has been of considerable interest to scientists ever since.

1.1.2 Biological role of DNA and RNA

Most of all DNA is contained within the nucleus of all cells except for bacteria and mitochondria, which do not have nuclei and their DNA moves freely throughout the cell. Within the cell DNA can be replicated or transcribed. Replication is where DNA is copied and transcription is the process where genetic code from DNA is used to produce mRNA as a template for protein synthesis. The first step in this process is the production of mRNA (messenger RNA) directly from the sequences of DNA. The DNA has encoded within it short sequences to indicate the beginning and end of a specific gene. The mRNA is assembled mirroring one of the DNA strands known as the template strand. The other strand is identical to the mRNA, except thymines are replaced by uridine, and is known as the coding strand. The mRNA then leaves the nucleus and moves into the cytoplasm where it is used as a template for the
production of proteins in a process called translation. Each three base sequence of the coding region of mRNA corresponds to an amino acid in the protein. Since proteins perform all functions required for growth and maintenance of the cell then DNA holds the code for all these functions.

Three separate classes of RNA are involved in the transcription and translation of a strand of DNA. Messenger RNA is the template onto which small (70 to 90 nucleotides) RNA molecules called tRNA (transfer RNA) dock. Lastly there are the ribosomal RNA's (rRNA) which form ribosomes along with ribosomal proteins. The ribosomes are the structure within which the mRNA and tRNA molecules combine to build proteins.

1.1.2.1 The RNA World

The question of how life evolved from inorganic lifeless matter to organic life has dogged science ever since people began doubting the creationist model. The gap between nonliving chemicals and bacterial cells is considered wider than that between bacteria and humans. Virtually all biologists agree that some sort of precellular life must have existed. The essence of the problem is a 'chicken and egg' paradox. DNA is a good store of genetic information but cannot carry out the functions required for a functioning cell. Proteins are good at carrying out the functions but cannot self-replicate and are therefore no use as stores of genetic information. The probability that both evolved spontaneously together seems unlikely so the question is "which came first?".

Between the competing camps of proteins first and DNA first has emerged the possibility of an RNA world, first proposed by Gilbert in 1986. In this model, RNA carried out both functions. In recent years it has been shown that RNA molecules can do more than simply create proteins. RNA molecules have been shown to behave as enzymes in RNA cleavage and ligation reactions. These molecules are termed
ribozymes and it has been suggested that RNA molecules play a more active, catalytic role in the production of proteins\textsuperscript{5} and crucially RNA can self replicate and modify its own sequences\textsuperscript{6}. Thus opening up the possibility that in the earliest life only RNA existed.\textsuperscript{5-11}

Mechanisms for recombination and the ability to produce new genetic sequences have been suggested which also explain how RNA systems could have evolved. For example the difference between an active store of information and an active ribozyme may be splicing out of some or all introns.\textsuperscript{5} Experiments have also shown that short random sequences of RNA can have function.\textsuperscript{12} By demonstrating the density of reactivity within the range of sequences it is easier to imagine the evolution of an RNA world. Then we could presume that over time DNA evolved as a more stable store for the genetic information and proteins developed as more efficient enzymes, relegating RNA to an intermediate role in most cases.

A problem of this research is that findings are mostly conjecture as there is no fossil record for analysis and any precellular life is unlikely to have left any trace of its existence. The earth came into existence $4.5 \times 10^9$ years ago and the first evidence of cellular life is $3.6 \times 10^9$ years ago. The window for precellular life is further shortened by the fact that for much of earth’s early life it was subject to meteoric bombardment and geological instability.\textsuperscript{13-14}

An early experiment to simulate primordial conditions was the first to shed light on this problem. A sealed flask containing methane, ammonia, hydrogen and water was subjected to an artificial lightning bolt for several weeks. The electrical discharge was used to form free radicals from which a complicated mixture of compounds was made. Analysis of the mixture at the end of the experiment revealed the presence of 13 of the 20 amino acids essential to life.\textsuperscript{15} Subsequent experiments and theory have shown the possibility of purines and pyrimidines, required for DNA and RNA, being present.\textsuperscript{16-17}
There are still problems with these theories. The fragility of RNA and the lack of a practical mechanism whereby oligoribonucleotides were made in the primordial soup cast doubt over the existence of the RNA world.\textsuperscript{13,14,18}

1.1.3 Structure of DNA and RNA

1.1.3.1 Primary Structure

The basic structure of DNA and RNA is that of a linear polyester where phosphoric acid constitutes the diacid component and a substituted D-ribose or 2'-deoxy-D-ribose is the diol. All the phosphodiester linkages are 3'-5' in the normal primary structure. On each ribose unit at the 1' position is a nucleobase. These are the basis of the genetic code and come in two forms. There are the purines adenine (A) and guanine (G) and the pyrimidines cytosine (C) and thymine (T). In RNA uracil (U) replaces thymine.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{DNA_RNA_structure.png}
\caption{The primary structure of DNA (left) and RNA (right)}
\end{figure}

In descending order the bases shown above are Guanine, Thymine (Uracil on the right), Adenine and Cytosine.
1.1.3.2 Secondary Structure

The secondary structure of DNA is directed by the hydrogen bonding of the nucleobase on one strand with the corresponding base on the other. Thymine couples with adenine and cytosine with guanine. The two strands form a right-handed spiral around a central axis and run anti-parallel to each other, shown on the left-hand side of figure 3.

![Diagram of DNA double helix structure showing the base pairs]

Figure 3. DNA double helix structure showing the base pairs
The main secondary structures are the A-DNA and B-DNA forms which both have right-handed helices and Watson-Crick base pairs. The base pairs are shown on the right side of figure three, which also shows the major and minor grooves which run down the side of the helix. Other types of helix with left-handed spirals, hairpin loops and triplexes are also known. Intramolecular base pairing causes hairpin loops. Z-DNA has a left-handed helical structure as opposed to the right-handed structure of A-DNA and B-DNA. The difference between the A and B forms is in the conformation of the sugar unit. In B-DNA it is the C-2' carbon which is out of the plane of the other carbons whereas in A-DNA it is the C-3' carbon.

RNA has the added complexity of the hydroxy function at the 2' position and as a result cannot easily adopt the helical structures of DNA due to the extra oxygen atom making too close a Van der Waals contact. As a consequence RNA has no general structure and has greater chemical reactivity than DNA.

RNA molecules can have some helical domains but they will also have hairpins and loops and other less defined motifs. The Watson-Crick base pairs still strongly influence the three-dimensional structure but now other less stable pairings (G-U) are also involved. The exact three-dimensional structure of the RNA molecule will greatly influence its biological function and activity.

1.1.4 DNA Triplex

The first triple helix was discovered in 1957 when it was noticed that polyuridine formed a 2:1 complex with polyadenosine in the presence of MgCl₂. Later in 1963 it was found that polycytidine also forms a 2:1 complex either with polyguanosine at pH 6.2 or with one of the cytosine strands existing in the protonated form. This triplex formation can be made pH independent by using 6-oxocytidine in place of cytidine on the third strand. Triplex formation has since been shown to inhibit activity at the bound sequences. For example inhibition of the restriction
endonuclease cleavage enzyme *Ksp632* can be achieved with micromolar concentrations of an oligonucleotide capable of forming a triplex with protein binding sites which regulate specific genes\(^22\) opening up a world of applications.

The structure of the triple helix was confirmed in the 1970's with x-ray diffraction data. In both the above pyrimidine cases the additional strand binds in the major groove of the DNA double helix by Hoogsteen (HG) hydrogen bonds. Binding causes distortion in the shape of the double helix to give a deeper major groove to accommodate the new strand.\(^23\) Cooperative binding effects are observed for triplex formation at adjacent sites.\(^24\)

![HG type, parallel T-A: T](image1)

![HG type, parallel C'G: C](image2)

Figure 4. Hoogsteen hydrogen bonds
There are two binding motifs. The pyrimidine (Y-R:Y) type mentioned above which uses the HG bonds and the more recently discovered (1988) purine (R-R:Y) which uses reverse HG bonding. The common features of both motifs are the central purine base and the fact that two hydrogen bonds are formed with the double helix. Only two out of the eight possible combinations with a pyrimidine as the central base are accommodated with reasonable stability. These triplexes (G-T:A) and (T-C:G) only form one hydrogen bond with the double helix and are much less stable, limiting their usefulness.\textsuperscript{24-29} A single mismatch in the 15 base pair cleavage probe of the type [DNA-EDTA-Fe(II)] produces a lowering of efficiency by at least a factor of ten.\textsuperscript{23}

Figure 5. Reverse Hoogsteen hydrogen bonds
This limitation of oligonucleotide triplex formation has lead to the investigation of unnatural base components designed to selectively interact with thymine and cytosine.\textsuperscript{30-32} Attempts to simply miss out a single pyrimidine interruption in a purine tract by introducing an abasic group opposite the pyrimidine caused considerable destabilisation of the triplex. Therefore groups capable of selective hydrogen bonding to pyrimidines are needed. The problem arises largely from the fact that the pyrimidines have only one potential site for hydrogen bonding. Several potential solutions have been tried to increase the number of hydrogen bonds and thus improve triplex stability.

![Diagram of modified bases](image)

**Figure 6.** Attempts to improve pyrimidine strand binding,

*(Top)* shows a modified cytosine designed to bind to both bases of the pair for the unnatural triplet C\textsuperscript{*}-C:G.\textsuperscript{30} *(Bottom)* shows an attempt to add an extra hydrogen bond to a pyrimidine within the double helix for the unnatural triplet A-U\textsuperscript{*}:A.\textsuperscript{31}
One approach is to design non-natural bases for the third strand, which can hydrogen bond with both bases of the pair. An extension of this is to use modified pyrimidines with additional functional groups capable of hydrogen bonding.\textsuperscript{30,31} Covalently attached intercalating agents are often added at the end of a sequence to increase triplex stability. A third approach was discovered by chance when a modified base was found to interact with the pyrimidine strand not by hydrogen bonding but sequence specific intercalation.\textsuperscript{32} Yet another method was the development of (3'-3') linked oligonucleotides for alternate strand binding.\textsuperscript{33} A final approach is to leave nucleotides aside and design another triplex forming system.\textsuperscript{34-38}

Figure 7. Shows a bidirectional pyrimidine oligonucleotide covalently attached to EDTA Fe designed to cleave a 4.05 kbp plasmid at the site specific to the sequence. Cleavage was observed with no cleavage at partly homologous sites.\textsuperscript{33}
Chapter 1 Introduction

1.2 Applications of Synthetic Oligomers of DNA and RNA

The advance of automated solid phase synthesis of oligonucleotides and the advent of molecular cloning technologies have opened up many applications throughout biology, chemistry and biochemistry. For example in DNA manipulation, synthesised oligonucleotides can be employed as primers or even to produce unnatural genes. Using chemical or enzymatic methods, oligonucleotides rich in $^{13}\text{C}$ or $^{15}\text{N}$ can be produced. Oligomers produced in this way can then be used as probes to examine the interaction between proteins and specific nucleotide sequences. Using the phenomena of triplexes, oligonucleotides can be used to determine DNA sequences or for site specific mutagenesis, cleavage or blocking. This can be achieved by producing an oligomer specific to a site on the natural sequence and attaching a chemical reagent at the end of the nucleotide. For example an iron-containing porphyrin will cleave the target at a specific site and an intercalating agent will lock the triplex in place and competitively inhibit replication or transcription of the targeted gene. Figure 7 shows how an Iron-EDTA complex attached to an oligomer can be used for site specific cleavage of a DNA plasmid. Non nucleotide triplexes have also been investigated for the same applications.

Perhaps the most significant application to date of oligonucleotides has been their use in recombinant DNA technology. This technology was developed in the 1970's to produce proteins as an alternative to making them synthetically or extracting from a natural source. The genes required for a protein are made chemically then introduced to a micro-organism. The organism then produces an excess of the corresponding protein in large recoverable yields. An example using this technology would be the production of the glucose regulating protein, insulin, in 1982. The gene for the protein was introduced to *Escherichia Coli*, insulin was produced by the bacteria and subsequently extracted on a large scale.
1.2.1 Antisense

Potentially the biggest application of synthetic polynucleotides and especially their analogues in medicine is in the field of antisense and antigene. This area holds the promise of drugs of such high selectivity as to be devoid of side effects, drugs that would only attack viral RNA or mRNA and not the host cell. Such drugs would require much lower doses than drugs that target proteins since they have a smaller number of targets within the body. These new pharmaceuticals would not only be able to block specific cell functions and gene expression but also promote them. But perhaps the greatest advance of antigene drugs would be the ability to design a new drug based on the primary structure of the target without the need to be concerned with protein active site geographies and the problems associated with designing a drug for it.49

![Figure 8. Antisense translation inhibition](image_url)
In the first step of the production of a protein the cell transcribes the required piece of DNA into several copies of mRNA and then ribosomes make several copies of the protein from each mRNA, a process called translation. The concept behind antisense drugs is to introduce a strand of antisense DNA to hybridise with the target mRNA and block translation. An antigene drug would bind with a specific site on the DNA of a cell and form a triple helix and block transcription.

In the formation of triplexes a strand of complementary DNA or DNA analogue binds in the major groove of the double helix structure. The triplex is stabilised by Hoogsteen hydrogen bonds. Hoogsteen hydrogen bonds are the same as Watson and Crick base pairs but have a third base positioned in the major groove and they are (C-G-C$^+$) and (T-A-T) where the third letter represents the extra nucleobase. Since they require the extra cytosine to be protonated these bonds are pH sensitive, forming at pH 6.2 and below.

![Figure 9: Triplex transcription inhibition](image)

Therefore, if the sequence of DNA is known and conserved then a drug would be very easy to design and potentially any part of the DNA could be targeted. However with RNA, since the structure is less predictable than DNA, similar problems to those of proteins arise. Namely not all of the structure will be accessible, some for example may have fixed structures incapable of triplex formation and therefore trial and error, computer modelling, crystal structures and other techniques will need to be employed to produce an active compound. The DNA triplex has also been exploited in terms of producing a higher affinity ligand for a single strand of DNA. By covalently binding together the two strands required to make the triplex, either at
both ends to form a circle or only at one to give a chain which folds over itself to form a loop, a new high affinity sequence specific ligand is created.\textsuperscript{51} Binding is tighter than with standard Watson-Crick base pairing, especially with the looped oligonucleotides and improved inhibition of enzymatic DNA synthesis has been reported.\textsuperscript{51}

There is precedence for the antisense approach in the natural world. Cells create their own mRNA’s which are used as repressors to gene expression and therefore regulate the levels of proteins in cells. There are several defined mechanisms by which these systems work. In some cases the target mRNA is permanently altered or destroyed.\textsuperscript{52-53} This also shows how an active antisense drug could promote gene expression by blocking the natural antisense inhibitor. However there is no such natural system using the antigene approach.

Statistically an oligonucleotide of between 11 and 15 residues would be specific to one sequence in the human genome. The spread of length arises from the fact that there are more A-T base pairs than G-C. Therefore a G-C rich sequence is less common and a shorter sequence would still be unique whereas an A-T rich sequence would need to be longer.\textsuperscript{54} In 1978 a synthetic oligodeoxynucleotide containing 13 residues was made and was the first to be used to selectively inhibit a virus by hybridising with the viral RNA.\textsuperscript{55}

However there are significant obstacles to be overcome if a practical drug is to be designed using these concepts. Measures need to be taken to stop the nucleotide sequence from being broken down by the cells’ own enzymes and lengthen the drug’s half-life in the body. One way to overcome these problems is to create a situation where the required oligonucleotide is continually produced within the target cell. Another solution is to modify the oligonucleotide to make it unrecognisable to the enzymes that breakdown such compounds. Backbone modifications at the phosphorus and at the sugar moiety as well as alterations of the bases have all been investigated. Generally improved resistance to nucleases is observed at the cost of poorer rates of triplex formation and hybridisation.\textsuperscript{54-61} Oligophosphoramidates
perform better than oligophosphodiester$^{62-63}$ and molecules where the phosphate backbone has been replaced by a peptide, N-(2-aminoethyl)glycine backbone perform well. These peptide nucleic acids (PNA's) are resistant to the nucleases that limit the half-life of oligonucleotides and are sufficiently different from $\alpha$-amino acid peptide bonds to be resistant to proteases and peptidases. PNA's were originally designed for sequence specific triplex formation and as such are good structural mimics of DNA. They can form double helices with DNA, RNA and PNA and they can bind with double helices by strand displacement.$^{64}$ PNA's have been shown to exhibit antisense activity in prokaryotic cells, with *E. Coli*, and eukaryotic cells, by injections into rat brains.$^{65}$

Drug delivery is another obstacle. Uptake by and transport within cells, moving through the blood brain barrier or transport into the nucleus for antigen gene therapy are all aspects of the same problem. Oligonucleotides delivered using liposomes, low-density lipoproteins, a composite system consisting of a stearyl-poly-L-lysine$^{66}$, biodegradable polyalkyl cyanoacrylate nanoparticles and other systems have all been investigated as potential solutions to this problem.

Several areas of medical research promise to yield antisense drugs in the very near future and some already have. The first antisense drug, Vitravene®, was launched in the USA in October of 1998 by the Isis pharmaceutical company after clearance from the FDA in August of the same year.$^{67}$ In August the following year the European commission granted a community marketing authorisation for Vitravene®. The drug works against the AIDS related eye infection cytomegalovirus retinitis. Human cytomegalovirus (HCMV) is a very common virus that is latent (present but inactive, like chicken pox) in most people. However those with compromised immune systems such as AIDS patients and transplant patients are vulnerable. HCMV attacks the light sensitive tissue of the eyes and ultimately leads to blindness, often in both eyes. Treatment involves injecting antisense oligonucleotides directly into the patient's eyeball. By targeting the mRNA's responsible for two proteins essential to the virus the sight of patients suffering from HCMV can be saved. RNA and DNA molecules can have physiological effects in other ways. Aptamers$^{68,69}$ are nucleotides
that can bind and inhibit proteins. Although there is debate as to the mechanism of
drugs like Vitravene™ an antisense mode of action seems the most likely.

Cancer is another area where considerable effort has been directed to produce an
antisense drug. One approach is to activate the natural tumour suppresser gene p53
by targeting its regulation. One target on this method is the oncoprotein MDM2 that
downregulates the expression of p53 by binding to the transactivation domain of p53
blocking transcription. In studies where MDM2 expression was inhibited in different
tumour types by antisense oligonucleotides, increased levels of p53 were noted
with concomitant depletion of the tumour mass. Other pathways have also been
investigated and targeted in attempts to encourage growth inhibition and apoptosis.

Further examples of the applicability of antisense technology are in the treatment of
genetic disorders and in modulating central nervous system functions. The classical
drugs for blocking the brain dopamine receptors bind to the receptors themselves,
whereas the new antisense drugs are designed to bind to the receptor-coding mRNA
and stop the production of the receptor in the first place. There is a range of
dopamine receptors encoded by different genes and the specificity inherent in the
antisense approach has the added advantage of being able to determine some of the
neurophysiological functions of individual receptors. Dominant genetic disorders
like Huntingdon’s disease and Marfan’s syndrome also appear to be amenable to the
antisense approach.

Once the problems of cost and delivery have been dealt with for one drug, the
lessons learned should potentially be easily transferred to every other possible
antisense or antigene drug. It is then the role of the organic chemist to design more
efficient and faster syntheses of oligonucleotides for mass screening and mass
production of drugs. At present each milligram of a typical unmodified
oligonucleotide cost around US$20. Someone weighing 50kg requires a dose costing
in the region of US$1000. If antisense drugs are to be widely used chemists need to
overcome these hurdles.
1.3 Chemical Synthesis of Oligodeoxyribonucleotides

Today DNA and RNA sequences can be synthesised either chemically on solid phase or using DNA strands as templates. The development of chemical synthesis started with solution chemistry but has since moved on to solid phase synthesis and several different approaches have been used. The variation between these competing methods lies largely in the type of activated phosphorus unit used.

Oligodeoxyribonucleotides possess a range of functionalities that complicates their synthesis. In the case of oligoribonucleotides the extra hydroxy group at the 2' position further complicates matters. The aim of oligonucleotide synthesis is normally to join the 5'-hydroxyl position on one nucleoside with the 3' position on another via a phosphodiester linkage to form a phosphodiester bridge, the backbone of DNA. In the case of RNA it is possible to have branched oligomers using the 2' position and occasionally DNA and RNA oligomers will require a 3'-3' linkage. Because of the wide range of functionalities present, selective blocking and deblocking strategies must be employed. For a completely orthogonal approach two different protecting groups for hydroxyl groups as well as groups for the primary amino groups and oxygens of the phosphate group are all needed.

Other problems in producing oligomers are the sensitivity of the glycosidic bonds of the purine and pyrimidine groups with the sugar group, which means only mild acids can be employed. Stereochemical and neighbouring group effects affect yields as the chain grows. With RNA the 2' protection causes even more steric problems further affecting yields. Protecting groups need to be removable under mild conditions and finally the product needs to be separable from truncated sequences. It’s clear that a robust and well designed strategy is needed to successfully produce oligomers of RNA and DNA.
The first nucleotide was synthesised by Todd\(^8\) in 1955 and since then considerable efforts have been made to refine and improve the chemistry through phosphodiester, H-Phosphonate and phosphoramidite methods.

### 1.3.1 The Phosphodiester Method

The phosphodiester method is perhaps the simplest of all coupling techniques for use in nucleic acid synthesis. Introduced in 1958, and further developed by Khorana \textit{et al}\(^8\), it was first used for the synthesis of a dinucleotide and then developed to synthesise larger oligomers.

The phosphodiester method involves the condensation of deoxynucleotides where the first nucleotide has a protected 5' hydroxyl and a free hydroxyl at the 3' position (1) ready to react as shown in figure 10 and the second nucleotide has a phosphate group at the 5' hydroxyl with a protected 3' hydroxyl (2). The exocyclic functional groups on the bases are also protected and the hydroxyl protecting groups for the two nucleotides are different. A condensing reagent such as dicyclohexylcarbodiimide (DCCI) or 2,4,6-triisopropylbenzenesulphonyl chloride (TPSCI) is then used to facilitate reaction between the nucleotides to produce the protected dinucleotide (3).

![Figure 10. Khorana’s phosphodiester approach](image)

The chain can be further extended by removing the 5' trityl protection followed by phosphorylation and introduction of another nucleotide with a protected 5' position.
and a free 3' position. However there are problems with this method. The negative charge on the phosphate group causes problems with solubility. Yields deteriorate and reaction times increase as the chain grows, thus making this method impractical for larger polynucleotides. In addition separation of the various condensation products required highly time consuming ion-exchange chromatography.

Despite these problems Khorana et al successfully produced the genes of alanine tRNA\textsuperscript{83} and of tyrosine suppressor tRNA\textsuperscript{84} but they needed to use block condensation methods. In these syntheses they produced short oligomers of 8 to 12 residues using the phosphodiester method. These oligomers had free 3' and 5' hydroxyis and those of the complementary strand overlapped by four or five residues to each side of those oligomers for the coding side. DNA polymerase enzymes were then employed to link together the oligomers to produce the whole 50 base pair gene.

1.3.2 Phosphotriester method

Although the phosphotriester method can be considered a development of the phosphodiester method, Michelson and Todd\textsuperscript{81} in fact used it first in 1955 to produce a dinucleotide. The advance from the diester method is the introduction of a phosphate protecting group. Removing the negative charge removed many of the problems of the diester method namely; solubility, and poor yields.

The original Michelson and Todd phosphotriester approach shown in figure 11 involved the activation of 5'-O-acetyltymidine-3'-O-benzyl hydrogen phosphonate (4) using N-Chlorosuccinimide (NSC) to produce 5'-acetyl-3'benzyolphosphochloridate (5). This activated molecule was then reacted with a nucleotide with a free 5' position and a protected 3' (6) to produce the corresponding dinucleotide phosphotriester (7).
Chapter 1 Introduction

Improving the phosphate and hydroxyl protecting groups has further developed this method. In 1965 Letsinger \textit{et al}^{85-88} introduced the \( \beta \)-cyanoethyl phosphate protecting group. Using the solid phase synthesis principle they succeeded in a stepwise synthesis of oligonucleotides up to tetramers. In the solid phase reactions cytidine was used as the anchor bound to the styrene-based solid support \textit{via} the amine group of the base. For oligomers larger than this, solution chemistry was required to achieve high enough yields.\textsuperscript{89,90}

![Figure 11. The Michelson and Todd phosphotriester approach](image1)

![Figure 12. The Letsinger phosphotriester approach](image2)
Oligodeoxynucleotides containing nucleosides other than thymidine were produced.\textsuperscript{88} The first step of Letsinger's approach, shown in figure 12, involved the phosphorylation of a 5'-monomethoxytrityl (MMTr) protected deoxyribonucleotide (1) with β-cyanoethyl protected phosphate and 2,4,6-trimethylbenzenesulfonyl chloride (MScI) to give the phosphodiester (8). The next step was the addition of thymidine in the presence of 2,4,6-triisopropylbenzenesulfonyl chloride (TPSCl) to give the fully protected dinucleotide phosphotriester (9). Addition of aqueous acetic acid to remove the monomethoxytrityl group (10) followed by treatment with aqueous ammonia to remove all remaining protecting groups gave the fully deprotected dinucleotide (11).

One problem with this approach is the production of side products, which are difficult to separate from the desired product. Incomplete or over phosphorylation is the major reason for the production of 3'-3' and 5'-5' by-products. In an effort to eliminate these undesired products Letsinger \textit{et al.}\textsuperscript{89} developed the β-benzoylpropionyl 3'-hydroxy protecting group. This group is removed using hydrazine hydrate in pyridinium acetate, reaction conditions mild enough to leave the phosphotriester unaffected.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{phosphate_protection.png}
\caption{Phosphate protection}
\end{figure}

(12) β-cyanoethyl (13) trichloroethyl (14) 2-chlorophenyl

Other chemists contributed to the search for better protecting groups, some of which are shown in figure 13. Eckstein \textit{et al.}\textsuperscript{91-93} introduced the trichloroethyl phosphate protecting group, Reese \textit{et al.}\textsuperscript{94-96} and Van Boom \textit{et al.}\textsuperscript{97} developed aryl phosphate protecting groups with electron withdrawing substituents for increased base lability. The best of these was found to be the 2-chlorophenyl group. It was
stable during synthesis and could be removed under mild conditions with minimal disruption to the oligonucleotide. Reese also suggested methoxytetrahydropyranyl as an alternative to the trityl group for 5’ protection.

In 1973 Cramer et al. introduced a two step sequential procedure shown in figure 14. In this method a nucleoside protected at the 5' position with the acid labile dimethoxy trityl group (DMTr) was first phosphorylated with 2,2,2-trichloroethylphosphodiimidazolidate then treated with triethylamine and water giving (16). β-Cyanoethanol is then added with TPSCI to protect the phosphate group to give the fully protected phosphotriester (17). Treatment with dilute aqueous trifluoroacetic acid (TFA) removes the DMTr to give (18). Compounds (16) and (18) are then condensed with TPSCI to give the protected dinucleotide (19). Treatment with TFA, then sodium hydroxide and finally Cu/Zn in ammonia yields the fully deprotected dinucleotide.

Figure 14. Cramer’s approach
In parallel with progress in protecting groups, the phosphorylation and condensing reagents were also developed. Phosphorylation was improved by addition of monofunctional reagents such as the phosphorochloridates where two of the oxygens are protected.\textsuperscript{101}

![Figure 15. Coupling reagent MSNT](image)

Improving the activation agent meant improving yields and minimising the sulfonylation of the free 5’ position of the condensing reagents. In 1975 Narang \textit{et al}\textsuperscript{102} introduced arylsulfonyltriazoles later refining this to arylsulfonyltetrazoles.\textsuperscript{103} In 1978 Reese \textit{et al} introduced the use of 2,4,6-trimethylbenzenesulfonyl-3-nitro-1,2,4-triazole (MSNT) (21).\textsuperscript{104} The fact that increasing steric bulk decreases the
sulfonylation side reaction and lead to other adaptations where arylsulfonyl triazoles and tetrazoles were used.\textsuperscript{105}

The rate of reaction for the condensation step can be increased by the addition of a nucleophilic catalyst. 1-Methylimidazole when introduced to the reaction produces a highly reactive intermediate (23) and this further reduces coupling times.\textsuperscript{106,107} Other imidazoles and tetrazoles have been used in this way, but since it is more acidic, the tetrazole is the better activator.\textsuperscript{106,107} Figure 16 shows how 1H-tetrazole is incorporated in the synthesis as a coupling catalyst.

All these refinements in protecting groups and reagents have now improved the yields and decreased coupling times for the phosphotriester approach beyond those of the phosphodiester method. Coupling steps originally taking several hours\textsuperscript{85} were reduced to five minutes with these advances.\textsuperscript{108} The synthesis of a 20 residue oligomer could now be accomplished in under 8 hours.\textsuperscript{109}

However there are still problems with side reactions, which ultimately limit the applicability of the phosphotriester method. Even with sterically hindered condensing reagents the competing side reaction of sulfonylation of the incoming nucleoside accounts for about 1\% of the reaction. Although not a problem for shorter sequences yields of around 97\% means that synthesis of longer sequences becomes impractical.\textsuperscript{110} Another side reaction involves the phosphorylation and substitution with triazole or tetrazole of the $O^6$- position of deoxyguanosine.

Despite these problems the phosphotriester method has been used successfully to produce large and biologically active oligonucleotides.\textsuperscript{111}
1.3.3 The Phosphite-triester and Phosphoramidite Methods

The phosphite-triester method, also known as the phosphoramidite method, was introduced by Letsinger \textit{et al} in 1975 and is based around the high reactivity of phosphorochloridite reagents with alcohols to give phosphite triesters. Coupling times between two nucleotides is greatly reduced from hours to minutes due to the increased reactivity of the P_3 system over the P_5 system. The resulting phosphite-triester is then easily oxidised up to the natural linker with aqueous iodine.

Letinger’s phosphite-triester approach, shown in figure 17, starts with 5′-O-phenoxyacetyl thymidine (24) reacted with \textit{ortho}-chlorophenyl phosphodichloridite in the presence of 2,6-lutidine to give the phosphitylated nucleoside (25). Then 3′ monomethoxytrityl protected thymidine (26) is added to give the phosphite dinucleotide (27) within 20 min at -78°C. Iodine and water are then used to rapidly oxidise the phosphite to give the protected dinucleotide (28). After removal of the phenoxyacetic ester the dinucleotide is ready to be rephosphitylated. However it was
found that the phosphate link was unstable with respect to the phosphitylating agent. The switch to 2,2,2,-trichloroethylphosphorodichloridite improved things enough to allow the production of tetramers.\(^{112}\)

Further refinement targeted the phosphate protecting group. Ogilvie \textit{et al.}\(^{113}\) investigated a range of groups including 2,2,2-tribromoethyl, \(p\)-chlorophenyl, 2-phenylethyl, 2-\(p\)-nitrophenylethyl, benzyl, methyl and \(\beta\)-cyanoethyl and found methyl and \(\beta\)-cyanoethyl to be the most effective. These groups remain the most widely used today. Matteucci \textit{et al.}\(^{114,115}\) demonstrated the solid phase synthesis of a dodecamer using methyl protection and tetrazole as a coupling catalyst. Coupling times to produce dimers were under two minutes with yields in excess of 90\%.\(^{114}\)

Although a successful method and a suitable one for solid phase synthesis, the phosphite-triester method using phosphochloridites still has problems. During preparation of phosphoramidites small amounts of 3'-3' dinucleotide are formed, causing purification problems and reduced yields. Phosphochloridites are very moisture, temperature and air sensitive necessitating low temperatures (-70\(^\circ\)C) and dry, inert conditions for their handling. In 1981 Beaucage and Caruthers introduced the deoxynucleotide phosphoramidites as an improvement. These phosphoramidites are more stable to hydrolysis and stable at room temperature while still being easily activated, forming the dinucleotide in high yields with minimal side reactions.\(^{116}\) This development greatly improved the transition from manual to semi-automated procedures.\(^{117}\)

In Caruther's phosphoramidite method, shown in figure 18, a 5'-dimethoxytrityl protected nucleotide (15) is reacted with chloro-\(N, N\)-dimethylaminomethoxy phosphine in the presence of \(N, N\)-diisopropylethylamine to give the phosphoramidite (29). This can be isolated as a powder and is stable enough to be stored. This compound is activated with tetrazole (30) and then coupled to 3'-\(O\)-levulinylthymidine to give the phosphite triester dinucleotide (31) in quantitative yield. Aqueous iodine is then used to oxidise to give the protected phosphate dimer (32).
This method was further improved by research into different dialkylaminomethoxy phosphines by McBride and Caruthers in 1983. The phosphitylating agents diisopropylaminomethoxy phosphine (33), morpholinomethoxy phosphine (34), pyrrolidinomethoxy phosphine (35) and 2,2,6,6-tetramethylpiperidinomethoxy phosphine (36) were evaluated. The diisopropylamino and morpholino derivatives were stable to silica gel chromatography with acetonitrile and, because of their stability and ease of handling, they have become the reagents of choice.
The removal of the methoxy phosphorus protection was still seen as a problem in this approach and in 1983 Sinha et al.\textsuperscript{122,123} introduced the β-cyanoethyl group. The advantage the β-cyanoethyl has over the methoxy protection is in its removal. Concentrated ammonia is used to remove the base protection and under these conditions the β-cyanoethyl group is also removed whereas the removal of methoxy protecting group requires another step using thiophenol. Other phosphate protecting groups have also been tried, including 2,2,2-trichloro-1,1-dimethyl (37),\textsuperscript{124} p-nitrophenylethyl (38)\textsuperscript{125} and allyl (39).\textsuperscript{126} Only the β-cyanoethyl group (40) meets all the criteria and therefore remains the protecting group of choice.

The only drawback of the β-cyanoethyl group is the relatively high production costs of the reagent. One alternative that has been suggested by Ravikumar et al.\textsuperscript{127} is the cyanobutenyl group (41). This group has all the advantageous properties of the β-cyanoethyl group of good phosphitylating properties and easy removal and has the added benefit of being 60% cheaper to produce.\textsuperscript{127}

A further development of the phosphoramidite approach was introduced by Uznanski et al.\textsuperscript{128} in 1987. In this approach the oxidation of the phosphite triester links is performed all at once at the end of the synthesis rather than after each reaction cycle.
In this approach, shown in figure 21, chlorodimorpholinophosphine (42) is reacted with a 5’ dimethoxytrityl protected nucleoside (15) to give the 5’-O-dimethoxytritylnucleoside-3’-O-phosphorodimorpholidite (43). This reagent is then activated with tetrazole and coupled to a solid support bound nucleoside to give the dimer (44). Mild acid treatment is used to remove the trityl 5’ protection and cleave the P-N bond ready for the addition of further monomer.

![Figure 21. Uznanski’s phosphoramidite approach](image)

The main advantage that the phosphoramidite method has over rival methods mentioned is its extremely short reaction times and high coupling efficiencies due to the highly reactive, but slightly unstable, P₃ reagents used. The phosphoramidite method is the only viable one for oligomers longer than 50 residues.
1.3.4 The H-Phosphonate method

This method was first described by Todd et al in 1957\textsuperscript{129} but was abandoned in favour of the phosphodiester and triester approaches. However in more recent years several laboratories have reinvestigated the H-phosphonate method for DNA and RNA synthesis.\textsuperscript{130-136} This renewed interest stems from the fact that this method offers the possibility of combining the advantages of the phosphotriester method, speed and efficiency of reaction due to the $P_3$ reactivity, with the stability of intermediates intrinsic in the phosphotriester approach.

In this method, outlined in figure 22, the H-phosphonate (46) is activated by rapid reaction with pivaloyl chloride to give a mixed anhydride intermediate (47). This is then coupled to a 5' unprotected nucleotide bound to a solid support (48) to give the dinucleotide (49). Mild acid treatment is employed to remove the trityl protection so that another monomer can be attached. Oxidation of all the internucleotide linkages is performed at the end of the synthesis using aqueous iodine. By avoiding the need for phosphinite protection reaction times are improved and the stability of the
phosphorus unit is also improved. H-phosphonate chemistry is also cheaper than phosphoramidite chemistry although for routine uses the phosphoramidite approach is still preferred.

1.4 Solid Phase Synthesis (SPS) of Oligodeoxyribonucleotides

Merrifield\textsuperscript{137} first developed solid phase synthesis in 1963 and the new approach virtually revolutionised peptide synthesis. The technique was quickly extended to nucleotides. Letsinger\textsuperscript{86} produced the first trinucleotides using this approach in 1965. Since then yields and reaction times have improved as the chemistry has been developed. The application of solid phase synthesis (SPS) technique in the production of DNA and RNA oligonucleotides is now widely accepted, usually utilising a phosphoramidite chemistry approach synthesising the polymers in the 3'-5' direction.

Essentially the SPS method is the heterogeneous reaction between a growing polymer, bound \textit{via} a linker to an insoluble solid support, and a solution-based monomer. At the end of each of the reactions performed it is easy to separate the monomers and other reagents in solution from the growing polymer by washing and filtration. This allows the use of large excesses of monomers and reagents thus optimising the yields. Furthermore, the solid support remains in the same reaction vessel through the course of the synthesis allowing for easy automation of the whole process.
1.4.1 The Solid Support and Linker

It is essential that solid supports must be inert and insoluble under all the reaction conditions to be used in the synthesis. Swelling properties of the support in solvents also influence suitability and the polymer must have a mechanically stable form to facilitate filtration. It must also have a functional group through which to attach the growing polymer via a linker. Merrifield settled on a copolymer of styrene and divinylbenzene,\(^\text{137}\) which is still in use today. Another widely used solid support is the controlled pore glass (CPG) support.\(^\text{138}\) These supports are manufactured to have different particle sizes between 500 and 1000Å in pore size. Automated synthesis methods typically use scales of between 10 and 50 micromol of nucleotide per gram of polymer. Lower level loadings give results that are difficult to reproduce and higher levels suffer from steric problems as the polymer grows.

In the SPS synthesis of oligonucleotides, the first nucleotide is normally attached through the 3' position to the solid support via a linker to a functional site on the solid support. The linker is incorporated to move the site of reaction away from the bulk of the polymer to ensure accessibility for reagents. The linker is therefore designed to be long, flexible and cleaved in base.

\[ \text{TrO-} \begin{array}{c} O \end{array} \text{B} + \text{Long Chain Alkylamine - CPG Support} \]

\[ \xrightarrow{\text{(46)}} \]

\[ \text{TrO-} \begin{array}{c} O \end{array} \text{B} + \text{Long Chain Alkylamine - CPG Support} \]

\[ \xrightarrow{\text{(47)}} \]

Figure 23. Attachment of the first monomer to CPG
1.4.2 Solid phase synthesis reaction cycle

Figure 24 shows a basic SPS oligonucleotide synthesis reaction cycle using the phosphoramidite approach. In step 1 a small column is packed with resin loaded with the first nucleotide linked through the 3' position and protected at the 5' position. This column is then inserted into the DNA synthesiser through which reagents and solvents are passed in an automated sequence. In step 2 mild acid is added to remove the 5' trityl protection. In step 3 the second phosphoramidite monomer is then added and activated with tetrazole to form the phosphite triester. In step 4 to remove the unreacted 5' positions from participating further acetic anhydride is added to cap them. In step 5 the phosphite triester is the oxidised to the phosphotriester using aqueous iodine. The cycle is then repeated (Steps 2-5) until the sequence is finished. The oligonucleotide is then cleaved from the resin under basic conditions, which simultaneously removes the exocyclic base protection.

1.4.3 Purification of Oligomers

Despite the high, near quantitative, yields for each step in the phosphoramidite SPS method, the finished product cleaved from the resin is generally not very pure. Since not every step goes to completion there will be many truncated sequences. The ammonium salts of the removed protecting groups can also often contaminate the product. The capping step is introduced to minimise the problem of failed coupling reactions. After each coupling reaction every free 5' positions is capped with an acetate group to ensure that it takes no further part in any of the synthesis cycle. When the polynucleotide is cleaved from the resin using base all protecting groups, including the acetates on the truncated sequences, are removed except for the acid labile trityl protection of the product sequence. The product sequence is the only sequence with the trityl group and the majority of the truncated sequences will be sufficiently different in size from the product to be separable by chromatography.
Figure 24. Solid phase general reaction cycle
Two effective separation methods have proved popular for the purification of the oligonucleotides; electrophoresis and chromatography.

Polyacrylamide gel electrophoresis (PAGE) is a highly sensitive method that uses charge to separate oligomers and can be used to separate fragments with only one charge difference. A polymerised, cross-linked acrylamide gel is held between two glass plates with each end immersed in buffer chambers containing an electrolytic salt solution through which an electric current is applied. The charged oligonucleotide fragments then move through the matrix at a speed inversely proportional to their mass although shape, size and hydrophobicity can also have an influence. UV can be used to follow the progress of the sample through the gel, however this requires the presence of standard samples for comparison. There are some drawbacks to PAGE in that it is only amenable to small scale work, product recovery can be low and it is very time consuming.

Ion exchange chromatography also separates compounds on the basis of charge and mass and in oligonucleotides that basically means the length of oligomer and any protecting groups still attached. Separation is achieved by slowly increasing the ionic strength of the mobile phases through a macroporous stationary phase. Truncated sequences of lower mass are eluted first with the desired oligomer being eluted last. This procedure is carried out after the 5' protection has been removed. Ion exchange columns have a higher capacity than reverse phase columns but their limitation is that compounds are separated solely on the basis of the charge to weight ratio and there is no distinction on other criteria. Ion exchange is often used before rp-HPLC when high purity is required.

Reverse phase high performance liquid chromatography (rp-HPLC) is normally carried out with the trityl groups still protecting the 5' position. After the final monomer has been coupled, the phosphorus bridges have been oxidised and the oligomer has been cleaved from the polymer by aqueous ammonia all protecting groups except the base stable trityl will have been removed. All the failure sequences will have free 5' positions and the only compound retaining the terminal 5' trityl
protection is the desired sequence. The hydrophobic nature of the trityl moiety compared to the more hydrophilic truncated oligonucleotides allows the product sequence to be more easily separated. As the oligonucleotide increases in length the difference in polarity enforced by the trityl group diminishes. In rp-HPLC with oligonucleotides, a water to acetonitrile gradient is usually used. Under these conditions the truncated sequences are eluted first with the lipophilic tritylated oligonucleotide being eluted last.

An alternative application of this approach has also been shown to work. Instead of using the lipophilic properties of a trityl-protecting group as the basis for separation, failure sequences are capped with a lipophilic group that will not be removed under resin cleavage conditions. As this cap need not be removed its stability to reaction and purification conditions can be ensured. After the 5' trityl protection of the desired oligonucleotide is removed, the desired product will be the only oligomer without a lipophilic group attached.139

A simplification of rp-HPLC is to use polymeric reverse phase cartridges140-141 for rapid and inexpensive purification. Using resins stable to a wide pH range the ammonia solution used in the deprotection can be added directly to the cartridge. Failure sequences are eluted then the trityl group is cleaved by the addition of acid and the product sequence is then eluted.

1.4.4 Protecting groups for solid phase synthesis

Developments in solid phase oligodeoxyribonucleotide synthesis have involved, in the main, the development of orthogonal protecting group strategies. An orthogonal protecting group strategy is one where different protecting groups can be added or removed without affecting protecting groups covering other functional groups. In the case of oligonucleotide synthesis this means using a protecting group for the 5' alcohol which can be removed without affecting the phosphorus or exocyclic protecting groups. The different types of phosphorus coupling strategies and
phosphorus protection have already been covered but the other protecting groups for the 5' primary alcohol, the exocyclic bases (and the 2' secondary alcohol in oligoribonucleotide synthesis) have been developed which have led to much improved yields and greater flexibility of the methodology.

It should be noted that protecting groups need to be designed with several factors in mind. Firstly they must be easily introduced with high regiospecificity. Secondly the conditions required to remove the group must be sufficiently mild as to leave the internucleotide and the glycosidic bonds unaffected. The groups also need to be designed to withstand reaction conditions during synthesis and conditions intended to remove other intermediate protecting groups. If possible protecting groups should be designed to confer other properties to the oligonucleotide they are attached to. Increased lipophilicity increases the ease of purification and increased stability improves the storage properties of the intermediates. Another extra attribute that a protecting group could confer is the addition of a specific chromophoric label that enables spectroscopic detection of protected compounds.

The orthogonal protecting group strategy has lead to the development of two categories of protecting groups. The first of these are the permanent protecting groups. These are groups designed to stay on the oligonucleotide throughout the solid phase synthesis cycles and are removed only at the end of the synthesis. The functional groups covered in this way are the exocyclic groups, the 2' ribose hydroxyl in RNA synthesis and phosphorus protection. These groups are usually designed to be removed at the same time as the oligomer is cleaved from the solid support to minimise the number of steps. The second types of protecting groups are the temporary protecting groups which are removed as part of the synthesis cycle. The trityl protection of the primary alcohol lies in this category.
1.4.4.1 **Exocyclic base protection**

Adenine, cytosine and guanine bases each have a primary amine group that has to be protected during oligonucleotide synthesis but thymidine and uracil bases do not require any protection. The amino groups have to be protected throughout the synthesis and protection is removed at the same time as the oligomer is cleaved from the resin. The amides of dG, dT and U may also have to be protected to avoid side reactions with coupling reagents (e.g. sulfonylation and phosphitylation).\(^{142-144}\)

![Figure 25. Nucleic Acid protection](image)

(48) iBu-dG, (49) Bz-dA, (50) Bz-dC, (51) dT.

The most common way of protecting base amino groups is to convert them to their amides, usually benzoyl (Bz) and isobutryl (iBu). Figure 25 shows each of the bases used in this research with their protection. For the production of more sensitive oligomers more labile amino protection is sometimes required. N-phenoxyacetyl derivatisation can be used for this purpose with all the bases except guanosine since N-phenoxyacetyl 2'-deoxyguanosine forms a gelatinous precipitate in acetonitrile and, if used, needs DMF to be added as a co-solvent. This is complicated by the fact that DMF frequently has free amine contaminants that interfere with the reaction.

In recent years coupling methods have been developed which avoid the need for nucleoside base protection\(^{145-147}\) in both the H-Phosphonate and the phosphoramidite methods. In parallel the photocleavable nucleobase protecting groups such as 6-
nitroveratryloxycarbonyl (NVOC) (52) and 2,2'-bis(2-nitrophenyl)ethoxycarbonyl (diNPEOC) (53) have also been used in the synthesis of base sensitive products. Both these protecting groups have been used in the synthesis of S-pivaloylthioethyl (t-Bu-SATE) (54) phosphotriester linkages shown in figure 26.\textsuperscript{148}

![Figure 26. Nucleic Acid photocleaveable protecting groups (52) NVOC and (53) diNPEOC, (54) The base sensitive t-Bu-SATE phosphate linker](image)

A long term problem associated with the handling and synthesis of DNA and RNA oligonucleotides is depurination and to a lesser extent depyrimidation. Depurination or depyrimidation usually results in chain cleavage at the apurinic, apyrimidinic site. The acid catalysed hydrolysis proceeds with a rapid initial protonation of the base moiety (N1 position for adenine and N7 for guanine). A second proton can then be taken up to form the dication (N7 position for adenine and N3 for guanine). The rate-limiting step is then the cleavage of the cation or dication to give the free purine base and a resonance stabilised glycosyl carbonium ion.\textsuperscript{149-152} Initially Lewis acids such as ZnBr\textsubscript{2} were used to limit depurination during the acidic cleavage of 5' protection. The improvement in reducing depurination was achieved at the expense of reaction times. Attempts to design exocyclic protecting groups to limit this reaction have been made as have attempts to optimise conditions for this step using protic acids.\textsuperscript{153-155}

Depurination is a natural occurrence and can be found in living cells. Depyrimidation also occurs but about one twentieth of that rate. Depurination is also of great interest in the field of biomolecular archaeology. This is the relatively young area of research where DNA and protein fragments are extracted from archaeological
remains to get information on genetic disease, migration and evolution. DNA degradation is the limiting factor governing the age to which DNA can be meaningfully interpreted, depurination being the major process. \(^{156}\)

\[
\begin{align*}
\text{(55)} & \\
\text{(56)} & \\
\text{(57)} & \\

\end{align*}
\]

Cleavage at point of depurination

Figure 27. Acidic depurination

1.4.4.2 Protection of the 5' position

Protection of the 5' ribose hydroxyl primary alcohol has changed little over the years. The mono and dimethoxy trityl groups introduced in the 1960's by Khorana et al\(^{157-158}\) continue to be widely used. The para-methoxy groups were introduced to the trityl system to improve the acid lability of the protecting group after it was found that the triphenylmethyl ethers were too stable to the acid conditions intended for their cleavage. The addition of a single methoxy group increases lability by a factor of ten and a second para methoxy group improves lability again up to a point suitable for solid phase synthesis.

4,4'-Dimethoxytrityl (DMTr) protection offers several other advantageous properties that have ensured its continued use. Even under mildly acidic conditions deprotection is fast and removal can be easily monitored using visible spectroscopy.
Under these conditions depurination is minimised. Additionally the steric bulk of the DMTr group ensures selective reaction with primary alcohols. The hydrophobicity of the three benzene rings increases the hydrophobicity of the protected nucleotide or oligomer to make purification by chromatography or solvent extraction much easier.

However this purification advantage diminishes with increasing oligomer chain length and once oligonucleotides get beyond thirty bases in length the difference in rp-HPLC retention time due to DMTr becomes negligible. Attempts have been made to increase the hydrophobicity of terminal protection and thus extend the purification advantage into longer oligonucleotides. Selinger and Gortz\textsuperscript{59} adapted MMTr by replacing the methoxy with longer alkoxy groups (58). Letsinger and Finnan\textsuperscript{160} substituted one phenyl ring with a napthyl group (59) but strong acid conditions were required to remove this group. Fourrey \textit{et al}\textsuperscript{161} adapted DMTr by replacing the phenyl with the fluorescent pyrenyl group (60) and Caruthers \textit{et al}\textsuperscript{162} have also investigated a large variety of triarylmethyl groups with different UV properties with the aim of using UV absorbance of the washings to characterise composition of mixed sequence probes. While these derivatives, shown in figure 28, have some advantages in certain circumstances the increased steric bulk of the reagents effectively reduces coupling yields and the derivatives have not been widely used.
Another acid labile 5' protecting group that has been examined is the 9-phenylxanthen-9-yl (pixyl) group.\textsuperscript{163-165} This is more labile than DMTr in acid hydrolysis but the conditions required for complete removal also result in partial deprotection of 2'-Thp based groups. Pixyl and to a greater extent 9-phenylthioxanthen-9-yl (S-Pixyl) groups can however be removed by photolysis. Although this is orthogonal to acid labile 2' protection (e.g. Thp) it does still have problems in that reactive by-products are formed and great care must be taken that natural light doesn’t deprotect the reagents before use.

Non acid labile groups have been examined with regards to their compatibility to Thp. Base labile groups have proven to be poor candidates because of the base lability of nucleoside protecting groups and of resin linker. One approach is to use silicon chemistry like that of the TBDMS group combined with some of the features of DMTr. The reagent 1,1,3,3-tetraisopropyl-3-(2-(triphenylmethoxy)ethoxy)disiloxane-1-yl chloride (TESCl) (61), shown in figure 29, modifies triphenylmethanol and connects it to a bulky, and therefore primary specific, silicon reagent. The incorporation of the triphenylmethanol means the UV monitoring qualities are retained and deprotection is achieved using fluoride ions. However in practice this group proved unsuccessful due to cleavage of the β - cyanoethyl phosphate protecting group and of the succinate linker used when attempting to deprotect.\textsuperscript{166} Moving to a terephthaloyl linker (62) solved the problem of cleavage from the support but the phosphate protection problem remains.
In 1992 Ramage et al. introduced a new N^a-amino protecting group derived from the fluorescent tetrabenzo[a,c,g,i]fluoromethyl moiety for the solid phase synthesis and subsequent purification of peptides. The peptides protected with tetrabenzo[a,c,g,i]fluorenyl-17-methanol (Tbfmoc) possess stronger hydrophobic properties and are retained on porous graphitised carbon (PGC). However the Tbfmoc group used in peptide synthesis is base labile and therefore unsuitable for 5' protection, although the 9-fluorenylmethoxycarbonyl (Fmoc) has been used for 5' protection in solid phase synthesis orthogonal to Mthp 2' protection.

Ramage and Wahl introduced 4-(17-tetrabenzo[a,c,g,i]fluoromethyl)-4',4''-dimethoxytrityl chloride (Tbf-DMTr-Cl) for 5' ribose hydroxyl protection. Here the phenyl group of DMTr had been alkylated at the para position with Tbf on a one-carbon spacer. This new protecting group exhibited some useful properties. It significantly influenced hydrophobicity to improve purification. However the inductive effect from the additional alkyl substitution had further increased the group’s lability. Also the group was still sterically restricted since the spacer to the bulky Tbf group was one carbon and as a result coupling yields to nucleotides were much lower than for DMTr.
1.5 Solid Phase Synthesis of Oligoribonucleotides

As the appreciation of the role RNA, both currently and historical in its evolution, has grown so has the interest in the chemical and enzymatic synthesis of RNA. The success of solid phase synthesis of oligoribonucleotides has however not matched that of oligodeoxyribonucleotides. The difference in efficiency stems from the additional 2' hydroxyl group on the ribose ring and the problems associated with its protection.

One approach different from the chemical solid phase methods mentioned for DNA is to use the natural pathway to RNA from DNA. The enzymatic approach involves using two chemically produced DNA sequences annealed together as a template for RNA polymerase from bacteriophage T7. This system can be used to produce milligram quantities but it does have some drawbacks. Success is highly dependent on length and sequence, for example oligomers of less than 12 units give poor yields and are difficult to purify to homogeneity. Other problems can occur with abortive initiations and faulty insertions. Sequences without a guanine at the 5' terminus have proved especially difficult to make and conditions often have to be customised for each particular sequence to optimise yields. The enzymatic method is also limited in the use of natural RNA monomers and cannot incorporate deoxyribonucleosides or unnatural monomers.

The solid phase synthesis of oligoribonucleotides has largely paralleled the methods used for the synthesis of oligodeoxyribonucleotides. In general however when the same methodology is applied to RNA the yields are poorer and the coupling times longer. This is due to the 2' hydroxyl group and the problems associated with its protection.
1.5.1 Protection of the 2’ position

There are several problems to be overcome in the successful protection of the 2’ position. The group chosen must be stable through all stages of the oligoribonucleotide synthesis, including successive acid washes. In addition the protocol for protection of the monomer units must allow complete separation of the 3’ and 2’ protected products to ensure no 5’-2’ links in the resulting oligoribonucleotide. The group should also be as sterically undemanding as possible to minimise the detrimental effect on coupling times for the adjacent hydroxyl group. The group should be cleaved in high yield at the very end of the cycle without nucleoside base modification or backbone migration or degradation, resulting in a RNA oligomer that is fully biologically active.

![Figure 31. Cleavage and migration of RNA linkages](image)

In 1956, Brown and Breslow et al\textsuperscript{174-175} showed that acid can catalyse RNA phosphoryl migration and that acid and base can cause RNA cleavage in experiments involving the partial hydrolysis of cytidine 3’-O-benzylphosphate and cytidine 3’-O-methylphosphate under acidic and basic conditions. Figure 31 shows how under acidic conditions 3’→2’ migration of the phosphoester can occur. Basic conditions produce no migration but both base and acid can hydrolyse the link. Both cleavage and migration proceed \textit{via} a cyclic pentacoordinate trigonal bipyramidal
oxyphosphorane transition state (66). This is a significant consideration since no purification techniques can differentiate between correctly linked oligomers and sequences containing 2'-5' links. Eliminating phosphoryl migration is therefore very important.

Figure 32. Acid labile 5' and 2' alcohol protection
(68) Thp (69) Mthp (70) DMTr (71) Pixyl

The development of groups for 2' hydroxyl protection began with attempts by Griffin and Reese\(^{176}\) to use tetrahydropyran-2-yl (Thp) ethers. This group was shown to be suitable since it was found to not be prone to migration from the 2' to 3' position and cleavage was achieved under conditions mild enough not to disrupt the oligonucleotide. While the Thp group was originally viewed as orthogonal to base labile 5' protection the acid lability of Thp was a problem as the acid labile trityl and 9-phenylxanthen-9-yl (pixyl) groups emerged as the standard 5' protection.\(^{177-179}\) Normally during the chain elongation phase of the solid phase cycle the 5' protection is removed under acidic conditions mild enough to leave Thp in place. However in solid phase synthesis the Thp group has to withstand repeated 5' deprotection cycles and the differential approach proved to be unsuitable for the synthesis of longer oligonucleotides.

Another problem with Thp is its chirality, which results in the formation of diastereoisomers. 4-Methoxytetrahydropyran-2-yl (Mthp) (69) was introduced as an achiral alternative to remove this problem.\(^{177-179}\) Although successful in removing
chiral problems Mthp is still moderately acid labile and therefore unsuitable for use in conjunction with the highly acid labile pixyl or trityl groups.

![Diagram](image)

Figure 33. Ctmp (72) and Fpmp (73)

The first protecting group for the 2' hydroxyl position designed to be compatible with acid labile 5' protection was the 1-[(2-chloro-4-methyl)phenyl] 4-methoxypiperidin-4-yl (Ctmp) (72) group.\(^{180}\) This group can be seen as a development of Mthp where an electron withdrawing group has been attached to stabilise the molecule to acid hydrolysis. For example changing the ring oxygen to a sulfur increases the rate of hydrolysis by a factor of five but changing it to the more electron withdrawing sulfone decrease hydrolysis by a factor of 400. In Ctmp a tertiary amine group has been introduced which remains unprotonated under mild acid (pH 2-2.5) allowing acid hydrolysis to proceed cleaving Ctmp but leaving 5' pixyl protection in place. At lower pH's the nitrogen becomes protonated and the electron withdrawing effect inhibits acid hydrolysis of Ctmp but allows removal of the 5' Pixyl group. Some acid hydrolysis still takes place at lower pH's however. The reaction rate is sufficiently reduced to make Ctmp a suitable protecting group for the 2' position of RNA using the phosphoramidite or H-phosphonate approach.\(^{181-183}\)

A drawback of the Ctmp group is its synthesis, which is long and inefficient. This led to the development of similar compounds that would have equivalent protecting group properties but also be easier to make. 1-(2-fluorophenyl)-4-methoxypiperidin-4-yl (Fpmp) was developed from this approach and emerged as an improvement to
Ctmp.\textsuperscript{184-185} This new group was found to be more stable to acid hydrolysis within the pH range required for 5' deprotection. RNA sequences can undergo migration and cleavage in the pH range 2 to 2.5 (0.01M HCl or dilute acetic acid) previously used for Ctmp and Fpmp cleavage. A refinement to Fpmp cleavage was to use a solution of sodium acetate (pH 3.25, 30°C, 36 hours). These conditions cleave Fpmp without any disruption to the internucleotide bonds.\textsuperscript{186-187} A further recent development of this system is the 1-(4-chlorophenyl) 4-ethoxypiperidin-4-yl (Cpep) group.\textsuperscript{178} This protecting group has slightly improved hydrolysis rates compared with Fpmp and Ctmp.

Another group, which uses completely different chemistry, that has been successfully applied to the 2' hydroxyl function is the t-butyldimethylsilyl (TBDMS) group. First introduced in 1973 by Ogilvie\textsuperscript{188} the TBDMS group has the advantage that it is relatively stable to acid and base and is therefore compatible with most existing protecting group strategies. As with all other silicon-based groups, TBDMS is cleaved rapidly by fluoride ions at room temperature.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{tbdms_migration.png}
\caption{TBDMS migration}
\end{figure}

One potential problem with TBDMS is 2'-3' migration of the protecting group.\textsuperscript{189-190} It has been reported that interconversion can occur in a wide variety of media. In methanol at 36°C the half-life of 2'-O-tert-butyldimethylsilyladenosine is only 57 minutes. The half-life increases to over six hours in aqueous pyridine with added base (0.01M benzylamine) and approximately 12 hours for aqueous pyridine alone. In 80\% acetic acid, 0.1M methanolic hydrochloric acid or in the solvents chloroform
and anhydrous DMF, interconversion is either non-existent or occurs at suitably low levels for the purposes of phosphoramidite and H-phosphonate solid phase chemistry.

The production of TBDMS protected monomers is performed by first reacting TBDMS chloride with the 5'-O-DMTr protected nucleoside in the presence of imidazole. The 5' protected nucleoside is used because TBDMS would react preferentially with the 5' position if it were left free. The reaction produces a mixture of 3' and 2' silylated products which must be separated by silica gel chromatography. Care must be taken here as silica can catalyse interconversion. The 2'-O-silyl product is then phosphitylated and is ready for use.

In 1987 Usman et al\textsuperscript{191} carried out the automated synthesis and purification of an oligoribonucleotide sequence using the silyl methodology. The oligomer, 43 nucleotides in length, was based on the 3'-half molecule of an \textit{Escherichia coli} N-formylmethionine tRNA and in this synthesis the modified bases were replaced by their unmodified counterparts. Coupling steps lasted 15 minutes and the silyl protection was removed by treatment with 1M tetrabutylammonium fluoride (TBAF) in THF for 4 hours. The strong basic conditions used to cleave the N-acyl protection and linker to the solid support were found to cause the removal of the 2' silyl protection and also chain cleavage. The problem is most severe when 30% ammonium hydroxide is used and was reduced significantly by adding 25% ethanol and conducting cleavage at room temperature over 2-3 days although under these conditions some cleavage is still detectable. The problem was overcome by using the more labile phenoxyacetyl amino protecting groups which can be removed at 55\degree C in 3:1 ammonium hydroxide-ethanol over 16 hours without detectable rates of chain cleavage\textsuperscript{192} or 4 hours at room temperature with TBAF.\textsuperscript{193}

Although silyl protection has been used successfully for the synthesis of large RNA molecules problems still exist. Cleavage with TBAF is highly sensitive to water and leaves fluoride salts that must be removed prior to analysis. An alternative to TBAF, triethylamine trihydrogen fluoride TEA.3HF, also has shortcomings and can cause
Chapter 1 Introduction

 degradation of some oligonucleotides. Another persistent problem with silyl protection is that coupling times are slower than their DNA equivalents. This has been attributed to steric issues resulting from the bulky, lipophilic TBDMS group.

Schwartz et al developed a protecting group designed to overcome the steric problems associated with TBDMS. Their new group, o-nitrobenzyloxymethyl, was developed from an earlier hydroxyl protecting group, o-nitrobenzyl, which had also suffered from steric problems in RNA synthesis. In the new group a carbon and oxygen have effectively been introduced as a spacer to move the bulky substituted benzene ring away from the 3’ hydroxyl function. This approach succeeded in reducing steric problems and yields greater than 98% over a two minute coupling cycle were observed; rates comparable with those in DNA synthesis. Photochemical cleavage is then affected by exposure to long wave UV light for 5 hours at pH 3.7. Care does have to be taken to avoid accidental cleavage introduced from ambient light. Gough et al introduced the p-nitrobenzyloxymethyl group. This group also overcame the steric problems and achieved coupling times of two minutes by using a short spacer. Deprotection here was achieved with TBAF in THF at 25°C over 24 hours.

Another protecting group developed for the 2’ position is the (trimethylsilyl)ethoxymethyl ether (SEM) group. This group can be thought of as an attempt to combine the silicon protection of TBDMS with the spacer moiety successfully used with the nitrobenzyl groups to reduce steric effects on coupling times. It also has the advantage over TBDMS in that because there is now a silicon – carbon bond, rather than the silicon – oxygen bond, so the group cannot migrate. Unlike TBDMS, SEM is stable to base and is removed by treatment with BF3.OEt2 in acetonitrile for 30 minutes.
Another group to be tried as a 2' protecting group is the 1,1-dianisyl-2,2,2-trichloroethyl group (DATE). Chosen for its orthogonal cleavage and high stability to acid and base it does however suffer from problems of steric bulk.

Rastogi and Usher introduced the first protecting group requiring a two-stage deprotection in 1995. 2-Hydroxyisophthalate formaldehyde acetal (HIFA), as the bis ester (81), is relatively stable to the acidic conditions used during repeated removal of 5' protecting groups. During the alkali conditions, for cleavage of the exocyclic protection and cleavage from the solid support, the bis ester is converted to the bis carboxylic acid (82) which can subsequently be easily removed using mild acid (83).
All 2' protection requires the synthesis of pure 2' protected product uncontaminated by 3' protected isomers. Most protection reactions produce mixtures of 3' and 2' protected products that are then purified by chromatography. Markiewicz\(^{200}\) offered a solution to this difficult and time-consuming problem in the form a reagent for the simultaneous protection of the 3' and 5' hydroxyl groups. Known as the Markiewicz reagent, 1,1,3,3-tetraisopropyldisiloxane (TIPDSi), makes use of the thousand fold higher reactivity of primary alcohols over secondary alcohols with silyl reagents. Introduced as 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane, TIPDSi reacts first with the 5' position then intramolecularly with the 3' position to give a 3'-5' protected nucleoside (85). The 2' hydroxyl group is then free to react with many of the protecting groups mentioned above. TIPDSi is then removed with TBAF to leave the 2' protected nucleotide (86).

Figure 38. Markiewicz protection
An additional procedure in silicon chemistry is the selective desilylation of multisilylated nucleosides at the primary 5' position.\textsuperscript{201} Although generally not of direct application for 2' protection this technique is important for the production of other modified nucleosides. A nucleoside protected by the Markiewicz reagent or by multiple TBDMS groups can be selectively deprotected at the 5' position leaving TBDMS protection on the 3' and 2' or leaving the Markiewicz reagent still attached at the 3' position.

The large volume of research into RNA and its chemical synthesis has meant that large highly modified oligoribonucleotides can now be chemically synthesised in the laboratory.\textsuperscript{202-203} However scope for refinement and improvement of protecting group strategies remains.
Chapter 2 Results and Discussion

2.1 Aims of this Research

Oligonucleotide synthesis has advanced a long way since its inception in 1955 when Michelson and Todd reported the first chemical synthesis of a dinucleotide. Reagents and protecting groups continue to be refined in an effort to improve speed and efficiency. The aims of this research were to design and synthesise novel protecting groups for the solid phase synthesis of oligonucleotides covering the 5' and 2' hydroxyl ribose positions.

2.1.1 5' Protection

Earlier work by Ramage and Wahl resulted in the introduction of 4-(17-tetrabenzo[a,c,g,i]fluoromethyl)-4',4''-dimethoxytrityl chloride (Tbf-DMTr-Cl) for the protection of the 5' hydroxyl position. This work had been successful in aiding purification but had areas capable of improvement. To overcome these problems we envisaged two modifications to the original design to produce a new protecting group.

The first modification was aimed at improving coupling efficiency. The coupling of Tbf-DMTr-Cl to thymidine was achieved in 45% yield compared to an average 78% yield for DMTr achieved by Khorana using the DMTr group in 1962 (although this was later improved to 93%). It was thought that the poor coupling yields for Tbf-DMTr-Cl were a result of steric effects. If the spacer between the Tbf and phenyl is increased from the one carbon previously employed then these steric effects should be removed and the reactivity of the new group should be brought closer to that of DMTr. Therefore the modification was to increase the length of spacer from one carbon to ten or eight.
The second modification was to change the substitution pattern on the benzene rings of the trityl system. The aim was to make the electronics of the system closer to DMTr and therefore subject to the same reaction conditions as DMTr. The \textit{para} methoxy groups were to be removed and replaced with methyl groups also the method of synthesis was to be altered such that the linker was to be attached through an oxygen atom rather than a carbon. This was done because the original system with two methoxy groups combined with the inductive effect of the carbon link to Tbf had proved too much. With three stabilising forces on the cation it had proved too stable and the group became too acid labile. By moving from one methyl and two methoxy groups to two methyls and one methoxy group we hoped to get the balance right so that the protecting group would have approximately the same lability as DMTr.

![Figure 39. Tbf-C_{10}-DMTr (87)](image)

The aim in this project was therefore is to produce a new 5' protecting group, shown in figure 39, based on Tbf-DMTr with the following properties:

- The protecting group should be removed under acidic conditions similar to those employed for the removal of DMTr or MMTr.
- The protecting group should be introduced under conditions mild enough to not affect the nucleotide or oligonucleotide and should be regioselective for primary alcohols in order to leave the secondary hydroxyl functions unprotected.
• The group should make use of Tbf’s lipophilic properties to enable purification of derivatised oligomers.

• Yields for coupling the new group to nucleotides should be higher than that for Tbf-DMTr-Cl.

• The group should contain no interfering reactive functionalities and should be accessible in reasonable yield.

• The new group should contain the UV/Visible properties of the trityl and Tbf groups so that its concentration could be easily measured and thus the extent of coupling calculated.

2.1.2 2' Protection

Three different approaches to preparing an improved protecting group for the 2' hydroxyl position were attempted. The first was a development on earlier work by Bremner\textsuperscript{204} based on propargyl alcohol; the second was based on 3-butyn-1-ol and the third on o-cresol.

![Chemical structures](88) (89) (90) (91)

Figure 40. Steric hindrance at the 2' position

The first common feature of the three approaches is the attempt at limiting steric interference at the point of nucleotide bond formation. Analysis by Kierzek \textit{et al}\textsuperscript{205} showed the profound effect on coupling efficiencies that bulky 2' groups can have. By looking at the coupling yields from the competitive condensation of four
ribonucleoside phosphoramidites (88-91) and a solid support bound deoxythymidine he showed the order to be size dependant. The order of reactivity was $88 > 89 > 90 > 91$ (from figure 40) with the relative yield of dimers being 1, 0.7, 0.4, 0.1 respectively which corresponds directly to the size of 2’ protection used. This shows the need to keep 2’ protection as unobtrusive as possible. All three systems are designed to be sterically undemanding at the $\alpha$ and $\beta$ position, with respect to the 2’ oxygen.

The second feature common to all three proposed systems is the two-stage deprotection strategy. In the final deprotection protocol use of base, to remove exocyclic and phosphate protecting groups as well as cleaving the oligomer from the support, first primes the system so that it can be cleaved under mild acidic conditions. Limiting the exposure of completely unprotected RNA and DNA to acid or base solutions should minimise sequence degradation.

The aim therefore is to produce a series of 2’ protecting groups with the following properties;

- The groups should be stable to repeated exposure to the acidic conditions used for the removal of 5’ protection.
- It should be stable to the alkaline conditions used for the removal of exocyclic deprotection and cleavage from the solid support.
- Conditions for removal should be mild so that there is minimal degradation of the oligonucleotide via cleavage, migration or depurination. Reagents used should also be easily removed from the oligomer during purification.
- The groups should have minimal steric bulk to keep coupling yields high.
- The groups should be prepared as alkyl halides for introduction using standard Williamson type reactions.
2.1.2.1 The Propargyl alcohol system

The first system designed by the Ramage group to fit the above criteria was introduced by Bremner and was based on propargyl alcohol. Figure 41 shows two of the many compounds investigated, 6-Hydroxy-hex-4-ynyl acetate (92) and -Hydroxy-hex-4-ynyl benzoate (93).

![Compounds 92 and 93](image)

Figure 41. Propargyl System

These groups were designed to be deprotected in a two-stage mechanism. During the addition of base, synchronous with cleavage from the solid support and removal of the exocyclic and phosphate protection, the ester is hydrolysed to give the free acid and the alcohol. During treatment of the intermediate with acid, the alcohol undergoes an intramolecular cyclisation to give the unprotected nucleoside.

The Baldwin Rules classify intramolecular cyclisation reactions that are favoured and those that are not. According to these rules Exo-Dig cyclisations are allowed for ring sizes 5 to 7. The cyclisation part of the deprotection for phenyl-7-hydroxy-hept-5-ynoate follows these rules and is a 5 Exo-Dig reaction.

![Baldwin Rules](image)

Figure 42. Baldwin Rules, 5 Exo-Dig

It has been shown that hydroxy groups can undergo cyclisation reactions with a triple bond to form enynols and furans in a 5 Exo-Dig fashion as shown in figure
Intramolecular reactions have also been shown using silyl protected enol ethers which react with triple bonds in an aldol fashion, an example of which is shown in figure (44).

To stop this cyclisation reaction happening prematurely the alcohol is protected throughout the reaction cycle as the ester. It was found however that cleavage of the acetates and benzoates was too slow and the conditions required would result in damage to the oligonucleotide. The benzoate was an improvement but cleavage conditions were still considered to be too harsh.

While Bremner proved that the basic premises of the propargyl protection strategy were correct, refinements were needed if the group was to be successfully employed for solid phase oligoribonucleotide synthesis. Two changes to his system to overcome the problems are evident. First of all that the benzoate is changed for a para-nitro benzoate group. Since the electron withdrawing nitro group should facilitate ester cleavage. The second change would be required to speed up the intramolecular cyclisation. If a methyl substituent was added to the backbone $\beta$ to the alcohol it should expedite ring closure by decreasing the entropic loss suffered when
the ring closes therefore lowering the free energy of reaction. However this addition does introduce the added complexity of a chiral centre to the molecule. Introducing two methyl substituents at the same position to remove the chiral problem would have resulted in the introduction of excessive steric bulk which would cause problems not only in synthesis of the group but may also have reduced coupling efficiency. The proposed protecting group is shown below in figure 45 and its deprotection in figure 46.

![Proposed new propargyl group](image1)

**Figure 45. Proposed new propargyl group**

![Deprotection of new propargyl system](image2)

**Figure 46. Deprotection of new propargyl system**
2.1.2.2 The 3-Butyn-1-ol System

Unlike the previous candidate, close analogues of this compound have not been made before as candidates for hydroxyl protection. It has been designed to conform to the criteria mentioned before and like the propargyl system does contain a triple bond close to the point of contact. Based on 3-butyn-1-ol it should be sterically undemanding enough and share a similar deprotection mechanism with the previous example, in that it would require base conditions followed by addition of acid and is stable to the acid conditions required for dimethoxy trityl cleavage.

![Figure 47. Butynol system](image)

The similarity of deprotection is only superficial however and the mechanism relies on a series of different reactions to the propargyl system. A potential deprotection mechanism is shown in figure 48, however this is just a proposed mechanism and other routes could be suggested. One point of diversity could be the ester cleavage. This could happen during the base steps to cleave from the solid support but could also happen during the acid step. The ways the molecule might fragment during cleavage could differ but the end result is the same.

After cleavage from the solid support by base the 2' protected oligomer is exposed to concentrated ammonia that should aminate the triple bond to give the imine or enamine.²¹¹

One alternative route to the enamine could be through a cycloaddition reaction of the triple bond with sodium or alkyl azide to give the 1,2,3 triazole. Heterocycles with three adjacent nitrogens are unstable and will degrade with the loss of N₂. A probable product from this degradation would be an imine²¹² corresponding to the
enamine shown in figure 48. The route through triazole is much more complicated and the actual mechanism of degradation would be difficult to follow.

The Schiff base then reacts with mild aqueous acid to give the β-keto acid, with the loss of ammonia. Imine hydrolysis is a rapid reaction that proceeds via a hemiaminal intermediate in mild acid but it should be noted that this is an equilibrium. The driving force in this case is the decarboxylation of the β-keto acid in the following step. β-Keto acids, which are unstable in acid but fairly stable in base, are prone to decarboxylation for two reasons. First of all the product of decarboxylation is an enol which is stabilised by resonance. Secondly when the β-keto acid decarboxylates it goes through a six membered cyclic transition state with the newly forming OH bond in the same plain as the C-C=O system thus producing the enol directly without going through a carbanion. In this example as there are no steric constraints to formation of this transition state and decarboxylation should proceed in acid at room temperature.

If this enol then isomerises by a 1,3-hydride shift the new enol could undergo a retro conjugate addition to give the free oligonucleotide. The materials released in the breakdown of the protecting group would be ammonia, carbon dioxide and methyl vinyl ketone which should all be easy to remove. There is a potential complication with methyl vinyl ketone in that it is prone to polymerisation however this should not be too problematic due to the low concentrations of potential monomer around during cleavage. This property may even be useful in driving the equilibrium for the final step.
Figure 48. Butynol deprotection
2.1.3 The o-Cresol System

The third system to be evaluated represents a departure from the acetylene-based systems and instead moves to a modified benzyl ether. Khorana\textsuperscript{214} first introduced the benzyl ether as a $2'$ protecting group for oligoribonucleotide synthesis in 1962, shown in figure 49, as an alternative to acid and base labile groups. In 1966 a $2'$-$5'$ dinucleotide was synthesised using benzyl ether protection.\textsuperscript{215} Removal of benzyl ethers was achieved using mild catalytic hydrogenolysis. One atmosphere of hydrogen using palladium black in aqueous ethanol has been successfully used to remove benzyl ether protection on a $2'$-$5'$ cyclic phosphate.\textsuperscript{216}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{benzyl_o-cresol.png}
\caption{Benzyl ether (96) and O-Cresyl system (97)}
\end{figure}

Figure 49. Benzyl ether (96) and O-Cresyl system (97)

Instead of using hydrogenolysis the new group will be based on $o$-cresol and will use a two-stage deprotection of base then acid similar to that used for the butynol and propargyl systems. The first reagent in deprotection, shown in figure 50, is the base used to remove the exocyclic protection and cleave from the support. During this step the ortho acetyl protection would be removed to give the ortho hydroxyl group.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{o-cresol_deprotection.png}
\caption{$O$-Cresyl deprotection}
\end{figure}

Figure 50. $O$-Cresyl deprotection
The second step to deprotection of this group would be acid treatment to give the ortho-quinone methide and the deprotected nucleotide.

### 2.2 Protection of 5' position

#### 2.2.1 Synthesis of Tbf C_{10} Tr

![Chemical structure of ethyl 4-(10 bromodecyloxy)-benzoate and Tetrabenzo[a,c,g,i]fluorene (Tbf)](image)

**Figure 51. Synthesis of ethyl 4-(10 bromodecyloxy)-benzoate**

The first step in our synthesis of Tbf-C_{10}-DMTrCl was the synthesis of the precursors ethyl 4-(10 bromodecyloxy)-benzoate (98) and tetrabenzo[a,c,g,i]fluorene (Tbf) (100) as shown in figures 51 and 52. The synthesis of the alkyl bromide (98) was achieved in 78% yield using a Williamson type reaction with an alkyl dibromide and ethyl 4-hydroxy-benzoate activated with sodium hydride. To ensure only mono alkylation, a 3 to 1 excess of dibromide was used but the dialkylated side product was still present at levels about 10% of product formed. The actual level of dialkylated product is probably higher as no extra effort was made to isolate and measure this byproduct.

Tetrabenzo[a,c,g,i]fluorene (100) was prepared using the procedure originally developed by Wahl and is shown below in figure 52. The Grignard reagent prepared from 9-bromophenanthrene was reacted with 0.5 equivalents of methyl formate to give bis-phenanthren-9-yl methanol (99) in 81% yield. Treatment with trifluoroacetic acid results in a [2+2] cyclisation to give tetrabenzo[a,c,g,i]fluorene (Tbf) (100) in 69% yield.
However the cyclisation product obtained is a mixture of two isomers (100) and (101) shown in figure 53. The product formed from the initial acid reaction with acid is the unsymmetrical isomer (100) as shown in Figure 53. Base, heat or ultraviolet light can then isomerise this to give the thermodynamically favoured symmetrical isomer (101). The presence of the symmetrical isomer is not problematic however as base treatment of both isomers gives the same anion.

Figure 53. Cyclisation mechanism for bis-phenanthren-9-yl methanol
The next step in the synthesis of Tbf C_{10} Tr was the coupling of the fragments (98) and (100) to make ethyl 4-(10-(tetrabenzo[a,c,g,i]fluorenyl) decyloxy)-benzoate (102) shown in figure 54. The procedure of Hay was followed with one alteration.\textsuperscript{217} Previously the initial step was formation, using tetrabutylammonium hydroxide, then isolation of the Tbf tetrabutylammonium salt precipitate by filtration under nitrogen. The anion was then dissolved in a second solvent and the alkyl bromide added. Although stable to water, the anion rapidly reacts with oxygen to give the Tbf ketone (103) so it was felt that the isolation of the anion solid represented an unnecessary exposure to air. The procedure was adapted by adding the alkyl bromide (98) after ten minutes and attempting the reaction as a suspension because of this high sensitivity of the Tbf anion to oxygen.

![Synthesis Diagram](image)

Figure 54. Synthesis of Ethyl 4-(10-(tetrabenzo[a,c,g,i]fluorenyl)decyloxy)-benzoate (102) and of 4-(10-(17'-tetrabenzo[a,c,g,i]fluorenyl)decyloxy)-4''',4''''-dimethyl trityl alcohol (104)

An excess of p-bromotoluene and magnesium were combined to produce the corresponding Grignard reagent and then reacted with the ester (102). This Grignard
reaction converted the Tbf ester into 4-(10-(17'-tetrabenzo[a,c,g,i]fluorenyl)decyloxy)-4'',4'''-dimethyl trityl alcohol (104) in 86% yield. Using reactions with ethyl benzoate, p-bromotoluene and magnesium as a model system it was found that excess Grignard reagent produced the highest yields.

Figure 55. Attempted synthesis of 4'-(10-(17'-tetrabenzo[a,c,g,i]fluorenyl)decyloxy)-dimethyl trityl-oxy-5'-thymidine (106)

Model studies for the chlorination of (104) and the coupling of the product to thymidine were carried out with 4''-4'''-dimethyl trityl alcohol as well as on trityl alcohol following the literature procedure.\textsuperscript{218} These studies were successful but the derivatives did show high sensitivity to moisture even under mild acidic conditions. Using flash column chromatography over silica the chloride was converted back to the alcohol. 4-(10-(17'-Tetrabenzo[a,c,g,i]fluorenyl)decyloxy)-4'',4'''-dimethyl trityl chloride (105) was successfully prepared using the same methodology as the models and was then used immediately in attempts, under several different sets of conditions, to couple to thymidine to produce bis-(-4-methylphenyl)-4-[10-(17-
tetrabenzo[a,c,g,i]fluorenyl)decyloxyj phenylmethoxy-5'-thymidine (106), as shown in figure 55.

This reaction was attempted many times using the literature procedure described by Strazzolini et al.\textsuperscript{218} with no success. Chlorine analysis and proton NMR as well as a visible change in colour (from pale yellow to bright orange) suggested that the alkyl chloride was formed but failed to react with the thymidine.

Incomplete removal of acetyl chloride after the chlorination and subsequent contamination of the coupling step was initially thought to be the cause. Removal of the excess acetyl chloride is achieved by repeated co-evaporation to dryness with a dry solvent after chlorination. Initially dry THF (3x10ml) was used as the solvent. Pyridine (3x10ml), benzene (3x10ml) and toluene (3x10ml) were all investigated but no reaction was observed in the coupling reaction. Due to the sensitivity of the chloride intermediate, recrystallisation or flash column chromatography are unsatisfactory so another method needed to be used to remove the acetyl chloride. However the evaporating to dryness technique has been used successfully in the literature, using benzene, so it seems unlikely that contamination with acetyl chloride is solely responsible for failure of the coupling reaction.\textsuperscript{159}

Another potential reason for failure of such reactions can be moisture reacting with the alkyl chloride to return the activated trityl system back to the alcohol. This seems unlikely given the stringent reaction conditions. However the thymidine was twice co-evaporated with dry pyridine to make sure moisture was removed.

When attempting to recover the trityl alcohol to recycle the material for another attempt it was noticed that there was a complicated mixture of compounds with Rf values close to that of the starting material. It was clear that the trityl system chosen was sensitive to the long exposure to pyridine used in the synthesis. A possible mechanism for this degradation is shown in figure 56.
Figure 56. Possible mechanism of methyltrityl degradation in base

First of all the chloride is easily lost due to the stability of the carbocation. The electron donating effect from the electron rich benzene rings stabilises the positive charge. Normally this electrophilic and sterically hindered carbon reacts with primary alcohols. However, in this case a faster reaction could be loss of a methyl proton to quench the positive charge giving the unsaturated compound shown in figure 56. The resulting compound could then undergo all kinds of reactions even polymerisation. To test if base sensitivity was the reason for the reaction failure, a quantity of alkyl chloride was stirred in pyridine without any other reagent for one week. After this time the pyridine was removed and the residue was analysed and was shown to have degraded.

Even if a small quantity of protected thymidine (106) was formed in the early stages of the coupling reaction it might still degrade by a similar mechanism. This observation led to the redesign of the system with the omission of the two aryl methyl groups. The new protecting group will now be analogous to monomethoxytrityl and will be less acid labile than the commonly used dimethoxy group. This should not present a significant problem however as monomethoxytrityl has been used in solid phase synthesis and since this group will only be removed once, the increased exposure to acid should not significantly increase depurination levels.
2.2.2 Shorter linker approach

Another possible explanation for the failure of the coupling reaction could be that the linker was long enough for the molecule to fold over on itself. It is possible that the highly aromatic and hydrophobic Tbf and trityl regions are attracted to each other and held in place in such a way as to obscure the active site to such an extent that the polar thymidine hydroxyl group is unable to penetrate and reach the site of reaction. To test this theory the protecting group was resynthesised with three and eight carbon linkers.

![Chemical structures and reactions](image)

Figure 57. C₈ and C₃ linker synthesis

The first step was synthesis of shortened precursors for coupling to Tbf, shown in figure 57. The C₈ fragment ethyl 4-(8 bromooctyloxy)benzoate (107) was produced
in 68% yield and the C₃ fragment ethyl 4-(8 bromopropyl)oxy-benzoate (108) was made in 75% yield. Coupling to Tbf was achieved in 83% yield for the C₈ linker but was unsuccessful for the C₃ linker. This might be explained by the fact that ethyl 4-(8 bromopropyl)oxy-benzoate is a low melting point solid and was harder to de-gas and purify than the other alkyl bromides used. The procedures used were identical to those used to prepare the ten carbon compounds.

This line of investigation was stopped at this point when the alternative strategy, where the aryl methyl groups had been removed, was used to produce a protected nucleotide. The C₈ fragment was used later for use in purifying 2' cresol protected compounds as is described in section 2.3.3.

2.2.3 Synthesis of Tbf C₁₀ DMTr

To test the theory that the methyl groups were responsible for the failure to produce the protected thymidine (106) monomethoxytrityl chloride was prepared and coupled to thymidine (using the same procedure unsuccessfully used with (104)). First the hydroxy group of ethyl 4-hydroxybenzoate was protected as the methyl ether (111) in 94% yield then a Grignard reaction with bromobenzene produced 4' monomethoxy trityl alcohol (112) in 90% yield. This was converted to the chloride (113) using acetyl chloride and purified by co-evaporation with benzene. Finally this was coupled to thymidine to give the 5' protected thymidine (114) in 57% yield.

With the success of the model system the synthesis of the new protecting group was begun and followed the same lines as the original plan, except that the Grignard reaction was carried out with bromobenzene producing (115) in 83% yield. The hydroxyl group was then converted into the chloride (116). Chlorine analysis gave poor results with values for the percentage of chlorine often well below the theoretical value for complete chlorination. There was a strong coloration between the age of a sample and the margin of deviation from the expected value. This was
not a concern since the combustion of Tbf is always incomplete and the alkyl chloride is highly sensitive and will revert to the alcohol. The NMR gave more encouraging results showing the loss of the hydrogen from the alcohol. Coupling to thymidine in pyridine was successful and 4''-(10-(17'-tetrabenzo[a,c,g,i] fluorenyldecyloxy)-trityl-oxy-5''-thymidine was produced in 77% yield, shown in figure 59.

The yield of 77% is identical with that originally achieved by Khorana\textsuperscript{57} with DMTr and is a distinct improvement on the 45% achieved by Wahl with the one carbon linker.\textsuperscript{170} Therefore the move from a one carbon spacer to a ten carbon spacer has removed the steric effects almost completely for coupling to thymidine. This yield represents unoptimised conditions and higher yields should be achievable.

Coupling to uridine to produce 4''-(10-(17'-tetrabenzo[a,c,g,i] fluorenyldecyloxy)-trityl-oxy-5''-uridine (118) was achieved in 46% yield. N\textsuperscript{2}-Isobutyryl-2'-deoxyguanosine (119), N\textsuperscript{4}-Benzoyl-2'-deoxycytidine (120), N\textsuperscript{6}-Benzoyl-2'-deoxyadenosine (121) were all protected in 68%, 83% and 70% yields respectively.
In early coupling reactions it was noticed that even after the extensive measures used to remove acetyl chloride the products were acylated at the 2' position. The probable reason for this lies in the nature of the solid chloride. It becomes a thick and sticky foam under vacuum and even after hours under a high vacuum there can be liquid, and therefore acetyl chloride, within the matrix.

Figure 59. Synthesis of 4’-(10-(17’-tetrabenzo[a,c,g,i]fluorenyldecyloxy)-trityl-oxy-5’-thymidine (117)

It was observed that there was no noticeable acylation in the production of protected deoxycytidine (120). The quality of starting material N^4-benzoyl-2’-
deoxycytidine was worse than those of the other nucleosides and a small excess had been added to compensate. This excess had been enough to remove the remaining acetyl chloride by acylation of the 5' position and therefore an excess of nucleotide was added to the procedures for the other deoxynucleotides. The excess used appears to relate to the yield produced, thymidine excess 14% gave a 77% yield, guanosine 21% excess gave a 68% yield, cytidine 37% excess gave an 83% yield and adenosine 12% excess gave 70%. The protected nucleotides are expensive so a refinement of this procedure would be the introduction of a simple alcohol to trap the acetyl chloride. This could be added before the nucleoside and would be sufficiently sterically demanding, a secondary or tertiary alcohol for example, to be unable to form an ether with the trityl chloride. This should be achievable since triphenylmethanol will not form an ester and trityl systems will not easily form ethers with secondary alcohols.

The next step was to convert the 3' hydroxyl groups into their phosphoramidites for use in solid phase synthesis. The 5' protected nucleosides were stirred in alcohol-free dichloromethane (DCM) with tetrazole diisopropylammonium salt (DIHT) and tetraisopropyl cyanoethylphosphoramidite. Sodium bicarbonate solution was then used to quench the reaction and the organics were extracted with more DCM. The DCM was washed with brine then dried and the solvent removed by vacuum. The solid residue was dissolved in the minimum of DCM and precipitated in hexane then dissolved in the minimum of ethyl acetate and precipitated in pentane. When using 5' MMTr thymidine as a test system the crude $^1$H and $^{31}$P NMR showed the reaction to be successful. It was clear that some oxidised P$^5$ compounds were also present but an attempt to purify by column chromatography destroyed the sample.
Figure 60. Synthesis of the protected nucleosides
The four deoxynucleotides were synthesised and purified using precipitation as described above. Mass spectroscopy and NMR were used to measure the purity of the products. 4'-(10-(17'-tetrabenzo[a,c,g,i]fluorenyl)decyloxy)-trityl-oxy-5'-N^4-benzoyl-2'-deoxycytidine-3'-O-N,N-diisopropyl-β-cyanoethyl phosphoramidite (123) was produced in 90% yield, 4'-(10-(17'-tetrabenzo[a,c,g,i]fluorenyl)decyloxy)-trityl-oxy-5'-thymidine-3'-O-N,N-diisopropyl-β-cyanoethyl phosphoramidite (124) in 72% yield, 4'-(10-(17'-tetrabenzo[a,c,g,i]fluorenyl)decyloxy)-trityl-oxy-5'-N^6-benzoyl-2'-deoxyadenosine-3'-O-N,N-diisopropyl-β-cyanoethyl phosphoramidite (125) in 91% and 4'-(10-(17'-tetrabenzo[a,c,g,i]fluorenyl)decyloxy)-trityl-oxy-5'-N^2-isobutyryl-2'-deoxyguanosine-3'-O-N,N-diisopropyl-β-cyanoethyl phosphoramidite (126) in 94%.

![Diagram of phosphoramidite synthesis]

**Figure 61. Phosphoramidite synthesis**

### 2.2.3.1 Solid phase Synthesis

The 5' Tbf C_{10}Tr nucleoside phosphoramidites were thus ready for testing in a solid phase synthesis. Four sequences of seven thymidines were synthesised with the final nucleoside being one of the four Tbf protected nucleosides. Standard monomer couplings last thirty seconds. The final step involved the addition of the Tbf trityl...
protected monomers, dissolved in 1:1 alcohol-free DCM and dry acetonitrile, and lasted ten minutes. The following sequences were prepared by this method:

(127) 5'-Tbf-DMTr-TT-TTT-TTT-3'
(128) 5'-Tbf-DMTr-CT-TTT-TTT-3'
(129) 5'-Tbf-DMTr-AT-TTT-TTT-3'
(130) 5'-Tbf-DMTr-GT-TTT-TTT-3'

At the end of each synthesis the sequences were cleaved from the solid support using concentrated aqueous ammonia in 20 minutes and the exocyclic amine protecting groups were removed by heating the solution overnight (16hrs) at 45°C. Standard dimethoxytrityl protocol is 40°C for 24hrs. The samples were then evaporated to dryness in vacuo at 45°C over three hours and the crude residue was analysed by HPLC at this point.

The samples were purified using a polypak, a pre-packed small column. The crude sample solutions were eluted (and the run off was reapplied), ammonium hydroxide solution and water were then used as eluants to remove truncated sequences. Trifluoroacetic acid (TFA) was added to cleave the 5' Tbf protection and, to elute the purified oligonucleotide, 2ml of 20% acetonitrile was washed through and collected. These samples were then analysed by HPLC and LCMS.

Figure 62a. HPLC traces for (127) before [left] and after [right] purification [A] shows the 7 thymidine sequence
Figure 62b. HPLC traces for (128) before [left] and after [right] purification[A] shows the 7 thymidine sequence.

Figure 62c. HPLC traces for (129) before [left] and after [right] purification[A] shows the 7 thymidine sequence.

Figure 62d. HPLC traces for (130) before [left] and after [right] purification[A] shows the 7 thymidine sequence.
The HPLC was an Aquapore reverse phase silica column (300Å particle size) 10cm C4, internal diameter 4.1mm and using a flow rate of 1ml/min. The gradient used was (1) 0-4min 50%A; 4-24min 50-90%A; 24-28min 90%A; 28-30min 90-50%A for reverse phase with samples dissolved in Acetonitrile and a minimum of Dioxan where (A) is Acetonitrile and (B) is water.

<table>
<thead>
<tr>
<th>Base</th>
<th>Final Step Yield</th>
<th>% of 7mer’s relative to 8mer’s</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Before purification</td>
</tr>
<tr>
<td>Thymidine</td>
<td>34%</td>
<td>47%</td>
</tr>
<tr>
<td>Deoxycytidine</td>
<td>55%</td>
<td>7mer peak off scale</td>
</tr>
<tr>
<td>Deoxyadenosine</td>
<td>12%</td>
<td>37%</td>
</tr>
<tr>
<td>Deoxyguanosine</td>
<td>33%</td>
<td>61%</td>
</tr>
</tbody>
</table>

Figure 63. Table containing yields for Tbf group coupling and levels of 7mer post and pre purification relative to 8mer

Despite the lengthened coupling times the final step yield is disappointing. The large variation between the bases is probably partially down to the quality of the phosphoramidite and repeated experiments should produce better results. The phosphoramidite samples were made two days before use and purified on the day before use. If the samples were made and purified on site and used immediately a better result might be seen, or alternatively stored under Argon before use. However the yield may be inherently limited due to the nature of the Tbf group. Solubility was a problem and DCM had to be added to the procedure to compensate. Investigation of different linker sizes should be able to find the optimal linker size where solubility in acetonitrile is improved but the steric problems are still eliminated.

The large amounts of the T7 truncated sequence observed in this reaction is a direct consequence of the poor coupling for the final step. Using the DMTr polypak technique a significant reduction was made in contamination of the final product sequence. Optimising the purification protocol should eliminate the presence of the
7mer material completely from the final product. Optimising purification could also raise the yield of the final step if it is found that there is incomplete cleavage of the Tbf 5' protecting group. Monomethoxytrityl esters are less acid labile than the dimethoxy esters and therefore Tbf C\textsubscript{10}Tr will also be less labile. Different deprotection conditions should be investigated to ensure maximum yield and minimal depurination.

2.2.4 Structural motif.

![Proposed structure derived from the NMR evidence](image)

Over the course of the synthesis of many Tbf containing compounds a common structural motif was noticed from the proton NMR. Every compound containing the moiety Tbf-CH\textsubscript{2}-CH\textsubscript{2}-R exhibited the same signal in the region $\delta \text{H} \ 0.3$ - $0.4$. Using two dimensional $^1$H - $^1$H correlated spectroscopy (COSY) NMR it was shown that in every case this two hydrogen multiplet signal was attributable to the hydrogen on the second carbon of the linker relative to the Tbf group. The first and third carbons in the chain could also be assigned using this technique. Attempts to discern the coupling constants between the hydrogens and therefore know the cis - trans
relationship between them were complicated but we propose the following structure to describe the NMR evidence.

The cis relationship from Tbf to C₁ was always clear, however the relationship from C₁ to C₂ was less clear and the coupling constant was more variable. Beyond this stage coupling constants couldn’t be individually assigned and C₂ is itself a complicated multiplet.

Therefore after the first two carbons the chain becomes flexible. This fixed cis then trans structure for Tbf to C₁ to C₂ is consistent with the low chemical shift for the hydrogens on the second carbon. The hydrogens are held in a position where they are slightly shielded by the induced field of the Tbf system.

2.3 Protection of 2' position of ribonucleotides

2.3.1 Propargyl system

The proposed synthesis for this compound followed the same route taken by Bremner but with two changes. First the bromochloropropane used by Bremner was replaced by bromochloro-2-methylpropane. Secondly a para-nitro group was introduced on the sodium benzoate used in the final step.

The first step is the protection of the propargyl alcohol function with dihydropyran to produce prop-2-yn-1-yloxytetrahydropyran (131) following a literature procedure. For this reaction pyridinium p-toluenesulfonate (PPTS) (132) was used as a catalyst and was prepared beforehand with p-toluenesulfonic acid monohydrate and pyridine. Removing acetylene hydrogen with butyl lithium has been achieved with high yield. But the second step proved harder to optimise and yields fell well below the 84% achieved by Bremner for his analogous system. The product, 1-chloro-2-methyl-6-tetrahydropyranloxy-hex-4-yne (133) proved difficult to separate
from the unreacted acetylene, although the reagent 1-bromo-3-chloro-2-methylpropane is easily removed. Yields were highly variable with no discernible reason for the variations and the best yield achieved was only 43%. One point noted was that partially active butyl lithium (samples which had lost >30% of their original activity) produced the worst results. Any unprotected alcohol would also hinder the reaction and excess dihydropyran was used in the first step to ensure the reaction went to completeness. Another point noted was that fresher samples of prop-2-yn-1-yl oxytetrahydropyran usually worked better than older samples. It may be that this reduced activity was due to the samples picking up moisture which hinders the reaction.

![Chemical Structure](image)

**Figure 65:** Synthesis of 6-Hydroxy-2-methyl-hex-4ynyl p-nitrobenzoate

The coupling of sodium p-nitrobenzoate and subsequent deprotection of THP was completed and gave (135) with 85% yield. The unreacted prop-2-yn-1-yl oxytetrahydropyran, which was not removed from the earlier reaction, was easily removed at this stage.

The next step towards coupling the protecting group to thymidine was to convert the alcohol into the alkyl iodide. Propargyl systems have been coupled to nucleosides before as the alkyl bromide and protecting groups are commonly coupled in their halide form. Bremner successfully made alkyl iodides and coupled them to thymidine. The same procedures were attempted on (135) with no success at all. In these reactions imidazole, triphenylphosphine and iodine were combined with (135)
in ether and acetonitrile under an atmosphere of nitrogen. The order of addition was changed, as were reaction times and temperatures with no effect.

A possible explanation for this failure is starting material contamination so every reagent was purified by recrystalisation and anhydrous solvents were used, the acetonitrile was distilled fresh and the diethyl ether was purchased in anhydrous form. Fresh highly pure iodine was used and compound (135) was purified by flash column chromatography many times. All these developments failed to produce any alkyl iodide (136).

![Figure 66. Synthesis of alkyl iodide tried and proposed (136)](image)

This reaction was repeated multiple times without positive results. I now feel that this reaction scheme should have been abandoned at an earlier stage. Attempts to take a different route through a mesylate or tosylate system, detailed in the next section, could perhaps have been investigated further and sooner.

### 2.3.2 Butynol system, 5-Hydroxy-pent-2-ynoate ethyl ester

The proposed route to compound (140) is shown above in figure 67. Protection of butynol with dihydropyran and PPTS proceeded in high yield following the same procedure as used for the propargyl system. As with the propargyl system removal of the acetylenic proton proved a difficult reaction. Treatment with butyl lithium and reaction with ethyl chloroformate to give (138) was a low yield reaction with
multiple side reactions. The butynol reaction was worse than the propargyl system and yields were variable. Even in larger scales the best yield achieved was only 14%. One reason for this low yield could be the quality of the ethyl-chloroformate used. Although it was freshly distilled it was an old sample and may have had reduced activity. It was also found that reduced activity butyl lithium had a negative effect on yields, even when it still showed strong activity. Despite the very low yields a low yield reaction at the beginning of the synthesis can be accommodated and the reaction was not investigated further.

Figure 67. Synthesis of 5-hydroxy-pent-2-ynoate ethyl ester (139), and attempted synthesis of iodide (140)

Deprotection to give (139) proceeded in high yield but the same problems with the iodination reaction as the propargyl system stopped the synthesis at this stage. Repeated attempts using increasingly pure reagents produced no iodide. Analysis of the products from the reaction showed the presence of possible elimination products.
If this is the case then iodine could be being successfully attached but in the conditions HI eliminates.

![Figure 68. Mesylate and Tosylate alternative pathways](image)

As with the propargyl system I feel that I spent too long trying to get the iodination reaction to work and attempts to work around the impasse should have started earlier. A different route to (140) through a mesylate or tosylate system was only attempted when stocks of pure (139) were limited and only one attempt at producing the mesylate could be attempted and gave inconclusive results. The reaction could only be run on a small scale and no pure product was obtained however the hydrogen NMR did show strong evidence of product. Figure 68 shows the route that would have been attempted had more (139) been produced and is based on a literature precedent for a butynol type system.\(^{223}\)

### 2.3.3 o-Cresol system

With the propargyl and butynol systems failing to reached the stage of producing a protected ribonucleoside a third system was investigated in which the chemistry involved in synthesis and coupling was known to work. This system was based on the simple bromination of cresol.\(^{224}\) In the synthesis for this linker ortho-cresol is
first protected with acetic anhydride to give (141). The methyl group was then brominated using N-bromosuccinimide activated with dibenzoyl peroxide to give (142).

Figure 69. O-Cresol System synthesis

Figure 70. Coupling to uridine
The next step after successful bromination is to attach the protecting group to the 2' position of a ribonucleoside. The method chosen followed the example of Bremner and other syntheses of benzyl ethers. Uridine is first activated by reaction with dibutyltin oxide to produce the complex (143). The solvent is then changed from benzene to DMF and the alkyl bromide (142) is added. This should give a mixture of 2' and 3' protected products (144).

Initial attempts used unprotected uridine and it was envisaged that the two isomers might be separable by column chromatography. If not then tritylating the 5' position or adding the Markiewicz reagent should allow separation of 3' and 2' protected nucleotides. Instead of the mixture of two isomers expected, the reactions produced a complicated mixture of at least four uridine-containing compounds. Attempts to isolate the isomers by chromatography were unsuccessful. An attempt to react the mixture with dimethoxytrityl chloride to produce the tritylated mixture (145) was unsuccessful and the mixture appeared unaltered.

The facts that dimethoxytrityl had not reacted with the mixture and that more isomers were present than there should be suggested that the acetyl group was mobile during the reaction and was transferring to the hydroxyl positions of the ribose. This explains why 5' protection had appeared to have little effect. The acetyl group may transfer inter or intra-molecularly but either way it is possible to get a large number of combinations. For example compound 144a could have an acetyl group, having transferred from (142), covering the 5' hydroxyl group or the 2' hydroxyl group or both. When taken with 144b this gives eight possible compounds, only half of which would have a free 5' position to react with a trityl group.

A small sample of 5' Tbf-C_{10}-Tr-Urdine was then used in the coupling reaction. By covering the 5' position at the start it should greatly reduce the number of isomers and correctly protected uridine might be recoverable. Unfortunately the small amount of product recovered (146) was difficult to characterise. As the stock of Tbf-C_{10}-Tr (115) and its precursors was very low the Tbf C_8 ester material (109) was used.
instead. The Grignard reaction with bromobenzene proceeded in good yield to give the alcohol (147). Coupling to uridine was completed in very good 75% yield to give 4'-(10-(17'-tetrabenzo[a,c,g,i]fluorenyloctyloxy)-trityl-oxy-5'-uridine (148). The marked improvement over the C_{10} system is probably an indication in improvement in technique and the addition of the excess nucleotide and not an inherent difference between the two linkers.

![Diagram](image)

Figure 71. Coupling to Tbf C_{10} Tr protected Uridine

Coupling of bromo cresol to (148) produced a much simpler mixture of isomers (149) but purification was still extremely difficult and a wide range of solvent mixtures were tried in an effort to achieve separation. Mass spectrometry was used to identify the correct molecular weight compound. It was noticed during this process that the major product was mono acetylated Tbf-C_{10}-Tr-uridine with a trace amount of diacetylated product present. This suggests that the transfer of acetate groups
could occur by an intermolecular process as well as intra-molecularly. Eventually 40mg of pure product having the correct molecular weight was recovered.

![Chemical structures and reactions](image)

Figure 72. Synthesis and coupling of Tbf C₆ Tr protected Uridine
An excess of the protecting group was used for most of these coupling reactions due to the reactivity of the alkyl bromide. Rapid deterioration is marked by discolouration and increased acidity. This excess would have hindered the reaction due to the fact that acetates were migrating and potentially blocking both free sites on the uridine. This probably accounts for the very low yields and in future reactions the level of uridine and protecting group should be equivalent.

Despite the potential doubt over the material collected being protected and acetylated in the correct position, the compound was subjected to a simulation of deprotection techniques, the results of which were followed by mass spectrometry. The compound was stirred as a suspension in 30% ammonia solution at 55°C overnight. After this time the mass spectrum showed the elimination of the acetate protection. The material was then added to 2% TFA in 50ml water for one hour at room temperature. Thin layer chromatography showed that the sample had been broken down further and included a spot consistent with uridine. Attempts to isolate uridine were unsuccessful, due to the very small quantity available. Although this is far from a rigorous proof of the mechanism, it does give encouragement that this system could be refined to produce a feasible protecting group.

2.4 Conclusion and Future work

2.4.1 5' Protection

5' Tbf C₁₀ trityl protected nucleosides have been successfully synthesised in good yields. They have been incorporated into an oligomer and used to purify that oligomer to a high degree in a single step using the existing deprotection protocol designed for the dimethoxy trityl group. Some further developments could be investigated to improve coupling yields and purification.
Introducing a simple alcohol to trap the traces of acetyl chloride and the use of a coupling catalyst such as N,N-dimethylaminopyridine (DMAP) should significantly improve the coupling yields and raise them from the 68-83% range and closer to the 90+ achieved for dimethoxy trityl.

Some alterations to the protocol may improve yields. Steps such as taking greater care with the phosphoramidites keeping the samples cold and oxygen free before their use on the automated instrumentation may improve the yield for addition to the growing solid phase chain. The effect on yield of the lengthening the coupling time for the final step could also be furthered investigated. These steps might improve the yield for this crucial reaction.

Optimising the purification protocol to take full advantage of the properties of Tbf should eliminate the presence of truncated sequences completely from the final product. The protocol used was designed to take advantage of the small increase in hydrophobicity from DMTr and not the larger influence of Tbf. Optimising purification could also raise the recovered yield of the final step if it is found that there is incomplete cleavage of the Tbf 5’ protecting group. If this were the case then some of the product sequence would have remained attached to the Tbf group and would not have been recovered. Monomethoxy trityl is less acid labile than the dimethoxy trityl and therefore Tbf C_{10}Tr will also be less labile. Different deprotection conditions should be investigated to ensure maximum yield and minimal depurination.

Finally, different linker sizes should be investigated with respect to the coupling of the protected oligonucleotide on the solid support. One carbon has been proved to be too short and ten carbons have been shown to work. An optimum chain length may exist where yields for coupling and synthesis are maximised.
2.4.2 2' Protection

The concept behind the butynol and propargyl systems was never tested due to the failure to solve the problem of converting the hydroxyl group to a leaving group suitable for coupling to a nucleotide. The decision to look for a way around the problem was taken too late when time and stocks were depleted. If this project was to be taken further, the tosylate or mesylate system should be investigated and then either used directly or first converted to the alkyl iodide. It would also be worth looking at the butyllithium step, as the yields were variable but constantly poor.

The cresol system has successfully been coupled to uridine but with disappointing results. A small amount of pure material was collected and its deprotection did appear to work but the scale was small and the results are inconclusive. If this system was to be reinvestigated an alternative protection should be placed on the hydroxyl group of the benzene ring. The acetate protection proved too labile under the conditions used and even with 5' protected nucleotides, produced low yields and multiple isomers. Before attempting to alter the hydroxyl protection, a test system should be made to evaluate the deprotection. For example coupling the protecting group to a 5' protected thymidine, where the acetate has no place to migrate, should allow higher yields of protected nucleoside to be obtained for testing the theory of deprotection in a more rigorous fashion. If this proves encouraging then various base labile groups could be used to protect the hydroxyl group and investigated.
Chapter 3 Experimental

3.1 Notes on Instrumentation and General Techniques

Nuclear Magnetic Resonance Spectroscopy

$^1$H NMR
Routine proton nuclear magnetic resonance spectra were measured on a Varian Gemini 200 (200MHz) spectrometer. Higher field spectra for characterisations were measured on Brüker AC250 (250MHz) spectrometer. Deuterated solvents were used as indicated and samples contained no external reference.

$^{13}$C NMR, $^{31}$P NMR and 2D ($^1$H-$^1$H and $^1$H-$^{13}$C) NMR
Carbon, Phosphorus and two dimensional nuclear magnetic resonance spectra were run on a Brüker AC250, DPX360 or AC200 spectrometers. Carbon spectra were run at 62MHz, Phosphorus at 100MHz, $^1$H-$^1$H COSY at 250MHz or 360MHz and $^1$H-$^{13}$C at 360MHz and 91MHz. Deuterated solvents were used as indicated and samples contained no external reference.

Infra-Red Spectroscopy
Infrared spectra were obtained using a Bio-Rad FTS-7 spectrophotometer. Samples were prepared as indicated as either Nujol mulls, KBr discs, between KBr discs neat if a liquid or dissolved in Bromoform if a solid.

Mass Spectrometry
High and Low Mass Spectra using Fast atom bombardment (FAB) techniques were recorded on a Kratos MS50 TC spectrometer. Electron impact (EI) analyses were recorded on a Kratos analytical profile spectrometer. Experiments were run using 3-
nitrobenzyl alcohol (NOBA) or α-monothioglycerol as the matrix. LCQ negative ionisations were recorded on a Finnigan MAT LCQ spectrometer.

**Liquid Chromatography Mass Spectroscopy**

Liquid Chromatography Mass Spectroscopy (LCMS) was performed on a Waters Alliance 2690 HPLC system with the gradient 100% water to 100% Acetonitrile over 30 minutes. Negative ionisation was created with 50 Volts. The Mass Spectrometry was a micromass platform 2.

**Ultraviolet Spectroscopy**

Ultraviolet spectra were run using dichloromethane as the solvent on a Perkin Elmer Lambda 11 spectrophotometer.

**Elemental Analysis**

Elemental analysis for carbon, hydrogen, nitrogen and chlorine was performed on a Perkin Elmer 2400. Compounds containing the polycyclic aromatic structure Tetrabeno[a,c,g,i]fluorene routinely afforded low carbon values due to incomplete combustion. Studies with extended combustion times and the use of catalysts such as vanadium oxide did not improve the results obtained for the carbon values of such systems.\(^{16}\)

**Melting Points**

Melting points were determined on a Büchi 510 apparatus using open capillaries and are reported uncorrected.

**High Pressure Liquid Chromatography**

Reverse-phase high performance chromatography (RP-HPLC) was used for all HPLC except for the testing of the oligonucleotides at Link Technologies Ltd where a normal phase Hypersil C18 4.6x150 mm HPLC column at a flow rate of 1ml/min was used. The RP-HPLC was performed using a Gilson system comprising of two
306 solvent delivery systems, an 811C dynamic mixer, an 805 manometric module, a 119 UV/VIS detector and a Gilson software driven gradient controller. An Aquapore reverse phase silica column (300Å particle size) 10cm C₄, internal diameter 4.1mm using a flow rate of 1ml/min was used.

Gradients used (A) Acetonitrile, (B) water.
(1) 0-4min 50%A; 4-24min 50-90%A; 24-28min 90%A; 28-30min 90-50%A for reverse phase with samples dissolved in Acetonitrile and a minimum of Dioxan and (2) 0-30min 10-100%A, 30-35min 100%A for normal phase with the samples dissolved in Acetonitrile DCM 10/1.

Thin Layer Chromatography and Flash Column Chromatography
All thin layer chromatography samples were run on precoated plastic sheets with Kiesogel 60 f254. Solvent systems used (a) DCM, (b) DCM/Hexane 3:1, (c) DCM/Hexane 1:1, (d) 10% Methanol in DCM, (e) 5% Methanol in DCM (f) 1:1 Ethyl Acetate and Hexane (g) 30% Hexane in Ethyl Acetate. Spots were identified using ultra violet light at 254nm or 352nm or using a Potassium Permanganate dip.
For flash column chromatography, Silica gel 60 (230-240 mesh) was used.

Solvents and Chemicals used
All chemicals were purchased from Acros Ltd or Aldrich Ltd and used without further purification unless stated, except for tetrazole diisopropylammonium salt (DIHT) and Tetraisopropyl cyanoethyl phosphoramidite which were obtained from Link Technologies Ltd and the protected nucleotides, which were from Raylo chemicals Ltd. Dry solvents were distilled in the presence of the following drying agents. THF (Sodium wire and Benzophenone as indicator), DCM (Calcium Hydride) and Pyridine (Phosphorus pentachloride).

The pre-packed polypack columns were proprietary products from Glen Research, Sterling, Virginia, USA.
### 3.2 Synthetic Procedures

**Ethyl 4-(10 bromodecyloxy)-benzoate (98)**

Ethyl 4-hydroxybenzoate (5.13g, 31mmol) in DMF (50ml) was added dropwise to a suspension of sodium hydride (1.69g, 60% by weight, 42mmol) in DMF (50ml). After 1.5 hours stirring at RT dibromodecane (31.2g, 1.04mmol) in DMF (140ml) was added and the reaction stirred at RT for a further 19 hours. The reaction was then quenched with 100ml of water and 100ml saturated solution of NH₄Cl. The products were extracted with 3x100ml of diethyl ether. The organics were dried with magnesium sulfate and the solvent removed under high vacuum. The product was then purified by flash column chromatography (using Hexane till the dibromodecane was removed, DCM/Hexane 3:1 then DCM) and recrystallised from ethanol to give a colourless powder. (9.3g, 78%); m/z HRMS (FAB) found 385.13660 C₁₉H₂₉O₃Br requires 385.13783; C.H.N. found C, 59.46 H, 7.98 required C, 59.4 H, 7.60; £max; (KBr Disc) 2992, 2926, 2850, 1708, 1605, 1467 cm⁻¹; λmax; 258.7nm; Rₜ (A) 0.60; δH (CDCl₃, 250 MHz); 7.98-7.94 (2H, m, aromatic), 6.89-6.86 (2H, m, aromatic), 4.36-4.28 (2H, q, COO-CH₂), 4.00-3.95 (2H, t, O-CH₂), 3.41-3.36 (2H, t, R-CH₂-Br), 1.86-1.72 (4H, m, alkyl), 1.4301.25 (15H, m, alkyl); δC (CDCl₃, 63 MHz); 166.3 (q), 162.7 (q), 131.3 (CH), 122.5 (q) 113.8 (CH), 68.0 (CH₂), 60.4 (CH₂), 33.8-28.0 (9 x CH₂), 14.2 (CH₃).

**Bis-phenanthren-9-yl methanol (99)**

9-Bromophenanthrene (50g, 194mmol) in dry THF (120ml) was added dropwise to magnesium turnings (5g, 207mmol) and some crystals of iodine. The solution was allowed to stir at RT for 2.5 hours. Methyl formate (4.3ml) was then added dropwise and the reaction stirred for 3 hours. The mixture was then poured into ~200ml of 2M HCl solution and ice. Diethyl ether (200ml) was added and the entire solution
filtered. A white solid was collected which was washed with more water and ether. It was then dried with magnesium sulfate to give a fine colourless powder (31g 83%); m/z (EI) 384 (M)+ HRMS (FAB) found, 384.15101 C_{29}H_{20}O calculated as 384.15142; C.H.N. found, C, 85.22 H, 5.77 required C, 90.625 H, 5.21; v_{\text{max}}; (DCM) 3583, 3063, 2963, 1495, 1450 cm^{-1}; \lambda_{\text{max}}: 255, 278, 299 & 331nm; Rf (A) 0.45; Mp ~240°C; \delta_{\text{H}} (CDCl_{3} 250 MHz); 8.80-8.79 (2H, dd, aromatic), 8.76-8.70 (2H, dd, aromatic), 8.14 (2H, dd, aromatic), 7.81-7.51 (12H, m, aromatic), 7.25 (1H, s, aromatic), 2.5 (1H, s, CH); \delta_{C} (CDCl_{3} 63 MHz); 136.3, 131.2, 130.8, 130.3, 130.0, 129.0, 126.9, 126.7, 126.4, 126.1, 124.3, 123.2, 122.3, 69.7, 67.0.

Tetrabenzo[a,c,g,i]fluorene (100)

Trifluoroacetic acid (30ml) was added to bis-phenanthren-9-yl methanol (31g) in DCM (200ml) and the suspension was stirred for 1.5 hours. After which time the solution was filtered and the precipitate was washed with more DCM and with water. The powder was then dried to give a fine yellow powder. (20.5g 69%); m/z (EI) 366 (M)+ HRMS (FAB) found, 366.14191 C_{29}H_{18} calculated as 366.14085; C.H.N. found C, 93.93 H, 4.86 C_{29}H_{18} required C, 95.08 H, 4.91; v_{\text{max}}; 3060, 1610, 1500 cm^{-1}; \lambda_{\text{max}}: 377, 338, 303, 251 nm; Rf (A) 0.82; Mp ~265°C; \delta_{\text{H}} (CDCl_{3} 250 MHz); 8.84-8.56 (2H, m, aromatic), 8.29-7.08 (15H, m, aromatic), 5.39 (1H, s, CH); \delta_{C} (CDCl_{3} 63 MHz) 148.3, 141.3, 138.0, 137.3, 136.7, 136.0, 135.4, 135.3, 134.1, 133.2, 132.4, 130.7, 130.7, 130.3, 129.4, 128.6, 128.4, 128.2, 127.9, 127.5, 127.5, 127.2, 126.9, 126.6, 126.5, 126.3, 126.2, 126.0, 125.8, 125.6, 125.3, 125.1, 124.9, 124.6, 124.2, 123.6, 123.5, 123.2, 121.2, 87.2, 53.1.

(Note: there are two isomers of Tbf present leading to a greater number of signals in the NMR)
Ethyl 4-(10-(tetrabenzo[a,c,g,i]fluorenyl)decyloxy)-benzoate (102)

Tetrabenzo[a,c,g,i]fluorene (2.00g 5.5mmol) was suspended in dioxan (100ml) and was heated to 80°C while being flushed with nitrogen for ~2hrs. Ethyl 4-(10 bromodecyloxy)-benzoate (2.07g 5.4mmol) was dissolved in dioxan (30ml) and Nitrogen was bubbled through for ~2hrs. A solution of NBu₄OH (3.88g 6.0mmol) was prepared in the same way with dioxan (30ml). All three solutions were kept under Nitrogen before being used.

The Tbf suspension was heated to reflux. The Tbf dissolved at ~70°C to give a clear deep blue solution. The NBu₄OH solution was then added via a syringe and the mixture allowed to reflux for ten minutes. The ethyl 4-(10 bromodecyloxy)-benzoate solution was then added in the same manner and the mixture was refluxed for a further 2 hours, after which time the reaction was allowed to cool overnight before being filtered. The solvent was removed in vacuo to leave a dark red oil. The product was isolated by flash column chromatography. Beginning with DCM/hexane 3:1 then progressing up to DCM then finally 5% methanol in DCM. The product was a foam and could not be recrystallised (3.10g 86%). m/z (FAB) 670 (M⁺) C₄₈H₄₆O₃; HRMS (MH⁺) found 671.35267 requires 671.352352; C.H.N. found C, 85.15 H, 6.95 C₄₈H₄₆O₃ required C, 85.97 H, 6.87; Vₘₐₓ ; (KBr) 3066, 2919, 2848, 1701, 1605, 1508, 1498 cm⁻¹; λₘₐₓ : 255, 289, 301, 365, 381nm; Rf (A) 0.6; Mp ~60°C; δₜ (CDCl₃ 250 MHz); 8.83-8.77 (4H, m, aromatic), 8.71-8.66 (2H, m, aromatic), 8.28-8.23 (2H, m, aromatic), 7.99-7.93 (2H, m, aromatic), 7.73-7.57 (8H, m, aromatic), 6.87-6.81 (2H, m, aromatic), 5.06-5.01 (1H, t, tfb alkyl CH), 4.40-4.29 (2H, q, COO-CH₂), 3.90-3.84 (2H, t, O-CH₂), 2.66-2.56 (2H, m, alkyl), 1.69-0.35 (19H, m, alkyl); δₜ (CDCl₃ 63 MHz); 166.2, 162.5, 144.1, 136.5, 135.8, 131.2, 130.1, 128.6, 127.8, 127.2, 126.5, 125.6, 125.4, 124.8, 124.2, 123.2, 122.3, 113.7, 67.8, 60.3, 47.0, 33.3, 29.1, 28.9, 28.8, 28.7, 28.5, 25.5, 21.9, 15.0, 14.1.
4-(10-(17'-Tetrabenzo[a,c,g,i]fluorenyl)decyloxy)-4',4'''-dimethyl trityl alcohol (104)

4-Bromotoluene (5.54g 32.4mmol) in THF (20ml) was added via a syringe to magnesium turnings (0.79g 32.5mmol) and a catalytic amount of iodine in THF (40ml). The syringe was then washed through with more THF (20ml). The solution was then refluxed for one hour. Ethyl 4-(10-(tetrabenzo[a,c,g,i]fluorenyl)decyloxy)benzoate (2.17g 3.24mmol) in THF (60ml) was then added dropwise. The dropping funnel was then washed through with a further 60ml of THF. The mixture was then refluxed for another three hours. The reaction was then quenched with a saturated solution of NH₄Cl (~100ml). The organics were extracted with dichloromethane and dried (MgSO₄). After removal of the solvent a yellow oil remained (3.7g). The product was then isolated by flash column chromatography using 1:1 DCM/hexane. The product was a foam which could not be recrystallised (2.23g 85%); m/z (EI) 808 (M)+ HRMS (EI) found 808.42587, C₆₀H₅₆O₂ calculated as 808.42803; C.H.N. found C, 86.56 H, 7.22 required C, 89.07 H, 6.98; Vmax; (KBr) 3382, 3057, 2921, 2850, 1605, 1503, 1436 cm⁻¹; λmax; 254, 289, 301, 365, 381 nm; Rf (A) 0.54; δH (CDCl₃ 250 MHz); 8.84-8.78 (4H, m, aromatic), 8.72-8.69 (2H, m, aromatic), 8.28-8.24 (2H, m, aromatic), 7.74-7.60 (8H, m, aromatic), 7.37-7.10 (10H, m, aromatic), 6.81-6.77 (2H, m, aromatic), 5.05-5.01 (1H, t, Tbf alkyl CH), 3.86-3.81 (3H, t, CH₃), 2.71 (1H, s, OH), 2.66-2.58 (2H, m, alkyl), 2.35-2.33 (6H, m, alkyl), 1.68-0.36 (16H, m, alkyl); δC (CDCl₃ 63 MHz); 158.0, 144.3, 144.3, 139.1, 136.7, 136.5, 131.1, 130.3, 128.9, 128.7, 128.2, 127.9, 127.6, 127.3, 126.7, 125.7, 125.5, 124.9, 124.3, 113.5, 81.3, 67.7, 47.1, 33.4, 29.2, 29.0, 29.0, 28.6, 25.7, 22.0, 20.9.
Chapter 3 Experimental

4-(10-(17'-Tetrabenzo[a,c,g,i]fluorenyl)decyloxy)-4',4'''-dimethyl trityl chloride (105)

4-(10-(17'-Tetrabenzo[a,c,g,i]fluorenyl)decyloxy)-4',4'''-dimethyl trityl alcohol (0.719g 0.89mmol) and acetyl chloride (1.7g 21.7mmol) were combined in dry DCM(20ml) and stirred under nitrogen overnight (18 hours) at room temperature, after which time the solution was evaporated to dryness. Dry THF (10ml) was added and the solution evaporated to dryness again. An orange yellow foam remained (0.78g N/A %). (The product is ~0.04g over a 100% yield). C,H,N. and Cl found C, 85.67, H, 6.68, Cl 4.24, C₆₀H₅₅OCl required C, 87.08, H, 6.70, Cl, 4.28; \( \nu_{max} \) (Nujol) 2919,2850, 1502, 1454 cm⁻¹; \( \lambda_{max} \); 254, 289, 301, 365 and 381nm; \( \delta_H \) (CDCl₃ 250 MHz); 8.84-8.78 (4H, m, aromatic), 8.72-8.69 (2H, m, aromatic), 8.28-8.24 (2H, m, aromatic), 7.74-7.60 (8H, m, aromatic), 7.37-7.60 (8H, m, aromatic), 7.37-7.10 (10H, m, aromatic), 6.81-6.77 (2H, m, aromatic), 5.05-5.01 (1H, t, Tbf alkyl CH), 3.86-3.81 (3H, t, CH₃), 2.66-2.58 (2H, m, alkyl), 2.35-2.33 (6H, m, alkyl), 1.68-0.36 (16H, m, alkyl); \( \delta_C \) (CDCl₃ 63MHz) 144.3, 142.7, 137.3, 137.0, 131.1, 130.8, 130.3, 129.5, 129.1, 128.8, 128.4, 128.2, 127.9, 127.6, 127.4, 126.7, 125.8, 124.9, 124.3, 123.4, 113.2, 67.8, 47.1, 33.4, 29.2, 29.02, 28.99, 28.6, 25.7, 22.0, 21.6, 20.9.

Ethyl 4-(10 bromooctyloxy)-benzoate (107)

Ethyl 4-hydroxybenzoate (8.611g 51.9mmol) in DMF (100ml) was added dropwise to a suspension of sodium hydride (2.566g 60% by weight 64.1mmol) in DMF (100ml). After 1.5 hours stirring at RT dibromoocctane (41.4g 151.6mmol) in DMF (100ml) was added and the reaction stirred at RT for a further 24 hours. The reaction was then quenched with 100ml of water and 100ml NH₄Cl saturated solution and the products were extracted with diethyl ether. The organics were dried with Magnesium Sulfate and the solvent removed under high vacuum. The product was then purified by flash column chromatography, beginning with DCM/Hexane 3:1 then progressing
up to DCM then finally 5% Methanol in DCM, and recrystallised from methanol to
give a colourless powder. (12.69g, 68%); m/z HRMS (El) found 356.09871 and
358.09790 C\textsubscript{17}H\textsubscript{25}O\textsubscript{3}Br requires 356.09939 and 358.09679; C.H.N. found C, 57.33 H, 7.35 required C, 57.15 H, 7.05; \textbf{M}\textit{p} 39°C; \textbf{V}_{\text{max}} \textit{; (DCM solution) 2932, 2855, 1705,}
1604cm\textsuperscript{-1}; R\textsubscript{f} (A) 0.73; \delta\textsubscript{H} (CDCl\textsubscript{3} 250 MHz) 8.00-7.94 (2H, dt, 9.00Hz and 2.43Hz),
6.91-6.85 (2H, dt, 8.99Hz and 2.42Hz), 4.37-4.29 (2H, q, 7.13Hz), 4.01-3.96 (2H, t, 6.49Hz), 3.42-3.37 (2H, t, 6.81Hz), 1.88-1.73 (4H, m), 1.46-1.34(11H, m); \delta\textsubscript{C} (CDCl\textsubscript{3} 63 MHz); 166.3, 162.7, 131.4, 122.5, 113.9, 67.9, 60.5, 33.9-25.8 (7 peaks), 14.3

**Ethyl 4-(10 bromopropyloxy)-benzoate (108)**

Ethyl 4-hydroxybenzoate (8.78g 52.9mmol) in DMF (100ml) was added dropwise
to a suspension of sodium hydride (2.589g 60% by weight 64.7mmol) in DMF (100ml). After 1 hour stirring at RT dibromopropane (32.2g 159mmol) in DMF (100ml) was added and the reaction stirred at RT for a further 48 hours. The reaction
was then quenched with 100ml of water and 100ml NH\textsubscript{4}Cl saturated solution and the
products were extracted with diethyl ether. The organics were dried and the solvent
removed. The high vacuum was required to remove all the DMF. The product was
then purified by flash column chromatography, beginning with DCM/hexane 3:1
then progressing up to DCM then finally 5% methanol in DCM. The final product
was a clear liquid. (11.34g, 74.7%) m/z HRMS (El) found 286.02036 and 288.01855
C\textsubscript{12}H\textsubscript{15}O\textsubscript{3}Br requires 286.02046 and 288.01854; C.H.N. found C, 50.11 H, 6.40
required C, 50.19 H, 5.27; \textbf{V}_{\text{max}} \textit{; (KBr Disc) 3076, 2977, 1709, 1606cm\textsuperscript{-1}; R\textsubscript{f} (A)
0.71; \delta\textsubscript{H} (CDCl\textsubscript{3} 250 MHz) 8.00-7.96 (2H, 9.00Hz), 6.92-6.88 (2H, 9.00Hz), 4.37-
4.29 (2H, q, 7.13Hz), 4.16-4.11 (2H, t, 5.8Hz), 3.61-3.56 (2H, t, 6.39Hz), 2.37-2.27 (2H, 6.11Hz), 1.39-1.33(3H, 7.12Hz); \delta\textsubscript{C} (CDCl\textsubscript{3} 63 MHz) 166.2, 162.2, 131.4,
123.0, 113.9, 65.3, 60.5, 32.0, 29.6, 14.2.
Ethyl 4-(10-(tetrabenzo[a,c,g,i]fluorenyl)octyloxy)-benzoate (109)

Tetrabenzo[a,c,g,i]fluorene (5.02g 13.7mmol) was suspended in dioxan (200ml) and was heated to 80°C while being flushed with nitrogen for ~2hrs. Ethyl 4-(10 bromooctyloxy)-benzoate (4.82g 13.5mmol) was dissolved in dioxan (50ml) and nitrogen was bubbled through for ~2hrs. A solution of NBu₄OH (9.73g 15.0mmol) solution was prepared in the same way with dioxan (50ml). All three solutions were prepared then kept under Nitrogen before being used.

The Tbf suspension was heated to reflux. The Tbf dissolved at ~70°C to give a clear deep blue solution. The NBu₄OH solution was then added via a syringe and the mixture allowed to reflux for ten minutes. The ethyl 4-(10 bromooctyloxy)-benzoate solution was then added in the same manner and the mixture was refluxed for a further 2 hours, after which time the reaction was allowed to cool overnight before being filtered. The solvent was removed in vacuo to leave a dark red oil. The product was isolated by flash column chromatography, beginning with DCM/hexane 3:1 then progressing up to DCM then finally 5% methanol in DCM, to give a foam which could not be recrystallised. (7.07g 81.6%); m/z (FAB) 642 (M⁺); HRMS (M⁺) found 642.31334 C₄₆H₄₂O₃ requires 642.31340 (MH⁺) found 643.32125 requires 643.32122; C,H,N. found 82.93 C, 6.45 H required 85.95 C, 6.59 H; Vₐₘₐₓ; (DCM) 3018, 2970, 1604 cm⁻¹; Rₜ (A) 0.77; δₜ (CDCl₃ 250 MHz); 8.82-8.75 (6H, m), 8.70-8.67(2H, dd, 1.48Hz and 8.00Hz), 7.98-7.92 (2H, d, 9.00Hz), 7.73-7.58 (8H, m), 6.81-6.75 (2H, d, 8.99Hz), 5.02-4.99 (1H, t, 4.37Hz), 4.39-4.31 (2H, q, 7.13Hz) 3.75-3.70 (2H, t, 6.59Hz), 2.64-2.56 (2H, dt), 1.49-0.75 (13H, m) 0.37-0.31 (2H, m); δₜ (CDCl₃ 63 MHz); 166.3, 162.6, 144.2, 136.7, 131.3, 131.1, 128.7, 127.9, 127.3, 126.7, 125.8, 125.5, 124.9, 124.3, 123.4, 122.4, 113.8, 67.8, 60.4, 47.1, 33.3, 29.1, 28.6, 28.5, 25.3, 22.0, 14.3. ¹H-¹H COSY Spectra (CDCl₃ 250MHz) See Appendix.
Chapter 3 Experimental

**Ethyl 4-(10-(tetrabenzo[a,c,g,i]fluorenyl)propyloxy)benzoate (110)**

Tetrabenzo[a,c,g,i]fluorene (2.01g 5.5mmol) was suspended in dioxan (100ml) and was heated to 80°C while being flushed with nitrogen for ~2hrs. Ethyl 4-(10 bromopropyloxy)-benzoate (1.611g 5.61mmol) was dissolved in dioxan (30ml) and Nitrogen was bubbled through for ~2hrs. A solution of NBu₄OH (3.87g 6.0mmol) solution was prepared in the same way with dioxan (30ml). All three solutions were prepared then kept under Nitrogen before being used.

The Tbf suspension was heated to reflux. The Tbf dissolved at ~70°C to give a clear deep blue solution. The NBu₄OH solution was then added via a syringe and the mixture allowed to reflux for ten minutes. The ethyl 4-(10 bromopropyloxy)-benzoate solution was then added in the same manner and the mixture was refluxed for a further 2.5hours, after which time the reaction was allowed to cool overnight before being filtered. The solvent was removed *in vacuo* to leave a dark red oil. Attempts to isolate the product by flash column chromatography, using a range of different solvent combinations. Crude NMR does however show product. Reaction repeated with the same result.

**Ethyl 4-methoxy benzoate (111)**

Potassium carbonate (50g, 363mmol) methyl iodide (45ml, 722mmol) and ethyl 4-hydroxybenzoate (12.0g 72.3mmol) were stirred together as a suspension in acetone (300ml) for 17 hours overnight at RT under Nitrogen. After this time the t.l.c. showed no Ethyl 4-hydroxybenzoate remaining. The acetone was removed before water and ether were added. The organic extracts were dried washed with Na₂S₂O₃ solution and solvent removed. (12.26g 94.2%) m/z HRMS (FAB) found 180.07792 C₁₀H₁₂O₃ requires 180.07864; ν max; (KBr plates) 3072, 2977, 2838, 1708, 1605cm⁻¹; Rf (A) 0.63; δH (CDCl₃ 250 MHz) 7.99-7.95 (2H, dd, 6.9Hz 2.1Hz) 6.91-6.85 (2H,
Chapter 3 Experimental

4- Methoxy triphenyl methanol (112)

Bromobenzene (7.64g 48.7mmol) in THF (40ml) was added dropwise to magnesium turnings (1.22g 50.2mmol) and a catalytic amount of Iodine also in THF (40ml) at RT under nitrogen. The solution was refluxed for 45mins then ethyl 4-methoxy benzoate was added in THF (40ml) and the solution was refluxed for a further 3.5 hours. The reaction was quenched by the addition of NH₄Cl solution and the organics were extracted with ether. The ether was dried and solvent removed to give the product which was recrystallised from propan-2-ol. (4.23g 89.8%); m/z HRMS (FAB) found 290.13147 C₂₀H₁₈O₂ requires 290.13068; C.H.N. found C, 82.63 H, 6.11 required C, 82.73 H, 6.25 Vₘₚₑₓₙₚ; (KBr Disc) 3434, 3017, 2969, 1605, 1507, 1444, 1247 cm⁻¹; Rₜ (A) 0.50; δₜ (CDCl₃ 250 MHz); 7.31-7.25 (10H, m) 7.20-7.16 (2H, d, 9.03Hz), 6.86-6.82 (2H, d, 9.03Hz), 3.80 (3H, s), 2.85 (1H, s); δₐ(CDCl₃ 62 MHz), 158.5, 146.9, 139.0, 129.1, 124.74, 127.71, 127.0, 113.0, 81.6, 55.1.

4 Methoxy triphenyl methyl chloride (113)²²⁶

4-Methoxy triphenyl methanol (2.00g 6.9mmol) and acetyl chloride (5ml 70mmol) were combined in dry Benzene (10ml) and stirred under Argon overnight (22 hours) at room temperature, after which time the solution was evaporated to dryness. Dry Benzene (10ml) was added and the solution evaporated to dryness again. An orange yellow powder remained. This was used without further purification or characterisation.
**5'-4-Methoxy trityl thymidine (114)**

Thymidine (1.60g 6.63mmol) was twice co-evaporated with dry Pyridine (~5ml) then added to 4-Methoxy triphenyl methyl chloride (6.90mmol) in dry pyridine (6ml). The reagents were then stirred under Argon for seven days. Solvent was then removed under high vacuum and the residue was dissolved in water and DCM. The organics were separated, dried with magnesium sulfate and purified by flash column chromatography, using DCH/hexane 3:1 then progressing to DCM and DCM with methanol from 1 to 5%. giving a white solid (3.90g 57%); m/z (FAB) 537 (M⁺ and Na) C₂₉H₂₈N₂O₆ calculated as 514; C,H,N. found, C, 68.94 H, 6.12 N, 4.99 required C, 70.02 H, 5.88 N, 5.44; Mp 96-98°C, literature Mp 102-105°C Vₓₒₓ (CBr₄, KBr plates) 3384, 3019, 1682, 1508, 1250cm⁻¹; δₓ (CDCl₃ 250 MHz) 9.47 (1H, s), 7.60-7.59 (1H, d, 1.08Hz), 7.42-7.14 (12H, m) 6.85-6.81 (2H, d, 8.91Hz), 6.46-6.40 (1H, t, 5.95Hz), 4.56 (1H, m), 4.08-4.07 (1H, m), 3.77 (3H, s), 3.49-3.32 (2H, m) 3.21 (1H, broad), 2.42-2.30 (2H, m), 1.43 (3H, d, 0.74Hz); δₓ (CDCl₃ 62 MHz) 163.9, 158.6, 150.5, 143.7, 143.6, 135.6, 134.7, 130.2, 129.0, 128.2, 127.9, 127.7, 127.1, 127.0, 113.1, 113.0, 111.2, 87.0, 86.1, 84.6, 72.3, 63.6, 55.1, 40.8, 11.7.

**4-(10-(17'-Tetrabenzo[a,c,g,i]fluorenyldecyloxy)-trityl alcohol (115)**

4-Bromobenzene (15.9g, 101mmol) in THF (50ml) was added via a syringe to magnesium turnings (2.40g 97.2mmol) and a catalytic amount of iodine in THF (50ml). The solution was then refluxed for 30mins. Ethyl 4-(10-(tetrabenzo[a,c,g,i]fluorenyldecyloxy)-benzoate (6.52g 9.73mmol) in THF (130ml) was then added dropwise. The mixture was then refluxed for another five hours. The reaction was then quenched with a saturated solution of NH₄Cl (~100ml). The organics were then extracted with dichloromethane and dried (MgSO₄). After removal of the solvent a yellow oil remained. The product was then purified by flash column chromatography using 1:1 DCM/Hexane. (6.29g 82.8%) m/z HRMS (FAB)
found 780.39841 C\textsubscript{58}H\textsubscript{52}O\textsubscript{2} calculated 780.39673; C.H.N. found C, 90.23 H, 7.02 required C, 89.19 H, 6.71; Mp 75-76°C; \textit{V}_{\text{max}}; 3419.6, 3017.6, 2970.0, 1640.1, 1140.7 cm\textsuperscript{-1}; \lambda_{\text{max}}; 288.7, 301.3, 365.1, 381.5 nm; \textit{R}_{f} (A) 0.76 (B) 0.59; \delta_{H} (CDCl\textsubscript{3} 250 MHz); 8.83-8.78 (4H, m), 8.71-8.67(2H, dd, 7.97Hz and 1.39Hz), 8.28-8.24 (2H, m), 7.74-7.59 (8H, m), 7.35-7.24 (10H, m), 7.17-7.11 (2H, d, 8.89Hz), 6.81-6.75 (2H, d, 8.91Hz), 5.07-5.03 (1H, t, 4.30Hz), 3.85-3.80 (2H, t, 6.56Hz), 2.77 (1H, broad), 2.66-2.58 (2H, dt, 11.85Hz and 4.47Hz), 1.67-1.56 (2H, m), 1.27-1.15 (2H, m), 1.05-0.73 (10H, m), 0.38-0.32 (2H, m) \delta_{C} (CDCl\textsubscript{3} 63 MHz); 158.1, 147.0, 144.3, 138.8, 136.7, 131.1, 130.3, 129.0, 128.7, 127.9, 127.7, 127.4, 127.0, 126.7, 125.8, 125.5, 124.9, 124.4, 123.4, 113.6, 81.6, 67.8, 47.1, 33.4, 29.2, 29.0, 28.6, 25.7, 22.0; \textsuperscript{1}H - COSY (CDCl\textsubscript{3} 360MHz) - See appendix.

4-(10-(17'-Tetrabenzo[a,c,g,i]fluorenyldecoxy)-trityl chloride (116)

4-(10-(17'-Tetrabenzo[a,c,g,i]fluorenyldecoxy)-trityl alcohol (1.00g 1.28mmol) and acetyl chloride (5ml 70mmol) were combined in dry Benzene (20ml) and stirred under nitrogen for 22 hours at room temperature. After which time the solution was evaporated to dryness. Dry benzene (10ml) was added and the solution evaporated to dryness again. An orange yellow foam remained (1.02g 100%). C.H.N. and Cl found C, 87.98 H, 6.40, Cl, 1.46, C\textsubscript{58}H\textsubscript{51}OCl required C, 87.13, H, 6.43, Cl, 4.43; \textit{V}_{\text{max}}; 3017, 2925, 2853, 1714, 1605, 1503 cm\textsuperscript{-1}; \delta_{H} (CDCl\textsubscript{3} 250 MHz); 8.83-8.78 (4H, m), 8.71-8.67(2H, dd, 7.97Hz and 1.39Hz), 8.28-8.24 (2H, m), 7.74-7.59 (8H, m), 7.35-7.24 (10H, m), 7.17-7.11 (2H, d, 8.89Hz), 6.81-6.75 (2H, d, 8.91Hz), 5.07-5.03 (1H, t, 4.30Hz), 3.85-3.80 (2H, t, 6.56Hz), 2.66-2.58 (2H, dt, 11.85Hz and 4.47Hz), 1.67-1.56 (2H, m), 1.27-1.15 (2H, m), 1.05-0.73 (10H, m), 0.38-0.32 (2H, m)

(Note: The t.l.c. of the product did show two spots, consistent with product and starting trityl alcohol being present. However, it was shown that in the solvent system used silica can convert the chloride back to the alcohol.)
4’-(10-(17’-Tetrabenzo[<a,c,g,i>]fluorenyldecyloxy)-trityl-oxy-5’-thymidine (117)

4-(10-(17’-tetrabenzo[a,c,g,i]fluorenyldecyloxy)-trityl alcohol (2.0179g 2.58mmol) and acetyl chloride (1ml 14mmol) were combined in dry benzene (10ml) and stirred under nitrogen for 24 hours at room temperature, after which time the solution was evaporated to dryness. Dry Benzene (10ml) was twice added and the solution evaporated to dryness again to leave 4-(10-(17’-tetrabenzo[a,c,g,i]fluorenyldecyloxy)-trityl chloride as an orange solid.

Thymidine (0.708g 2.94mmol) was twice co-evaporated with dry pyridine (~5ml) then added to the 4-(10-(17’-tetrabenzo[a,c,g,i]fluorenyldecyloxy)-trityl chloride (~2.58mmol) in dry pyridine (15ml). The reagents were then stirred under argon for seven days. Solvent was then removed under high vacuum. The residue was dissolved in water and DCM. The organics were separated, dried with calcium carbonate and purified by flash column chromatography, using DCM and methanol 0 to 10%, to give a colourless powder as product, which could not be recrystallised. (2.0g 77%); m/z HRMS (FAB) found 1004.47666 C_{68}H_{64}N_{2}O_{6} requires 1004.47644; Mp 123-125°C; C.H.N. found C, 78.19 H, 6.33 N, 2.64 required C, 81.25, H, 6.42 N, 2.79; V_{max} (DCM) 3387, 3018, 2924, 2855, 2360, 1683, 1247 cm^{-1}; V_{max} (CBr_{4}, KBr plates) 3379, 3187, 3017, 2924, 2851, 1692, 1679, 1504, 1468, 1248cm^{-1}; λ_{max}; 301.4, 364.94, 380.8 nm; R_{f} (A) 0.00 (D) 0.73; δ_{H} (CDCl_{3} 250 MHz); 9.06 (1H, s), 8.81-8.76 (4H, m), 8.69-8.65 (2H, dd, 7.97Hz and 1.27Hz), 8.27-8.23 (2H, m), 7.72-7.57 (9H, m), 7.41-7.19 (12H, m), 6.79-6.75 (2H, d, 8.90Hz), 6.44-6.38 (1H, t, 5.91Hz), 5.06-5.02 (1H, t, 4.28Hz), 4.59-4.57 (1H, m), 4.08-4.02 (1H, m), 3.83-3.78 (2H, t, 6.52Hz), 3.52-3.32 (2H, m), 2.65-2.56 (2H, m), 2.45-2.29 (2H, m), 1.65-1.53 (2H, m), 1.41-0.71 (17H, m) 0.33 (2H, m); δ_{C} (CDCl_{3} 63MHz) 163.7, 158.2, 150.4, 144.2, 143.8, 143.7, 136.7, 135.6, 134.3, 131.2, 130.25, 128.7, 128.2, 127.9, 127.8, 127.7, 127.3, 127.1, 126.7, 125.8, 125.5, 124.9, 124.3, 123.4, 113.6, 111.1, 87.1, 86.0, 84.5, 72.2, 67.8, 64.3, 63.6, 62.9, 52.7, 47.1, 40.8, 33.4, 29.2, 29.0, 28.6, 25.7, 25.2, 22.0,
Chapter 3 Experimental

11.6, 7.7; $^1$H - COSY (CDCl$_3$, 360MHz) and $^1$H / $^{13}$C Correlation Spectra (CDCl$_3$, 360MHz and 90MHz) - See appendix.

4'-(10-(17'-Tetabenzo[a,c,g,i] fluorenyldecyloxy)-trityl-oxy-5'-uridine (118)

4-(10-(17'-teterbenzo[a,c,g,i]fluorenyldecyloxy)-trityl alcohol (0.508g 0.65mmol) and acetyl chloride (3ml 42mmol) were combined in dry benzene (10ml) and stirred under Nitrogen for 24 hours at room temperature, after which time the solution was evaporated to dryness. Dry benzene (10ml) was twice added and the solution evaporated to dryness again to leave 4-(10-(17'-teterbenzo[a,c,g,i] fluorenyldecyloxy)-trityl chloride as an orange solid.

Uridine (0.162g 0.66mmol) was twice co-evaporated with dry pyridine (~5ml) then added to the 4-(10-(17'-teterbenzo[a,c,g,i]fluorenyldecyloxy)-trityl chloride (0.65mmol) in dry pyridine (10ml). The reagents were then stirred under Argon for seven days. Solvent was then removed under high vacuum. The residue was dissolved in water and DCM. The organics were separated, dried with calcium carbonate and purified by flash column chromatography using DCM and methanol 0 to 10%, to give a colourless powder as product, which could not be recrystallised. (0.30g 46%); m/z HRMS (FAB) found 1007.46331 C$_{67}$H$_{62}$N$_2$O$_7$ requires 1007.46318; C.H.N. found C, 74.03 H, 6.42 N, 2.78 required C, 79.92 H, 6.46 N, 2.78; (CBr$_4$ KBr Disc) 3388, 3018, 2923, 2851, 1681, 1461, 1247, 1141cm$^{-1}$; Rf (A) 0.00 (D) 0.57; $\delta$$_H$ (CDCl$_3$, 250 MHz); 8.83-7.18 (29H, m), 6.79-6.76 (2H, d, 8.90Hz), 5.85-5.81 (1H, d 2.4Hz) 5.06-5.03 (1H, t, 4.3Hz), 4.41-4.37 (1H, m), 4.25-4.21 (1H, m), 4.13-4.11 (1H, d, 4.33Hz), 3.84-3.77 (2H, m), 3.46 (2H, m), 2.62-2.56 (2H, m), 2.17-0.71 (16H, m), 0.35-0.25 (2H, m); $\delta$$_C$ (CDCl$_3$, 62 MHz) 163.5, 158.2, 150.8, 144.2, 140.2, 136.7, 134.0, 131.8, 131.1, 130.3, 130.2, 129.7, 128.7, 128.2, 127.9, 127.8, 127.7, 127.3, 127.3, 127.0, 126.7, 126.1, 125.8, 125.5, 124.9, 124.3, 123.4, 121.9, 113.6,
4'-{(10-(17'-Tetrabenzo[a,c,g,i] fluorenyldecyloxy)-trityl-oxy-5'-N²-isobutyryl-
2'-deoxyguanosine (119)

4-(10-(17'-Tetrabenzo[a,c,g,i]fluorenyldecyloxy)-trityl alcohol (1.5368g 1.9mmol) and acetyl chloride (1.5ml 21mmol) were combined in dry benzene (5ml) and stirred under Nitrogen for 24 hours at room temperature, after which time the solution was evaporated to dryness. Dry benzene (10ml) was twice added and the solution evaporated to dryness again to leave 4-(10-(17'-tetrabenzo[a,c,g,i] fluorenyldecyloxy)-trityl chloride as an orange solid.

N²-Isobutyryl-2'-deoxyguanosine (0.76g 2.3mmol) was twice co-evaporated with dry pyridine (~5ml) then added to 4-(10-(17'-tetrabenzo[a,c,g,i]fluorenyldecyloxy)-trityl chloride (1.9mmol) in dry pyridine (15ml). The reagents were then stirred under Argon for seven days. Solvent was then removed under high vacuum. The residue was dissolved in water and DCM. The organics were separated, dried with calcium carbonate and purified by flash column chromatography, using DCM and methanol 0 to 10%, to give a colourless powder as product, which could not be recrystallised. (1.4g 68.2%); m/z HRMS (FAB) found 1100.53541 C₇₂H₇₀N₅O₆ requires 1100.53261; Mp 152-153°C; C.H.N. found C, 76.42 H, 6.48 N, 6.04 required C, 78.59 H, 6.32 N, 6.36; \( \lambda_{max} \) (CBr₄, KBr plates) 3361, 3187, 3017, 2925, 2851, 1947, 1673, 1605, 1559, 1248cm\(^{-1}\); \( \delta_{max} \) 301, 365, 381nm; R₆ (A) 0.0 (E) 0.75; \( \delta_{H} \) (CDCl₃ 250 MHz) 12.30 (1H, s, NH), 9.9 (1H, s, NH), 8.78-8.64 (6H, m), 8.25-8.19 (2H, m), 7.78-7.11 (21H, m), 6.70-6.66 (2H, d, 8.89Hz), 6.15 (1H, broad), 5.00-4.97 (1H, tbf alkyl CH, t, 4.21Hz), 4.70 (1H, m), 4.48 (1H, broad), 4.17 (1H, m), 3.75-3.70 (2H, t, 6.16Hz), 3.29 (2H, m), 2.58-2.24 (5H, m), 1.56-1.50 (2H, m), 1.26-0.67 (18H, m), 0.31 (2H, m). \( \delta_{C} \) (CDCl₃ 63Hz) 179.8, 158.0, 155.7, 149.9, 148.3, 147.6, 144.2,
Chapter 3 Experimental

4'-\((10-\text{-Tetrabenzo}[a,c,g,i] \text{ fluorenyldecyloxy})\text{-trityl-oxy-5}'\text{-N}^4\text{-benzoyl-2'}\text{-deoxycytidine}\) (120)

4-(10-\((17'\text{-Tetrabenzo}[a,c,g,i] \text{fluorenyldecyloxy})\text{-trityl alcohol}\) (1.517g 1.9mmol) and acetyl chloride (1.5ml 21mmol) were combined in dry benzene (5ml) and stirred under nitrogen for 24 hours at room temperature, after which time the solution was evaporated to dryness. Dry benzene (10ml) was twice added and the solution evaporated to dryness again to leave 4-(10-(17'-tetrabenzo[a,c,g,i] fluorenyldecyloxy)-trityl chloride as an orange solid.

N^4-Benzoyl-2'-deoxycytidine (0.86g 2.6mmol) was twice co-evaporated with dry pyridine (~5ml) then added to the 4-(10-(17'-tetrabenzo[a,c,g,i]fluorenyldecyloxy)-trityl chloride (1.9mmol) in dry pyridine (10ml). The reagents were then stirred under Argon for seven days. Solvent was then removed under high vacuum. The residue was dissolved in water and DCM. The organics were separated, dried over calcium carbonate and purified by flash column chromatography, using 0 to 10% methanol in DCM, to give a colourless powder as product, which could not be recrystallised. (1.72g 83.2%); m/z HRMS (FAB) found 1094.51081 C_{74}H_{68}N_{3}O_{6} requires 1094.51082; Mp 136°C; C.H.N. found C, 78.72 H, 6.22 N, 3.74 required C, 81.22 H, 6.17 N, 3.84; V_{\text{max}} (CBr₄, KBr plates) 3649, 3389, 3060, 3018, 2925, 2852, 1949, 1811, 1697, 1649, 1481, 1391, 1310, 1249 cm\(^{-1}\); λ_{\text{max}}; 301.4, 365.0, 381.7nm; R_{f} (A) 0.0 (E) 0.78; δ_{H} (CDCl₃, 250 MHz); 8.81-8.76 (4H, m) 8.69-8.65 (2H, dd, 7.99Hz and 1.47Hz), 8.27-8.23 (2H, m), 7.92-7.11 (27H, m) 6.83-6.80 (2H, d, 8.95Hz), 6.33-6.24 (1H, m), 5.05-5.02 (1H, t, 4.36Hz) 4.37-4.36 (1H, m), 4.26-4.25 (1H, m), 3.86-3.80
(2H, t, 6.49Hz), 3.47-3.46 (2H, m), 2.88-2.75 (2H, m), 2.64-2.56 (2H, m), 1.65-1.53 (2H, quintet, 7.02Hz), 1.37-0.70 (8H, m), 0.33 (2H, m); δ (CDCl₃ 62 MHz); 162.1, 158.2, 144.6, 144.2, 143.7, 143.6, 136.6, 134.4, 132.8, 131.1, 130.2, 128.7, 128.2, 127.9, 127.4, 127.3, 127.0, 126.6, 125.7, 125.5, 124.9, 124.3, 123.3, 113.6, 96.5, 87.2, 87.0, 86.3, 70.7, 67.7, 65.7, 62.6, 47.0, 41.9, 33.3, 29.2, 29.0, 28.6, 25.7, 22.0, 15.1. ¹H-COSY (CDCl₃ 360MHz) - See appendix

4'-(10-(17'-Tetrabenzo[a,c,g,i] fluorenyldeoxyloxy)-trityl-oxo-5'-N⁶-benzoyl-2'-deoxyadenosine (121)

4-(10-(17'-Tetrabenzo[a,c,g,i]fluorenyldeoxyloxy)-trityl alcohol (2.00g 2.57mmol) and acetyl chloride (1ml 14mmol) were combined in dry benzene (10ml) and stirred under Nitrogen for 24 hours at room temperature, after which time the solution was evaporated to dryness. Dry benzene (10ml) was twice added and the solution evaporated to dryness again to leave 4-(10-(17'-tetrabenzo[a,c,g,i]fluorenyldeoxyloxy)-trityl chloride as an orange solid.

N⁶-Benzoyl-2'-deoxyadenosine (0.95g 2.8mmol) was twice co-evaporated with dry pyridine (~5ml) then added to the 4-(10-(17'-tetrabenzo[a,c,g,i]fluorenyldeoxyloxy)-trityl chloride (2.6mmol) in dry pyridine (15ml). The reagents were then stirred under Argon for seven days. Solvent was then removed under high vacuum. The residue was dissolved in water and DCM. The organics were separated, dried with calcium carbonate and purified by flash column chromatography, using 0 to 10% methanol in DCM, to give a colourless powder as product, which could not be recrystallised. (2.0g 70.4%); m/z HRMS (FAB) found 1118.52248 C₇₅H₆₈N₅O₅ requires 1118.52205; Mp 130°C; C.H.N. found, C, 75.30 H, 5.94 N, 8.17 required C, 80.55 H, 6.04 N, 6.26; Vₘₐₓ (CBr₄, KBr plates) 3662, 3373, 3017, 2925, 2852, 1949, 1816, 1692, 1681, 1608, 1504, 1452, 1249cm⁻¹; λₘₐₓ 301.0, 365.3, 381.0nm; Rₐ (A) 0.0 (E) 0.73; δH (CDCl₃ 250 MHz); 8.80-8.64 (8H, m), 8.30-7.94 (8H, m), 7.71-7.14 (19H,
Chapter 3 Experimental

4-Methoxy-trityl-oxy-5'-thymidine-3'-O,N,N-diisopropyl-β-cyanoethyl phosphoramidite (122)

4-Methoxy-trityl-oxy-5'-thymidine (0.39g 0.755mmol) and tetrazole diisopropylammonium salt (DIHT) (0.068g 0.40mmol) were dried by co-evaporating with dry acetonitrile (~10ml) three times and then dissolved in dry alcohol-free DCM (6ml) and stirred under Argon. Tetraisopropyl cyanoethyl phosphoramidite (0.38g 1.25mmol) was then injected and the solution was stirred at RT for 48 hours. HPLC monitoring showed the reaction had gone to completion. Sodium Bicarbonate solution was added to quench the reaction then the organics were extracted with DCM. The DCM was washed with brine then dried and the solvent removed by vacuum. The solid residue was dissolved in the minimum of DCM and precipitated in hexane then dissolved in the minimum of ethyl acetate and precipitated in pentane. $^{31}$P and $^1$H NMR were taken at this point. An attempt to further purify using flash column chromatography, using 0 to 10% methanol in DCM, was undertaken which destroyed the sample. Crude $\delta_H$(CDCl$_3$ 200 MHz); 7.58 (1H, s), 7.42-7.26 (12H, m), 6.86-6.82 (2H, d, 8.79Hz), 6.41 (1H, t), 4.57 (1H, m), 4.06-4.05 (1H, d, 2.92Hz), 3.79 (3H, s), 3.45-3.38 (2H, dq, 11.4Hz and 2.4Hz), 2.37 (2H, m), 1.62-0.82 (21H, m); $\delta_p$(CDCl$_3$ 101MHz) 149.64, 149.21.
4'-[(10-(17'-tetrabenzo[a,c,g,i]fluoren-yl)decyloxy)-trityl-oxy-5'-N^4-benzoyl-2'-deoxycytidine-3'-O-N,N-diisopropyl-β-cyanoethyl phosphoramidite (123)

4'-[(10-(17'-tetrabenzo[a,c,g,i]fluorenyl)decyloxy)-trityl-oxy-5'-N^4-Benzoyl-2'-deoxycytidine (1.54g 1.41mmol) and tetrazole diisopropylammonium salt (DIHT) (0.128g 0.748mmol) were dried by co-evaporating with dry acetonitrile (~10ml) three times and then dissolved in dry alcohol-free DCM (8ml) and stirred under argon. Tetraisopropyl cyanoethyl phosphoramidite (0.679g 2.26mmol) was then injected and the solution was stirred at RT for 24 hours. Sodium bicarbonate solution was added to quench the reaction then the organics were extracted with DCM. The DCM was washed with brine then dried and the solvent removed by vacuum. The solid residue was dissolved in the minimum of DCM and precipitated in hexane then dissolved in the minimum of ethyl acetate and precipitated in pentane to give a colourless powder that was then dried at RT under vacuum. (1.64g 89.9%); m/z (FAB) 1293 (M+) C_{83}H_{84}N_{5}O_{7}P; HRMS (MH^+) found 1294.61869 requires 1294.61869; Mp 112°C; C.H.N. found C, 72.59 H, 6.60 N, 5.73 required C, 77.01 H, 6.54 N, 5.41; V_{max} (CBr_4, KBr plates) 3018, 2925, 2253, 1696, 1656, 1609, 1555, 1480, 1393 cm^{-1}; λ_{max}; 301.4, 365.0, 381.1nm; δ_{H} (CDCl_3, 250 MHz); 8.77-8.66 (6H, m), 8.34-8.22 (3H, m) 7.88-7.85 (2H, d, 7.27Hz), 7.66-7.25 (24H, m), 6.83-6.28 (2H, d, 5.92Hz), 6.29-6.28 (1H, d, 4.82Hz), 5.01 (1H, br.s), 4.65-4.63 (1H, m), 4.34-4.08 (1H, m), 3.94-3.30 (8H, m), 2.83-2.30 (6H, m), 1.60 (2H, m), 1.36-0.71 (24H, m), 0.33 (2H, m); δ_{C} (CDCl_3, 62 MHz); 168.8, 162.1, 158.3, 155.0, 144.3, 143.7, 143.5, 136.7, 134.4, 132.9, 131.2, 130.3, 128.8, 128.3, 127.9, 127.5, 127.4, 127.2, 127.0, 125.8, 125.5, 124.9, 124.4, 123.4, 117.8, 115.0, 113.6, 96.5, 87.1, 85.7, 72.5, 71.9, 67.8, 62.2, 62.1, 60.6, 58.3, 58.0, 47.1, 46.5, 45.6, 43.3, 41.1, 40.9, 40.8, 33.4, 29.3, 29.0 (m), 28.6, 24.5, 22.4, 22.1, 20.2, 20.1, 19.1; δ_{p} (CDCl_3, 101MHz) 150.00, 149.43.
4'-(10-(17'-Tetrabenzo[a,c,g,i]fluorenyl)decyloxy)-trityl-oxy-5'-thymidine-3'-O-N,N-diisopropyl-β-cyanoethyl phosphoramidite (124)

4'-(10-(17'-Tetrabenzo[a,c,g,i]fluorenyl)decyloxy)-trityl-oxy-5'-thymidine (1.526g 1.519mmol) and tetrazole diisopropylammonium salt (DIHT) (0.130g 0.761mmol) were dried by co-evaporating with dry acetonitrile (~10ml) three times and then dissolved in dry alcohol-free DCM (8ml) and stirred under Argon. Tetraisopropyl cyanoethyl phosphoramidite (0.680g 2.261mmol) was then injected and the solution was stirred at RT for 24 hours. HPLC monitoring showed the reaction had gone to completion. Sodium Bicarbonate solution was added to quench the reaction then the organics were extracted with DCM. The DCM was washed with brine then dried and the solvent removed by vacuum. The solid residue was dissolved in the minimum of DCM and precipitated in hexane then dissolved in the minimum of ethyl acetate and precipitated in pentane to give a colourless powder that was then dried at RT under vacuum. (1.324g 72.3%); m/z (FAB) 1293 (M+) C_{77}H_{81}N_{4}O_{7}P; HRMS (MH+) found 1205.5921 requires 1205.59212; Mp 108°C; C.H.N. found C, 72.76 H, 6.69 N, 4.72 required C, 76.72 H, 6.77 N, 4.65; V_{\text{max}} (CBr_{4}, KBr plates) 3019, 2926, 2853, 2256, 1687cm^{-1}; λ_{\text{max}} (i); 301.3, 365.1, 381.2 nm; δ_{H} (CDCl_{3} 250 MHz); 8.81-8.67 (6H, m), 8.27-8.23 (2H, m), 7.72-7.58 (9H, m), 7.43-7.24 (12H, m), 6.81-6.77 (2H, dd, 8.89Hz and 2.58Hz), 6.46-6.38 (1H, m), 5.04-5.01 (1H, t, 4.24Hz), 4.66 (1H, m), 4.17-4.11 (1H, m), 3.85-3.30 (8H, m), 2.64-2.27 (6H, m), 1.64-1.56 (2H, m), 1.43-0.73 (27H, m) (prominent peaks at 1.18 and 1.15), 0.34 (2H, m); δ_{C} (CDCl_{3} 63MHz) 163.7, 158.2, 150.3, 144.2, 143.7, 143.6, 136.6, 135.4, 134.2, 131.1, 130.2, 128.7, 128.2, 127.8, 127.3, 127.1, 126.6, 125.7, 125.5, 124.9, 124.3, 123.4, 117.4-117.2 (N≡C-C-C-O-P, d, 14.8Hz), 113.5, 111.1, 87.0, 85.5-85.2 (CH-C-O-P, d, 14.2Hz), 84.5, 73.4-73.1 (CH-O-P, m, 16.3Hz), 67.8, 63.2-63.0 (P-O-CH_{2}, d, 15.2Hz), 60.3, 58.3-57.8 (CH_{2}-O-P, m), 47.1, 43.2-43.0 (CH-N-P, m), 33.4, 29.2, 29.0, 28.6, 25.7, 24.4 (CH_{3}-C-N-P), 22.0, 20.3-20.1 (P-O-C-CH_{2}-CN, d, 12.3Hz), 11.6 (CH_{3}-C-CO); δ_{p} (CDCl_{3} 101MHz) 149.57, 149.14; ^{1}H - COSY (CDCl_{3}, 360MHz) - See appendix.
4′-(10-(17′-Tetrabenzo[a,c,g,i]fluorenyl)decyloxy)-trityl-oxy-5′-N₆-benzyol-2′-deoxyadenosine-3′-O,N,N-diisopropyl-β-cyanoethyl phosphoramidite (125)

4′-(10-(17′-Tetrabenzo[a,c,g,i]fluorenyl)decyloxy)-trityl-oxy-5′-N₆-Benzyol-2′-deoxyadenosine (1.56g 1.39mmol) and tetrazole diisopropylanmonium salt (DIHT) (0.130g 0.763mmol) were dried by co-evaporating with dry acetonitrile (~10ml) three times and then dissolved in dry alcohol-free DCM (8ml) and stirred under Argon. Tetraisopropyl cyanoethyl phosphoramidite (0.705g 2.34mmol) was then injected and the solution was stirred at RT for 24 hours. Sodium Bicarbonate solution was added to quench the reaction then the organics were extracted with DCM. The DCM was washed with brine then dried and the solvent removed by vacuum. The solid residue was dissolved in the minimum of DCM and precipitated in hexane then dissolved in the minimum of ethyl acetate and precipitated in pentane to give a colourless powder that was then dried at RT under vacuum. (1.66g 90.6%); m/z (FAB) 1317 (M⁺) C₈₄H₇₄N₇O₆P; HRMS (MH⁺) found 1318.62992 requires 1318.62990; Mp 103-105°C; C.H.N. found C, 72.43 H, 6.85 N, 8.22 required C, 76.51 H, 6.42 N, 7.44; V_max (CBr₄, KBr plates) 3193, 3018, 2927, 2853, 2255, 1682, 1605, 1557, 1249cm⁻¹; λ_max; 301.2, 365.1, 381.2nm; δ_H (CDCl₃, 250 MHz); 8.80-8.65 (6H, m), 8.31-7.92 (4H, m), 7.80-7.19 (25H, m), 6.77-6.71 (2H, m), 6.49 (1H, m), 5.03-5.00 (1H, t, 4.16Hz), ~4.8 (1H, m), 4.32 (1H, m), 3.85-3.35 (10H, m), 2.75-2.55 (4H, m), 1.61-1.56 (2H, m), 1.34-0.71 (24H, m), 0.33 (2H, m); δ_C (CDCl₃, 63 MHz); 158.0, 125.4, 153.0, 151.2, 149.3, 144.2, 143.8, 141.3, 136.6, 134.5, 133.5, 133.2, 132.5, 131.1, 130.2, 128.9, 128.6, 128.2, 127.9, 127.7, 127.3, 126.8, 126.6, 125.7, 125.5, 124.8, 124.3, 123.3, 123.2, 117.4-117.3 (C≡N, d, 8.5Hz), 113.4, 86.6-84.6 (CH-C-O-P and N-CH-C-C-O-P, m), 73.3-73.0 (CH-O-P, m), 67.7 (CH₂-O-Ph), 63.2-63.0 (CH₂-C-C-O-P, m), 58.3-57.9 (CH₂-O-P, m), 47.1, 43.43.0 (CH-N-P, d, 12.2Hz), 39.3 (CH₂-C-O-P), 33.9-25.7 (9 alkyl chain CH₃), 20.0-19.7 (CH₂-CN), 14.0-13.9 (CH₃); δ_p (CDCl₃, 101MHz) 149.94-148.53 (m).
4'-{(10-(17'-Tetrabenzo[a,c,g,i]fluorenyl)deoxyloxy)-trityl-oxy-5'-N2-isobutyryl-2'-deoxyguanosine-3'-O,N,N-diisopropyl-β-cyanoethyl phosphoramidite (126)

4'-{(10-(17'-Tetrabenzo[a,c,g,i]fluorenyl)deoxyloxy)-trityl-oxy-5'-N2-Isobutyryl-2'-deoxyguanosine (1.528 g 1.389 mmol) and tetrazole diisopropylammonium salt (DIHT) (0.136 g 0.793 mmol) were dried by co-evaporating with dry acetonitrile (~10 ml) three times and then dissolved in dry alcohol-free DCM (8 ml) and stirred under Argon. Tetraisopropyl cyanoethyl phosphoramidite (0.686 g 2.278 mmol) was then injected and the solution was stirred at RT for 23 hours. Sodium bicarbonate solution was added to quench the reaction then the organics were extracted with DCM. The DCM was washed with brine then dried and the solvent removed by vacuum. The solid residue was dissolved in the minimum of DCM and precipitated in hexane then dissolved in the minimum of ethyl acetate and precipitated in pentane to give a colourless powder that was then dried at RT under vacuum. (1.701 g 94.1%); m/z (FAB) 1299 (M+) C81H86N7O7P; HRMS (MH+) found 1300.64016 requires 1300.64016; Mp 83-85°C; C.H.N. found C, 72.13 H, 6.80 N, 7.45 required C, 74.80 H, 6.67 N, 7.54; V_max (CBr4, KBr plates) 30147, 2926, 2853, 2254, 1700, 1687, 1607, 1507, 1452, 1248 cm\(^{-1}\); λ_max; 301.2, 365.0, 381.0 nm; δ_H (CDCl3 250 MHz) 8.80-8.66 (6H, m), 8.26-8.21 (2H, dd), 7.80-7.19 (21H, m), 6.77-6.71 (2H, dd), 6.21-6.16 (1H, t, 6.68 Hz), 5.02-4.95 (1H, t, 4.21 Hz), 4.81-4.66 (1H, m), 4.28-4.23 (1H, m), 3.88-3.25 (8H, m), 2.97-2.89 (1H, m), 2.76-2.24 (6H, m), 1.62-1.54 (2H, m), 1.40-0.72 (30H, m), 0.33 (2H, m); δ_C (CDCl3 63 MHz) 178.61-178.58 (NH-CO-R, d, 1.64 Hz), 158.1-158.0 (O-C aromatic, d, 3.77 Hz), 155.53-155.48 (NH-CO-C-N, d, 3.08 Hz), 147.7-147.0 (1C, m), 147.7-147.0 (1C, m), 144.2, 144.1, 144.0, 143.9, 138.0, 137.1, 136.6, 134.9, 134.6, 131.1, 130.2, 130.1, 128.6, 128.3, 128.2, 128.1, 127.9, 127.7, 127.6, 127.3, 126.9, 126.8, 126.6, 125.7, 125.4, 124.8, 124.3, 123.34, 123.31, 122.2, 121.6, 117.57-117.47 (CN, d, 6.8 Hz), 113.44-113.34 (aromatic CH, d, 6.29 Hz), 86.44-84.67 (2C, m), 74.43-73.27 (1C, m), 67.7 (CH2-O-Ph), 57.69-57.17 (CH2-O-P, m), 47.0 (tbf-CH-alkyl), 43.07-42.87 (CH-N-P, d, 12.49 Hz), 39.80-39.32 (CH2-C-O-P, d, 10.33 Hz), 35.76-35.63 (CO-CH-C, d, 8.28 Hz) 33.3, 30.8, 29.18-22.16 (6C, m),
22.0, 20.90-19.84 (CN-CH₂-C-O-P, m), 18.61-18.57 (CH₃-C-CO, d, 2.18Hz); δₚ (CDCl₃ 101MHz) 149.2, 124.8.

Prop-2-yn-1-yloxytetrahydropyran (131)

Dihydropyran (4.31g 51.3mmol) and propargyl alcohol (3.36g 59.9mmol) and a catalytic amount (~200mg) of pyridinium p-toluenesulfonate were stirred in an open flask with DCM (50ml) for 2.5 hours. The reaction was then added to 200ml of 2M KOH solution and extracted with ether. The ether extracts were then dried and the solvent removed in vacuo to leave a clear oil (6.6g 93%); m/z 357, 179 (M⁺ C₈H₁₂O₂ requires 140; C,H,N. found C, 49.41 H, 6.31 C₈H₁₂O₂ required C, 68.54 H, 8.63 (sample losing weight); Vₘₐₓ: (KBr plates) 3289, 2942, 2853, 1441 cm⁻¹; δₜ (CDCl₃ 250 MHz); 4.81-4.78 (1H, t, thp), 4.32-4.13 (2H, q, C≡C-CH₂), 3.87-3.75 (1H, m, thp), (3.56-3.45 (1H, m, thp), 2.41-2.38 (1H, t, 2.43Hz C≡CH), 1.83-1.48 (6H, m, thp); δₜ (CDCl₃ 63 MHz); 96.7 (CH), 79.6 (=CH), 73.8(-C≡), 61.8 (CH₂), 53.8 (CH₂), 30.0(CH₂), 25.1(CH₂), 18.8(CH₂).

Pyridinium p-toluenesulfonate (132)

p-Toluene sulfonic acid monohydrate (2.32g 12.2mmol) was added to distilled pyridine (5.12g 65mmol) and the mixture was stirred at RT for 30 minutes. The excess pyridine was then removed in vacuo to leave a colourless powder. This powder was then recrystallised from acetone to give white flakes which were then dried (2.21g 72%). δₜ (CDCl₃ 250 MHz) 8.97-8.94 (2H, d, pyr), 8.43-8.35 (1H, t, pyr), 7.96-7.89 (2H, t, pyr), 7.79-7.75 (2H, d, aromatic), 7.14-7.11 (2H, d, aromatic), 2.30 (3H, s, alkyl). Mp 116°C, literature Mp 117-119°C.
1-Chloro-2-methyl-6-tetrahydropyranyloxy-hex-4-yne (133)

Each of the reagents had been purged of air by bubbling Nitrogen through for \(\sim 2\) hours before use. Prop-2-yn-1-yloxy tetrahydropyran (8.05g 57.5mmol) in THF (10ml) was stirred under nitrogen in an acetone / dry ice bath. Butyllithium (22ml of 2.2M solution 48.4mmol) in THF (10ml) was then added dropwise. After 20 minutes the acetone / dry ice bath was removed. After another 50 minutes 1-chloro-3-bromo-2-methylpropane (10.52g 61.5mmol) in THF (10ml) was added dropwise. The solution was then refluxed for 20 hours. After which time 30ml of ether and 30ml of water were added and the layers separated. The water was washed with more ether and the extracts were combined and dried. The solvent was removed \textit{in vacuo} to give an orange solution (9.6g). NMR analysis showed this to be a mix of starting materials and the product. Column chromatography, using hexane/DCM progressing to just DCM, removed the chloro-3-bromo-2-methylpropane to leave a pale yellow oil (6.62g) this was analysed as (71% product and 29% unreacted starting material, (prop-2-yn-1-yloxy tetrahydropyran) this was carried through to the next stage without further purification. (4.7g 42%), \textit{m/z} (EI) 230 (M-H)\(^+\) 229.09947 found C\(_{12}\)H\(_{18}\)O\(_2\)Cl requires 229.09953; \(\nu\) max; (KBr plates) 2941, 2869, 1442, 1347, 1264, 1202, 1120, 1025, 901cm\(^{-1}\); \(\delta\)\(_H\) (CDCl\(_3\), 250 MHz); 4.79-4.76 (1H, m), 4.29-4.12 (2H, m), 3.84-3.75 (1H, m), 3.53-3.39 (3H, m), 2.32-2.27 (2H, m), 2.08-1.98 (1H, m) 1.84-1.43 (6H, m), 1.05-1.03 (3H, d, 6.73 Hz); \(\delta\)\(_C\) (CDCl\(_3\), 63 MHz); 96.4, 83.4, 73.8, 61.8, 54.3, 49.3, 34.8, 30.0, 25.2, 23.2, 18.9, 17.2.

6-Hydroxy-2-methyl-hex-4ynyl p-nitrobenzoate (134) and (135)

Unpurified 1-Chloro-2-methyl-6-tetrahydropyranyloxy-hex-4-yne (2.00g \(\sim 5.8\)mmol) and \(p\)-nitro sodium benzoate (1.02g 5.4mmol) were refluxed together under Nitrogen in DMF (40ml). After six hours the reaction was allowed to cool. Ether was added and the solution was filtered. The solvents were removed \textit{in vacuo}
(High vac required) to give a dark red oil (1.7g). The oil was partially purified by flash column chromatography at this stage, using hexane/DCM progressing to just DCM, and characterised. \( m/z \) (EI) 362 (M\(^+\)) HRMS found 361.15187 C\(_{19}\)H\(_{23}\)NO\(_6\) requires 361.15254; \( \nu_{\text{max}} \), (KBr plates) 3109, 2942, 2869, 2223, 1724, 1604, 1528 cm\(^{-1}\); \( \delta_\text{H} \) (CDCl\(_3\) 250 MHz); 8.30-8.15 (4H, m), 4.78-4.76 (1H, t, 3.2Hz), 4.31-4.13 (4H, m), 3.85-3.76 (1H, m); 3.54-3.45 (1H, m); 2.42-2.32 (2H, m), 2.26-2.15 (1H, m), 1.83-1.47 (6H, m), 1.11-1.09 (3H, d, 6.7Hz); \( \delta_\text{C} \) (CDCl\(_3\) 63 MHz); 164.4, 150.4, 130.5, 123.4, 83.3, 77.7, 69.1, 61.9, 54.3, 32.1, 30.1, 25.2, 22.9, 18.9, 16.3.

The oil (1.7g) was then dissolved in methanol (80ml) and added to 15ml of 5% solution of oxalic acid. This mixture was then stirred for 50 hours. The methanol was removed before ethyl acetate and water were added and the products extracted. The organic layer was dried (MgSO\(_4\)) and the solvent removed \textit{in vacuo} to give an orange oil which was then purified by flash column chromatography, using hexane/DCM progressing to just DCM, to give a pale orange oil. (1.37g 4.9mmol); \( m/z \) (FAB) 279 (M\(^+\)) C\(_{14}\)H\(_{15}\)N\(_3\)O\(_5\) calculated as 277 C.H.N. found C, 59.21 H, 5.62 N, 4.75 C\(_{14}\)H\(_{15}\)N\(_3\)O\(_5\) required C, 60.64 H, 5.45 N, 5.05; \( \nu_{\text{max}} \), (KBr plates) 3418, 3109, 2963, 2925, 2874, 2221, 1721, 1604, 1527 cm\(^{-1}\); \( \delta_\text{H} \) (CDCl\(_3\) 250 MHz); 8.31-8.16 (4H, m, aromatic), 4.31-4.28 (2H, d, CH\(_2\)-CO\(_2\)R), 4.24-4.23 (2H, t, O-CH\(_2\)-C=C), 2.38-2.32 (2H, m, C=C-CH\(_2\)-R), 2.30-2.11 (1H, m, CHR\(_3\)), 1.77 (1H, s, OH), 1.13-1.10 (3H, d, CH\(_3\)); \( \delta_\text{C} \) (CDCl\(_3\) 63 MHz); 164.5 (C=O), 150.4 (q), 135.4 (q), 130.6 (ar), 123.4 (ar) , 83.2 (C=C), 80.2 (C=C), 69.0 (CH\(_3\)), 51.1 (CH\(_2\)), 32.1 (CH), 22.8 (CH\(_2\)), 16.3 (CH\(_3\)).

\textbf{But-3-yn-1-yloxytetrahydropyran (137)}

Dihydropyran (6.73g 80mmol) and but-3-yn-1-ol (5.25g 75mmol) and a catalytic amount (~200mg) of pyridinium \( p\)-toluenesulfonate were stirred in an open flask with DCM (40ml) for 6 hours, after which time a t.l.c. showed butynol to still be present so an extra 1.0g of dihydropyran was added and the reaction allowed to run
overnight (another 18 hours). The reaction was then added to 200ml of 2M KOH solution and extracted with ether. The ether extracts were then dried with calcium carbonate and the solvent removed in vacuo to leave a yellow oil (11.39g 98.6%). m/z 369, 269 (M+) C₉H₁₄O₂ requires 154; C.H.N. found C, 58.08 H, 7.66 C₉H₁₄O₂ required C, 70.10 H, 9.15 (sample losing weight); \( \nu_{\max} \) (KBr plates) 3291, 2941, 2872, 2119, 1465, 1351 cm\(^{-1}\); Rf (A) 0.30; \( \delta_H \) (CDCl\(_3\) 250 MHz); 4.63 (1H, t, thp) 3.84-3.83 (2H, m, thp), 3.57-3.51 (2H, m, thp), 2.51-2.43 (2H, dt, CH₂-CC), 1.97-1.95 (1H, t, C=CH₂), 1.59-1.49 (6H, m, alkyl); \( \delta_C \) (CDCl\(_3\) 63 MHz); 98.6, 81.3, 69.0, 65.3, 62.0, 30.4, 25.2, 19.8, 19.2.

**Pent-2-yn-5-oxytetrahydropyran ethyl ester (138)**

Butyllithium (21ml of 2M soln 42mmol) was added in 1ml portions slowly to but-3-yn-1-yloxytetrahydropyran (6.5g 42mmol) in THF (30ml) with a dry ice / acetone bath. After addition of the butyllithium and solution was stirred for 2 hours then the dry ice bath was removed and the reaction was allowed to warm to RT. Then freshly distilled ethyl chloroformate (4.9g 45mmol) was added dropwise. The solution was left stirring for 1 hour at RT. Ethyl Acetate and brine were then added and the layers separated. Organic extracts were dried over MgSO₄. The solvent was then removed in vacuo to give a dark oil. (1.29, 5.7mmol, 13.5%); m/z HRMS (EI) found 226.12071 C₁₂H₁₈O₄ requires 226.12051; C.H.N. found, C 62.88, H 8.07 required C 63.70, H 8.02; \( \nu_{\max} \) 2942, 2873, 1710, 1445, 1364, 1253, 1128, 1076, 1039cm\(^{-1}\); Rf (A) 0.56 (D) 0.92; \( \delta_H \) (CDCl\(_3\) 250 MHz); 4.63-4.60 (1H, t, 3.3Hz), 4.22-4.13 (2H, dd, 7.14Hz), 3.89-3.44 (4H, m), 2.64-2.58 (2H, t, 6.9Hz), 1.81-1.47 (6H, m) 1.29-1.24 (3H, 7.14Hz) \( \delta_C \) (CDCl\(_3\) 63 MHz); 153.5, 98.6, 86.0, 73.7, 64.2, 62.1, 61.7, 30.0, 25.2, 20.1, 19.1, 13.9.
5-Hydroxy-pent-2-ynoate ethyl ester (139)

5-Hydroxy-pent-2-ynoate ethyl ester (1g) was dissolved in methanol (40ml) and added to 20ml containing oxalic acid (1g). This mixture was stirred for ~50 hours. The methanol was removed before ethyl acetate and water were added and the products extracted. The organic layer was dried with magnesium sulfate and the solvent removed in vacuo to give an oil. (0.6g 93%); m/z HRMS (EI) found 142.06318 C_7H_{10}O_3 requires 142.06299; \nu_{max}: 3400, 2938, 2237, 1708, 1437, 1367, 1253 cm^-1; R_f (A) 0.1, (D) 0.52; \delta_H (CDCl_3 250 MHz); 4.22 - 4.14 (2H, q, 7.15Hz), 3.77 - 3.72 (2H, t, 6.36Hz), 2.84 - 2.81(1H, broad), 2.58 - 2.53 (2H, t, 6.46Hz) 1.39 - 1.23 (3H, t, 7.14Hz); \delta_C (CDCl_3 63MHz) 153.5, 86.1, 74.1, 61.8, 59.7, 22.8, 13.8.

O-Cresyl acetate (141)

ortho-Cresol (47.7g 0.42mol) and acetic anhydride (60ml 0.64mol) were stirred together in pyridine (50ml) at RT under air for 48hours. Volatiles were then removed by vacuum and the product was distilled (~20mm Hg at 105°C) to give a clear oil (61.6g 93%); m/z HRMS (FAB) found 150.6806 C_9H_{10}O_2 requires 150.6808; \nu_{max}; (KBr plates) 3060, 3029, 2930, 2863, 1759, 1585 cm^-1; \delta_H (CDCl_3 360 MHz); 7.31-7.18 (3H, m), 7.08-7.06 (1H, m), 2.37 (3H, s), 2.25 (3H, s); \delta_C (CDCl_3 63 MHz) 169.7, 149.8, 131.6, 130.5, 127.4, 126.5, 122.3, 21.2, 16.6.

\alpha-Bromo o-cresyl acetate (142)

A trace of dibenzoyl peroxide was added to o-Cresyl acetate (20.0g 134mmol) and N-Bromosuccinimide (23.8g 134mmol) in carbon tetrachloride (60ml) then refluxed under air for 1.5hours. The solution was then cooled, filtered and the solvent removed by vacuum. The product was then distilled. (25.5g 82.7%) m/z HRMS (EI)
found 229.97665 and 227.97843 \text{C}_9\text{H}_9\text{O}_2\text{Br} requires 229.97667 and 227.97859; V_{max}; \text{(KBr Plates)} 3493, 3303, 3062, 3033, 2933, 1763, 1584\text{cm}^{-1}; R_f \text{ (A)} 0.58 \text{ (D) 1.0} ; \delta_h  \text{ (CDCl}_3\text{ 250 MHz)} ; 7.43-7.10 (4H, m), 4.42 (2H, s), 2.36 (3H, s) ; \delta_c  \text{ (CDCl}_3\text{ 63 MHz)} 168.8, 148.8, 130.6, 129.7, 129.4, 126.1, 122.9, 27.5, 20.8.

**Mixture of 3'-O and 2'-O-o-cresyl acetate uridine (143)**

Uridine (2.68g 11mmol) and dibutyltinoxide (2.79g 11.2mmol) were refluxed together in benzene for 3.5 hours with continuous extraction of water. The solvent was removed by vacuum and bromo o-cresyl acetate (2.90g 12.65mmol) and DMF (15ml) were added and the suspension heated to 85°C for 18 hours under Argon. Solvent was removed and the product purified by flash column chromatography, using hexane/DCM progressing through DCM to DCM/methanol combinations. (1.62g 37%) After columning a complicated mixture of isomers still remained. 2D NMR showed the presence of at least four compounds containing Uridine.

**Mixture of 2'-O and 3'-O-o-cresyl acetate-5'-4', 4''dimethoxy-trityl-oxy-uridine (145)**

A mixture of 3'-O and 2'-O-o-cresyl acetate uridine (1.04g 2.6mmol) and 4', 4'' dimethoxy trityl chloride (1.04g 3.2mmol) were combined with pyridine (5ml) at 0°C under Argon. The solution was allowed to warm to RT and left stirring for 18 hours. Methanol was added and then the solvent removed by vacuum. The residue was dissolved in DCM and washed with NaHCO$_3$ solution, dried with calcium carbonate then purified by flash column chromatography, using hexane/DCM progressing through DCM to DCM/methanol combinations. Resulted in a complicated mixture of compounds largely unchanged from the initial mixture.
Mixture of 2'-O and 3'-O-o-cresyl acetate 4'-(10-(17'-tetrabenzo[a,c,g,i] fluorenyldcyloxy)-5'-trityl-oxy-uridine (146)

4'-(10-(17'-tetrabenzo[a,c,g,i] fluorenyldcyloxy)-trityl-oxy-5'-uridine (0.14g 0.14 mmol) and Dibutyltinoxide (0.33g 1.31mmol) were refluxed together in Benzene (20ml) for 3.5hours with continuous extraction of water. The solvent was removed by vacuum and Bromo o-Cresyl acetate (4.8mmol) and DMF (10ml) were added and the suspension heated to 85°C for 20 hours under Argon. Solvent was removed and the product purified by flash column chromatography (0.104g 66%). Material difficult to characterise. Remade with different linker group.

4-(10-(17'-Tetrabenzo[a,c,g,i]fluorenyloctyloxy)-trityl alcohol (147)

4-Bromobenzene (19.0g 121mmol) in THF (50ml) was added via a syringe to magnesium turnings (2.97g 121mmol) and a catalytic amount of iodine in THF (50ml). The solution was then refluxed for 30mins. Ethyl 4-(10-(tetrabenzo[a,c,g,i]fluorenyloctyloxy)-benzoate (7.93g 12.4mmol) in THF (50ml) was then added dropwise. The mixture was then refluxed for another four hours after which the reaction was then quenched with a saturated solution of NH₄Cl and ice (200ml). The organics were then extracted with dichloromethane and dried (MgSO₄). After removal of the solvent a yellow oil remained. The product was then isolated by flash column chromatography using 1:1 DCM/Hexane. (8.70g 93.6%) m/z (FAB) 752, 364. HRMS found 752.36533 C₅₆H₄₈O₂ calculated 752.36543; C.H.N. found C, 88.63 H, 6.53 requires C, 89.33 H, 6.43; Mp 82-85°C; V max; cm⁻¹; λ max, nm; Rf (A) 0.72; δH(CDCl₃ 250 MHz); 8.83-8.70 (6H, m), 8.27-8.23 (2H, m), 7.74-7.60 (8H, m), 7.38-7.25 (10H, m), 7.17-7.13 (2H, d, 9.00Hz), 6.76-6.71 (2H, d, 8.98Hz), 5.01-4.98 (1H, t, 4.30Hz), 3.73-3.67 (2H, t, 6.62Hz), 2.79 (1H, s), 2.66-2.57 (2H, m), 1.57-0.76 (10H, m), 0.38-0.37 (2H, m); δc (CDCl₃ 63 MHz); 158.0, 147.0, 144.2, 138.7, 136.6, 131.1, 130.2,129.0, 128.7, 127.9, 127.7, 127.3, 127.0, 126.6, 125.7, 125.5, 124.9,
124.3, 113.5, 81.6, 67.6, 47.0, 33.3, 29.1, 28.8, 28.7, 28.5, 25.4, 22.0; $^1$H - COSY (CDCl$_3$ 360MHz) - See appendix.

**4'-**(10'-(17'-Tetrabenzo[a,c,g,i]fluorenyloctyloxy)-trityl-oxy-5'-uridine (148)**

Uridine (1.764g 7.2mmol) was twice co-evaporated with dry Pyridine (~5ml) then added to 4-(10-(17'-tetrabenzo[a,c,g,i]fluorenyloctyloxy)-trityl chloride (2.49g 3.3mmol) in dry pyridine (10ml). The reagents were then stirred under Argon for seven days. Solvent was then removed under high vacuum. The residue was dissolved in water and DCM. The organics were separated, dried with magnesium sulfate and purified by flash column chromatography, using DCM/methanol going from DCM to 10% methanol. (2.44g 75.3%); m/z 978, 735, 364 HRMS (FAB) found 978.42498 C$_{65}$H$_{58}$N$_2$O$_7$ requires 978.42440; Mp 134°C; C.H.N. found 75.03 C, 5.80 H, 2.23 N requires 79.73 C, 5.97 H, 2.86 N; Rf (D) 0.44 (F) 0 (H) 0.25; $\delta_H$ (CDCl$_3$, 250 MHz) 10.38 (1H, s), 8.84-8.71 (6H, m), 8.29-8.27 (2H, m), 8.05-8.02 (1H, d, 8.05Hz), 7.74-7.25 (20H, m), 6.78-6.76 (2H, d, 8.89Hz), 5.92 (1H, s), ~5.5 (1H, broad), 5.37-5.34 (1H, d, 8.04Hz), 5.08-5.06 (1H, t, 4.25Hz), 4.46 (1H, br.s), 4.35 (1H, br.s), 4.19-4.17 (1H, t, 4.90Hz), 3.73-3.69 (2H, t, 6.35Hz), 3.57-3.51 (2H, m), 2.64-2.62 (2H, m), 1.48-0.77 (10H, m), 0.38 (2H, m); $\delta_C$ (CDCl$_3$, 62 MHz) 163.8, 158.2, 150.1, 144.2, 144.0, 143.6, 140.3, 136.7, 133.9, 131.1, 130.3, 130.2, 129.0, 128.7, 128.2, 127.9, 127.7, 127.3, 127.1, 126.7, 125.8, 125.5, 124.9, 124.3, 123.4, 113.6, 102.1, 90.3, 87.1, 83.4, 75.2, 69.4, 67.6, 61.7, 47.1, 33.36, 29.1, 28.8, 28.7, 28.5, 25.5, 22.0. $^1$H-COSY (CDCl$_3$, 360MHz) - See appendix
**Mixture of 2'-O and 3'-O-o-cresyl acetate 4'-(10-(17'-tetrabenzo[a,c,g,i]fluorenyloctyloxy)-trityl-oxy-5'-uridine (149)**

4'-(10-(17'-Tetrabenzo[a,c,g,i]fluorenyloctyloxy)-trityl-oxy-5'-uridine (1.00g 1.02mmol) and Dibutyltinoxide (0.265g 1.06mmol) were refluxed together in Benzene (15) for 3 hours with continuous extraction of water. The solvent was removed by vacuum and Bromo o-Cresyl acetate (0.320g 1.40mmol) and DMF (10ml) were added and the suspension heated to 85°C for 20 hours under Argon. Solvent was removed and the product purified by flash column chromatography, using DCM/methanol going from DCM to 10% methanol, leaving (0.372g 32.4%) a complex mixture of products. After repeated purification 40mg of pure product was isolated (3.5%). \( m/z \) HRMS (FAB) found 1127.48588 \( C_{14}H_{67}N_2O_9 \) requires 1127.48466; \( R_f \) (F) 0.27 (H) 0.76; \( \delta_H \) (CDCl\_3, 250 MHz); 10.17 (1H, br.s), 8.83-8.72 (6H, m), 8.31-8.29 (2H, m), 8.01-6.76 (28H, m), 6.03-5.87 (1H, m), 5.41-5.30 (1H, m), 5.10 (1H, m), 4.60-4.18 (3H, m), 3.91-3.53 (6H, m), 2.66 (2H, m), 2.37-2.10 (3H, m), 1.71-0.80 (10H, m), 0.40 (2H, m).

**3.2.1 Deprotection Test**

Only 40mg of (149) was collected so deprotection studies were limited in scope. To prove the basic theory this sample was subjected to the acid and base conditions associated with the intended solid phase synthesis deprotection protocol.

The mixture of 2'-O and 3'-O-o-cresyl acetate 4'-(10-(17'-tetrabenzo[a,c,g,i]fluorenyloctyloxy)-trityl-oxy-5'-uridine was stirred as a suspension in 30% ammonia solution at 55°C overnight. The sample was then vacuumed to dryness. Dissolved in a minimum of ethyl acetate then precipitated in hexane. T.l.c. showed that the majority of starting material had been broken down. The solid
collected was analysed by mass spectrometry. m/z 1126 (starting material), 1084 (Phenol), 1067 (Phenol minus OH).

The sample was then stirred in 2% TFA 50ml for one hour at room temperature. T.l.c. showed that the sample had been broken down further and included a spot consistent with Uridine. Precipitation in hexane from ethyl acetate produced a solid as before. Mass spectrometry showed that the phenol has been mostly removed. Attempts to isolate Uridine were unsuccessful.

### 3.3 Oligodeoxyribonucleotide Synthesis

#### 3.3.1 Synthesiser details and procedure

The synthesis of oligodeoxyribonucleotides was performed on an Applied Biosystems 380B DNA Synthesiser. The solid support was the silica based Link Technologies SynBase support. The linker to the support was a standard succinyl group. Thymidine monomers were prepared as their phosphoramidites these were then activated with 0.45M tetrazole / acetonitrile solution. Each monomer coupling lasted thirty seconds except the final step of addition of the Tbf trityl protected monomers, which lasted ten minutes. The Tbf trityl protected monomers were dissolved in 1:1 alcohol-free DCM and dry acetonitrile. Each synthesis was run on a 0.2 μmol scale. Acetic anhydride and lutidine in THF were used as the capping reagents. Oxidation was achieved using a 0.1M Iodine / THF / Pyridine and water solution. Acid deprotection was performed using 3% trichloroacetic acid in DCM. The cycle details are listed in the appendix.

At the end of each synthesis the sequences were cleaved from the solid support using 1ml concentrated aqueous ammonia for 20 minutes and the exocyclic amine
protecting groups were removed by heating the solution overnight (16hrs) at 45°C. The standard protocol designed for dimethoxy trityl suggests 40°C for 24hrs. The samples were then vacuumed to dryness at 45°C over three hours and the crude residue was analysed by HPLC.

3.3.2 Purification procedure

The sample was then dissolved in 1.5ml of 0.1M triethylammonium acetate (TEAA). The polypak was prepared by running through 2ml of acetonitrile then 2ml of TEAA. The sample was then loaded onto the polypak. The run off was collected and added again to ensure that the entire sample was loaded. The polypak was then eluted with 3ml of ammonium hydroxide (1:20) solution then 2ml of HPLC water. Trifluoroacetic acid 2ml was then added and allowed to elute over two minutes, this was repeated three times. Next 2ml of HPLC water was added and washed through. Finally 2ml of 20% acetonitrile was eluted and collected to wash off the purified oligonucleotide. This sample was then analysed by HPLC and LCMS.

3.3.3 Results

Overall yield and the average yield per-cycle were measured by analysing the absorbance at 490nm of the acid cycle washes, thus measuring the quantity of dimethoxy trityl removed. These figures therefore do not include the final addition of Tbf trityl protected monomer, which was not removed in this way. Concentration of the final oligomer is measured using the absorbance at 260nm using the known absorbance of the monomers. Using this figure for the final concentration of oligomer and knowing the average yield per-cycle for the dimethoxy trityl steps allows the yield for the final step to be calculated. Crude refers to samples before purification and therefore still contains the Tbf trityl-protecting group. Purified refers to samples after purification and therefore have had the group removed.
Thymidine (127)

**Crude HPLC;** (Area %, retention time in minutes) 7mer sequence (40.8%, 4.53), 8mer sequence plus protecting group (45.0%, 24.78); **Purified HPLC;** 7mer sequence (1.18%, 6.60), 8mer sequence (96.69%, 6.86); **Overall yield 95.2%;** **Average yield per cycle 98.8%;** yield of 8mer sequence 0.065 µmols; **Final step yield 34.0%;** **Purified m/z 2370.98 (M⁺), 2067.27 (7mer).**

Cytidine (128)

**Crude HPLC;** (Area %, retention time in minutes) 8mer sequence (73.5%, 23.49); **Purified HPLC** 7mer sequence (16.6%, 6.57), 8mer sequence (75.3%, 6.88); **Overall yield 96.3%; Average yield per cycle 99.1%;** yield of 8mer sequence 0.1062 µmols; **Final step yield 55.1%;** **Purified m/z 2355.87 (M⁺), 2067.20 (7mer).**

Adenosine (129)

**Crude HPLC;** (Area %, retention time in minutes) 7mer sequence (30.3%, 4.57), 8mer sequence (10.0%, 23.13 and 25.3%, 23.67), solvent peak (21.7%, 33.10-33.22); **Purified HPLC** 7mer sequence (11.7%, 6.63), 8mer sequence (76.4%, 6.88); **Overall yield 94.3%; Average yield per cycle 98.5%;** yield of 8mer sequence 0.0234 µmols; **Final step yield 12.4%;** **Purified m/z 2380.14 (M⁺), 2067.04 (7mer).**

Guanidine (130)

**Crude HPLC;** (Area %, retention time in minutes) 7mer sequence (29.4%, 4.59), 8mer sequence (18.9%, 23.19), solvent peak (42.8%, 33.16); **Purified HPLC** 7mer sequence (23.2%, 6.58), 8mer sequence (67.1%, 6.83); **Overall yield 95.1%; Average yield per cycle 98.8%;** yield of 8mer sequence 0.062 µmols; **Final step yield 32.7%; crude m/z 3158.25 (M⁺), 2066.38 (7mer); Purified m/z 2396.00 (M⁺), 2066.99 (7mer).
Part 4: References

67. Isis Pharmaceuticals, Annual report 1998
Chapter 5 Appendices

5.1 Abbreviations

2D NMR  2 dimensional NMR
A       Adenine
AIDS    Acquired Immune Deficiency Syndrome
Bz      Benzoyl
br.s    Broad singlet
C       Cytosine
CPG     Controlled pore glass
Ctmp    1-[(2-chloro-4-methyl)phenyl] 4-methoxypiperidin-4-yl
Cpep    1-(4-chlorophenyl) 4-ethoxypiperidin-4-yl
d       Doublet
dA      Deoxyadenine
DATE    1,1-dianisyl-2,2,2-trichloroethyl group
DBTO    Dibutyltin oxide
DCCI    Dicyclohexylcarbodiimide
DCM     Dichloromethane
dG      Deoxyguanidine
DIHT    Tetrazole diisopropylammonium salt
diNPEOC 2,2'-bis(2-nitrophenyl)ethoxycarbonyl
DMAP    N,N-Dimethylaminopyridine
DMF     N,N-Dimethylformamide
DMTr    4/4' Dimethoxytrityl
DNA     Deoxyribonucleic acid
dT      Deoxythymidine
EDTA    Ethylene diamine tetraacetic acid
EI      Electron impact
FAB     Fast atom bombardment
FDA     Food and drug administration of the USA
Fmoc    9-Fluorenylmethoxycarbonyl
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<th>Abbreviation</th>
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<td>G</td>
<td>Guanine</td>
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<td>HCMV</td>
<td>Human Cytomegalovirus</td>
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<td>Hoogsteen</td>
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<td>HIFA</td>
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S-Pixyl 9-Phenylthioxanthen-9-yl
SPS Solid phase synthesis
t Triplet
T Thymidine
TFA Trifluoroacetic Acid
TBAB Tetrabutylammonium bromide
TBAF Tetrabutylammonium fluoride
TBDMS t-butyldimethylsilyl
Tbf 4-(17-tetrabenzo[a,c,g,i]fluoromethyl)
Tbf C₈ Tr 4-(8-(17'-tetrabenzo[a,c,g,i]fluorenyloctyloxy)-trityl
Tbf C₈ Tr Cl 4-(8-(17'-tetrabenzo[a,c,g,i]fluorenyloctyloxy)-trityl chloride
Tbf C₁₀ Tr 4-(10-(17'-tetrabenzo[a,c,g,i]fluorenyldecyloxy)-trityl
Tbf C₁₀ Tr Cl 4-(10-(17'-tetrabenzo[a,c,g,i]fluorenyldecyloxy)-trityl chloride
Tbf C₁₀ DMTr 4-(10-(17'-tetrabenzo[a,c,g,i]fluorenyldecyloxy)-4',4''-dimethyl trityl
Tbf C₁₀ DMTr Cl 4-(10-(17'-tetrabenzo[a,c,g,i]fluorenyldecyloxy)-4',4''-dimethyltrityl chloride
Tbfmoc Tetrabenzo[a,c,g,i]fluorenyl-17-methanol
TEA.3HF Triethylamine trihydrogen fluoride
TEAA Triethylammonium Acetate
TESCl 1,1,3,3-Tetraisopropyl-3-(2-
(Triphenylmethoxy)ethoxy)disiloxane-1-yl chloride
TFA Trifluoroacetic acid
THF Tetrahydrofuran
Thp Tetrahydropyran-2-yl
TIPDSi 1,1,3,3-Tetraisopropyldisiloxane
Tlc Thin layer chromatography
TPSCl 2,4,6-trisopropylbenzenesulphonyl chloride
tRNA Transfer RNA
Uridine

Ultra violet

5.2 2D NMR

All NMR experiments were conducted using CDCl₃ as the solvent and samples contained no external reference. Frequencies were 250 MHz for hydrogen, ¹H - COSY at 360MHz, carbon at 63 and ¹H / ¹³C Correlation Spectra used 360MHz and 90MHz.

Ethyl-4-(10-(tetrabenzo[a,c,g,i]fluorenyl)octyloxy)-benzoate (109)

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4-(10-(17'-tetrabenzo[a,c,g,i]fluorenyldecyloxy)-trityl alcohol (115)

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<td>C$_7$ and C$_9$</td>
</tr>
<tr>
<td>1.05-0.73</td>
<td>10</td>
<td>C$_3$-C$_7$</td>
<td>C$_2$ to C$_8$</td>
</tr>
<tr>
<td>0.38-0.32</td>
<td>2</td>
<td>C$_2$</td>
<td>C$_1$ and C$_3$</td>
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</tbody>
</table>
5'-O-4'-(10-(17'-tetrabenzo[a,c,g,i]fluorenyldcyloxy)-trityl-thymidine (117)

<table>
<thead>
<tr>
<th>H's</th>
<th>Assignment</th>
<th>$^1$H – COSY Correlations</th>
<th>$^1$C from $^1$H/$^{13}$C Correlations</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.06</td>
<td>M</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8.81-8.76</td>
<td>A</td>
<td>A</td>
<td>~124</td>
</tr>
<tr>
<td>8.69-8.65</td>
<td>A</td>
<td>A</td>
<td>~128</td>
</tr>
<tr>
<td>8.27-8.23</td>
<td>A</td>
<td>A</td>
<td>~126</td>
</tr>
<tr>
<td>7.72-7.57</td>
<td>8A and K</td>
<td>A to A, K to L</td>
<td>A: 126 to 130, L, ~136</td>
</tr>
<tr>
<td>7.41-7.19</td>
<td>10e and 2D$_2$</td>
<td>E to E, D$_1$ to D$_2$</td>
<td>128 to 132</td>
</tr>
<tr>
<td>6.79-6.75</td>
<td>D$_1$</td>
<td>D$_2$</td>
<td>113.6</td>
</tr>
<tr>
<td>6.44-6.38</td>
<td>J</td>
<td>I</td>
<td>~85</td>
</tr>
<tr>
<td>5.06-5.02</td>
<td>B</td>
<td>C$_1$</td>
<td>47.1</td>
</tr>
<tr>
<td>4.59-4.57</td>
<td>H</td>
<td>G and I</td>
<td>72.2</td>
</tr>
<tr>
<td>4.08-4.02</td>
<td>G</td>
<td>F and H</td>
<td>87.1</td>
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<td>3.83-3.78</td>
<td>C$_{10}$</td>
<td>C$_9$</td>
<td>67.8</td>
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<tr>
<td>3.52-3.32</td>
<td>F</td>
<td>G</td>
<td>64.3</td>
</tr>
<tr>
<td>2.65-2.56</td>
<td>C$_1$</td>
<td>B and C$_1$</td>
<td>33.4</td>
</tr>
<tr>
<td>2.45-2.29</td>
<td>I</td>
<td>H and J</td>
<td>40.8</td>
</tr>
<tr>
<td>1.65-1.53</td>
<td>C$_9$</td>
<td>C$_7$ and C$_9$</td>
<td>~30</td>
</tr>
<tr>
<td>1.41-0.71</td>
<td>C$_3$ to C$_8$</td>
<td>C$_2$ to C$_8$</td>
<td>~30</td>
</tr>
<tr>
<td>0.33</td>
<td>C$_2$</td>
<td>C$_1$ and C$_3$</td>
<td>22.0</td>
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![Diagram of the molecule](image-url)
5'-O-4'-(10-(17'-tetrabenzo[a,c,g,i]fluorenlydecyloxy)-trityl-N^4-Benzoyl-2'-deoxycytidine (120)

<table>
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<th>δ_H</th>
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<th>Assignment</th>
<th>COSY correlations</th>
</tr>
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<tbody>
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<td>8.81-8.76</td>
<td>4</td>
<td>A</td>
<td></td>
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<td>8.69-8.65</td>
<td>2</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>8.27-8.23</td>
<td>2</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>7.92-7.11</td>
<td>27</td>
<td>8A, 10E, 5N, L, K, 2D₂</td>
<td>A to A, E to E, L to K, D₂ to D₁</td>
</tr>
<tr>
<td>6.83-6.80</td>
<td>2</td>
<td>D₁</td>
<td>D₂</td>
</tr>
<tr>
<td>6.33-6.24</td>
<td>1</td>
<td>J</td>
<td>I</td>
</tr>
<tr>
<td>5.05-5.02</td>
<td>1</td>
<td>B</td>
<td>C₁</td>
</tr>
<tr>
<td>4.37-4.36</td>
<td>1</td>
<td>H</td>
<td>G and I</td>
</tr>
<tr>
<td>4.26-4.25</td>
<td>1</td>
<td>G</td>
<td>F and H</td>
</tr>
<tr>
<td>3.86-3.80</td>
<td>2</td>
<td>C₁₀</td>
<td>C₉</td>
</tr>
<tr>
<td>3.47-3.46</td>
<td>2</td>
<td>F</td>
<td>G</td>
</tr>
<tr>
<td>2.88-2.75</td>
<td>1</td>
<td>I</td>
<td>H and J</td>
</tr>
<tr>
<td>2.64-2.56</td>
<td>2</td>
<td>C₁</td>
<td>B and C₂</td>
</tr>
<tr>
<td>1.65-1.53</td>
<td>2</td>
<td>C₉</td>
<td>C₈ and C₁₀</td>
</tr>
<tr>
<td>1.37-0.70</td>
<td>8</td>
<td>C₃ to C₈</td>
<td>C₂ to C₉</td>
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<tr>
<td>0.33</td>
<td>2</td>
<td>C₂</td>
<td>C₁ and C₃</td>
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5'-O-4'-(10-(17'-tetrabenzo[a,c,g,i]fluorenyldecyloxy)-trityl-N^6-Benzoyl-2'-deoxyadenosine (121)

<table>
<thead>
<tr>
<th>δ_H</th>
<th>H's</th>
<th>Assignment</th>
<th>COSY correlations</th>
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</thead>
<tbody>
<tr>
<td>8.80-8.64</td>
<td>8</td>
<td>16A, 2D_2</td>
<td>A to A, D_2 to D_1</td>
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<tr>
<td>8.30-7.94</td>
<td>8</td>
<td>10E, K</td>
<td>E to E, K to L</td>
</tr>
<tr>
<td>7.71-7.14</td>
<td>19</td>
<td>L and 5N</td>
<td>and N to N</td>
</tr>
<tr>
<td>6.75-6.71</td>
<td>2</td>
<td>D_1</td>
<td></td>
</tr>
<tr>
<td>6.50-6.42</td>
<td>1</td>
<td>J</td>
<td></td>
</tr>
<tr>
<td>5.06-5.02</td>
<td>1</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td>4.40-4.32</td>
<td>1</td>
<td>G</td>
<td>F and H</td>
</tr>
<tr>
<td>3.80-3.76</td>
<td>2</td>
<td>C_{10}</td>
<td>C_9</td>
</tr>
<tr>
<td>3.44-3.36</td>
<td>2</td>
<td>F</td>
<td>G</td>
</tr>
<tr>
<td>2.97-2.75</td>
<td>2</td>
<td>I</td>
<td>H and J</td>
</tr>
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<td>2.69-2.50</td>
<td>2</td>
<td>C_1</td>
<td>B and C_2</td>
</tr>
<tr>
<td>1.60-1.51</td>
<td>2</td>
<td>C_9</td>
<td>C_8 and C_{10}</td>
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<tr>
<td>1.26-0.69</td>
<td>12</td>
<td>C_3</td>
<td>C_2 to C_9</td>
</tr>
<tr>
<td>0.33</td>
<td>2</td>
<td>C_2</td>
<td>C_1 and C_3</td>
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</tbody>
</table>

[Diagram of the molecular structure of the compound]
5'-O-4'-(10-(17'-tetrabenzo[a,c,g,i]fluorenyl)decyloxy)-trityl-thymidine-3'-O-N,N-diisopropyl-β-cyanoethyl phosphoramidite (124)

<table>
<thead>
<tr>
<th>$\delta_H$</th>
<th>H’s</th>
<th>Assignment</th>
<th>COSY correlations</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.81-8.67</td>
<td>6</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>8.27-8.23</td>
<td>2</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>7.72-7.58</td>
<td>9</td>
<td>8A and K</td>
<td>A to A, K to L</td>
</tr>
<tr>
<td>7.43-7.24</td>
<td>12</td>
<td>10E and 2D$_2$</td>
<td>E to E, D$_2$ to D$_1$</td>
</tr>
<tr>
<td>6.81-6.77</td>
<td>2</td>
<td>D$_1$</td>
<td>D$_2$</td>
</tr>
<tr>
<td>6.46-6.38</td>
<td>1</td>
<td>J</td>
<td>I</td>
</tr>
<tr>
<td>5.04-5.01</td>
<td>1</td>
<td>B</td>
<td>C$_1$</td>
</tr>
<tr>
<td>4.66</td>
<td>1</td>
<td>H</td>
<td>G (faint) and I</td>
</tr>
<tr>
<td>4.17-4.11</td>
<td>1</td>
<td>G</td>
<td>H (faint) and F (faint)</td>
</tr>
<tr>
<td>3.85-3.30</td>
<td>8</td>
<td>C$_{10}$, F, N and Q</td>
<td>C$_{10}$ to C$_9$, F, N and Q to R</td>
</tr>
<tr>
<td>2.64-2.27</td>
<td>6</td>
<td>I, C$_1$ and P</td>
<td>I to H and J, C$_1$ to B and C$_2$, P to N</td>
</tr>
<tr>
<td>1.64-1.56</td>
<td>2</td>
<td>C$_9$</td>
<td>C$<em>8$ and C$</em>{10}$</td>
</tr>
<tr>
<td>1.43-0.73</td>
<td>27</td>
<td>C$_3$ to C$_8$, L and R</td>
<td>C$_2$ to C$_9$, L to K and Q to R</td>
</tr>
<tr>
<td>0.34</td>
<td>2</td>
<td>C$_2$</td>
<td>C$_1$ and C$_3$</td>
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</table>
4-(10-(17'-tetrabenzo[a,c,g,i]fluorenyloctyloxy)-trityl alcohol (147)

<table>
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<tr>
<th>$\delta_H$</th>
<th>H's</th>
<th>Assignment</th>
<th>COSY correlations</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.83-8.70</td>
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<td>A</td>
</tr>
<tr>
<td>8.27-8.23</td>
<td>2</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>7.74-7.60</td>
<td>8</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>7.38-7.25</td>
<td>10</td>
<td>E</td>
<td>E</td>
</tr>
<tr>
<td>7.17-7.13</td>
<td>2</td>
<td>$D_2$</td>
<td>$D_1$</td>
</tr>
<tr>
<td>6.76-6.71</td>
<td>2</td>
<td>$D_1$</td>
<td>$D_2$</td>
</tr>
<tr>
<td>5.01-4.98</td>
<td>1</td>
<td>B</td>
<td>$C_1$</td>
</tr>
<tr>
<td>3.73-3.67</td>
<td>2</td>
<td>$C_8$</td>
<td>$C_7$</td>
</tr>
<tr>
<td>2.79</td>
<td>1</td>
<td>F</td>
<td>-</td>
</tr>
<tr>
<td>2.66-2.57</td>
<td>2</td>
<td>$C_1$</td>
<td>B and $C_2$</td>
</tr>
<tr>
<td>1.57-0.76</td>
<td>10</td>
<td>$C_3$ to $C_7$</td>
<td>$C_2$ to $C_8$</td>
</tr>
<tr>
<td>0.38-0.37</td>
<td>2</td>
<td>$C_2$</td>
<td>$C_1$ and $C_3$</td>
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</table>

![Diagram of the molecule](image-url)
5'-O-4'-(10-(17'-tetrabenzo[a,c,g,i]fluorenyloxy)-trityl-uridine (148)

<table>
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<tr>
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<th>Assignment</th>
<th>COSY correlations</th>
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<td>A</td>
</tr>
<tr>
<td>8.29-8.27</td>
<td>2</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>8.05-8.02</td>
<td>1</td>
<td>K</td>
<td>L</td>
</tr>
<tr>
<td>7.74-7.25</td>
<td>20</td>
<td>8A, 2D₂, 10E</td>
<td>A to A, D₂ to D₁ and E to E</td>
</tr>
<tr>
<td>6.78-6.76</td>
<td>2</td>
<td>D₁</td>
<td>D₂</td>
</tr>
<tr>
<td>5.92</td>
<td>1</td>
<td>J</td>
<td>I</td>
</tr>
<tr>
<td>5.5</td>
<td>1</td>
<td>N or P</td>
<td>-</td>
</tr>
<tr>
<td>5.37-5.34</td>
<td>1</td>
<td>L</td>
<td>K</td>
</tr>
<tr>
<td>5.08-5.06</td>
<td>1</td>
<td>B</td>
<td>C₁</td>
</tr>
<tr>
<td>4.46</td>
<td>1</td>
<td>H</td>
<td>I and G</td>
</tr>
<tr>
<td>4.35</td>
<td>1</td>
<td>I</td>
<td>H and J</td>
</tr>
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<td>4.19-4.17</td>
<td>1</td>
<td>G</td>
<td>F and H</td>
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<td>C₈</td>
<td>C₇</td>
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<td>F</td>
<td>G</td>
</tr>
<tr>
<td>2.64-2.62</td>
<td>2</td>
<td>C₁</td>
<td>B and C₂</td>
</tr>
<tr>
<td>1.48-0.77</td>
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<td>C₃ to C₇</td>
<td>C₂ to C₈</td>
</tr>
<tr>
<td>0.38</td>
<td>2</td>
<td>C₂</td>
<td>C₁ and C₃</td>
</tr>
</tbody>
</table>
5.3 Courses Attended


Organic Chemistry Department Colloquia, University of Edinburgh, 1997-2000, various speakers.

Demonstrating course, Teaching and Learning Association, University of Edinburgh 1997.


Industrial Inorganic Chemistry Course, University of Edinburgh, Professor P. Tasker, 1997


Edinburgh Centre for Protein Technology Lectures, University of Edinburgh, 1997-1999, various speakers.

Walker Memorial Lectures, University of Edinburgh, Prof J.M. Lehn (Pasteur Institute, Strasbourg) 1998, Dr T. McKillop (Zeneca Pharmaceuticals) 1999.
NMR Spectroscopy Course, University of Edinburgh Drs Sadler, Barlow, Reed, Uhrin, Hewage and Parkinson.


Synthons in Organic Chemistry, Prof. E. Vilsmeir (University of Kaiserslautern), 1998.

Romanes Symposium, University of Edinburgh, Prof K.C. Nicolaou (Scripps Research Institute), Dr K. Hale (University College London), Prof P.J. Kocienski (Glasgow University) 1998, Prof A.I. Scott (Texas A&N University), Dr C. Schofield (Oxford University), D. W. Young (Sussex University), 2000.


Amino Acids in Organic Chemistry, Dr J. Podlech (University of Stuttgart), 1999.

5.4 Presentations


Poster presented at Royal Society of Chemistry, Perkin Division, Scottish Meeting, University of Aberdeen 1999.
### 5.5 DNA Synthesiser Cycle

Synthesis cycle for the 0.2 mircomole β-cyanoethyl phosphoramidite method.

<table>
<thead>
<tr>
<th>Step Number</th>
<th>Function</th>
<th>Time (secs)</th>
<th>Active for Bases</th>
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</thead>
<tbody>
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<td>5</td>
<td>Yes Yes Yes Yes Yes</td>
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<tr>
<td>2</td>
<td>#18 to column</td>
<td>60</td>
<td>Yes Yes Yes Yes Yes</td>
</tr>
<tr>
<td>3</td>
<td>Reverse flush</td>
<td>10</td>
<td>Yes Yes Yes Yes Yes</td>
</tr>
<tr>
<td>4</td>
<td>Block Flush</td>
<td>5</td>
<td>Yes Yes Yes Yes Yes</td>
</tr>
<tr>
<td>5</td>
<td>Phos Prep</td>
<td>3</td>
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<tr>
<td>6</td>
<td>Tet to column</td>
<td>5</td>
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</tr>
<tr>
<td>7</td>
<td>B+ tet to column</td>
<td>3</td>
<td>Yes Yes Yes Yes Yes</td>
</tr>
<tr>
<td>8</td>
<td>Tet to column</td>
<td>3</td>
<td>Yes Yes Yes Yes Yes</td>
</tr>
<tr>
<td>9</td>
<td>B+ tet to column</td>
<td>3</td>
<td>Yes Yes Yes Yes Yes</td>
</tr>
<tr>
<td>10</td>
<td>Tet to column</td>
<td>3</td>
<td>Yes Yes Yes Yes Yes</td>
</tr>
<tr>
<td>11</td>
<td>B+ tet to column</td>
<td>3</td>
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<td>Tet to column</td>
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<td>13</td>
<td>#18 to column</td>
<td>4</td>
<td>Yes Yes Yes Yes Yes</td>
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<td>14</td>
<td>wait</td>
<td>30</td>
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<td>15</td>
<td>Cap prep</td>
<td>3</td>
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<td>Reverse flush</td>
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<td>17</td>
<td>Block Flush</td>
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<td>Cap to column</td>
<td>10</td>
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<tr>
<td>19</td>
<td>#18 to waste</td>
<td>5</td>
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<tr>
<td>20</td>
<td>Reverse flush</td>
<td>10</td>
<td>Yes Yes Yes Yes Yes</td>
</tr>
<tr>
<td>21</td>
<td>Block Flush</td>
<td>5</td>
<td>Yes Yes Yes Yes Yes</td>
</tr>
<tr>
<td>22</td>
<td>#15 to waste</td>
<td>5</td>
<td>Yes Yes Yes Yes Yes</td>
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<tr>
<td>23</td>
<td>#15 to column</td>
<td>12</td>
<td>Yes Yes Yes Yes Yes</td>
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<tr>
<td>24</td>
<td>#18 to waste</td>
<td>5</td>
<td>Yes Yes Yes Yes Yes</td>
</tr>
<tr>
<td>25</td>
<td>wait</td>
<td>30</td>
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<tr>
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<td>Reverse flush</td>
<td>10</td>
<td>Yes Yes Yes Yes Yes</td>
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<tr>
<td>27</td>
<td>Block Flush</td>
<td>5</td>
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Reagent 15: 0.1M Iodine / THF / Pyridine / H₂O
Reagent 14: 3% TFA / DCM
Reagent 18: Acetonitrile
Cap A: THF / Lutidine / Acetic anhydride
Cap B: methylimidazole / THF 1:9
Activate: 0.45M tetrazole / Acetonitrile
### 5.6 Diagram list

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<td>Figure 1. The DNA to protein pathway</td>
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<td>Figure 2. The primary structure of DNA and RNA</td>
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<td>Figure 3. DNA double helix structure showing the base pairs</td>
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<td>Figure 4. Hoogsteen hydrogen double bonds</td>
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<td>Figure 5. Reverse Hoogsteen hydrogen double bonds</td>
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<td>Figure 6. Attempts to improve pyrimidine strand binding</td>
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<td>Figure 7. Representation of alternate strand binding</td>
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<td>Figure 8. Antisense translation inhibition</td>
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<td>Figure 10. Khorana’s phosphodiester approach</td>
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<td>Figure 11. The Michelson and Todd phosphotriester approach</td>
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<td>Figure 12. Letsinger’s phosphotriester approach</td>
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<td>Figure 13. Phosphate protection</td>
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<td>Figure 14. Cramer’s approach</td>
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<td>Figure 15. Coupling Reagent MSNT</td>
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<td>Figure 16. 1H-tetrazole as a catalyst</td>
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<td>Figure 17. Letsinger’s phosphite triester approach</td>
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<td>Figure 18. Caruthers’s Phosphoramidite method</td>
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<td>Figure 19. Phosphitylating agent</td>
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<td>Figure 21. Uzanaski’s phosphoramidite approach</td>
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<td>Figure 22. H-Phosphonate approach</td>
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<td>Figure 23. Attachment of the first monomer to CPG</td>
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<td>Figure 27. Acidic depurination</td>
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