The Development of Methodology for the Chemical Synthesis of Oligonucleotides

by

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To my family

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

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Abstract

The development of novel protecting group strategies for the 2'-hydroxyl function of ribonucleosides has been reviewed and a new design has been formulated. The design is based upon a two stage deprotection stategy which is compatible with the final steps in RNA synthesis using solid phase methods. Central to this is the intramolecular addition to an acetylenic system leading to an acid-labile enol ether system, collapse of which results in liberation of the 2'-hydroxyl function.

A series of propargyl based systems have been synthesised, incorporated and their suitability evaluated for the automated chemical synthesis of oligoribonucleotides using the phosphoramidite approach.

Also discussed are the fully automated syntheses of branched oligodeoxynucleotides (ODNs), accomplished using a 5',2'-di(dimethoxytrityl)-protected *arabino*-uridine derivative as the branching monomer. The synthesis and incorporation of the branching monomer has been described. The affinities of the branched ODNs towards complementary single stranded ODNs were studied using UV thermal denaturing experiments.

Contents

Abbreviations

A	Adenine
BDDDP	2-*tert.*-Butylimino-2-diethyl-amino-1,3-dimethylperhydro-1,3,2-diazaphosphorin
br	Broad
C	Cytosine
CPG	Controlled pore glass
Ctmp	1-[(2-Chloro-4-methyl)phenyl]4-methoxypiperidin-4-yl
d	Doublet
DATE	1,1-Dianisyl-2,2,2-trichloroethyl
DBTO	Dibutyltin oxide
DCCI	1.3-Dicyclohexylcarbodiimide
DCM	Dichloromethane
DMAP	N,N-Dimethylaminopyridine
DMF	N,N-Dimethylformamide
DMSO	Dimethylsulphoxide
DMTr	Dimethoxytrityl
DNA	Deoxyribonucleic acid
EI	Electron impact
FAB	Fast atom bombardment
Fpmp	1-(2-Fluorophenyl)4-methoxypiperidin-4-yl
G	Guanine
HIFA	2-Hydroxyisophthalate formaldehyde
HPLC	High performance liquid chromatography
HRMS	High resolution mass spectrometry
LCAA	Long chain alkyl amine
LHMDS	Lithium hexamethyldisilazane
m	Multiplet

Me	Methyl
MMTr	Monomethoxytrityl
m.p.	Melting point
mRNA	Messenger RNA
MS	Mass spectrometry
Mthp	4-Methoxytetrahydropyran-4-yl
NCS	N-Chlorosuccinimide
NMR	Nuclear magnetic resonance
ODNs	Oligodeoxynucleotides
PCR	Polymerase Chain Reaction
Ph	Phenyl
pixyl	9-Phenylxanthen-9-yl
ppm	Parts per million
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
rp-HPLC	Reverse phase HPLC
s	Singlet
SEM	(Trimethylsilyl)ethoxymethyl ether
t	Triplet
T	Thymine
TBAB	tetra-n-butylammonium bromide
TBAF	tetra-n-butylammonium fluoride
TBDMS	*tert*-Butyldimethylsilyl
Tbf	4-(17-Tetrabenzo[a,c,g,I]fluoromethyl)
Tbf-DMTr	4-(17-Tetrabenzo[a,c,g,I]fluoromethyl)-4',4"-dimethoxytrityl
Tbf-DMTr-Cl	4-(17-Tetrabenzo[a,c,g,I]fluoromethyl)-4',4"-dimethoxytrityl chloride
TCA	Trichloroacetic acid
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
Thp	Tetrahydropyran-2-yl

tlc	Thin layer chromatography
TPSCl	Triisopropylbenzenesulfonyl chloride
tRNA	Transfer RNA
U	Uracil
UV	Ultra-violet

1. Introduction

1.1. Biological Importance

The basic structure and chemical nature of deoxyribonucleic acid (DNA) has been known since Watson and Crick's[1] publication, in Nature of April 1953. The model of the double helix created huge interest in the importance of nucleic acids, particularly because of the biological implications. It was obvious that within the sequences of nucleotide bases in the DNA of the cell lay the information required to specify the diversity of biological molecules needed to carry out the functions of that cell. These molecules hold the key to life by carrying the genetic code for each organism, through which the sequence of DNA could be translated into protein.

Ribonucleic acid (RNA) which, among other functions, transports the genetic information from DNA to the site of protein production, the ribosome. The flow of genetic information is called the central dogma of molecular biology[2] in which DNA is transcribed into RNA, which is then translated into protein (Figure 1).

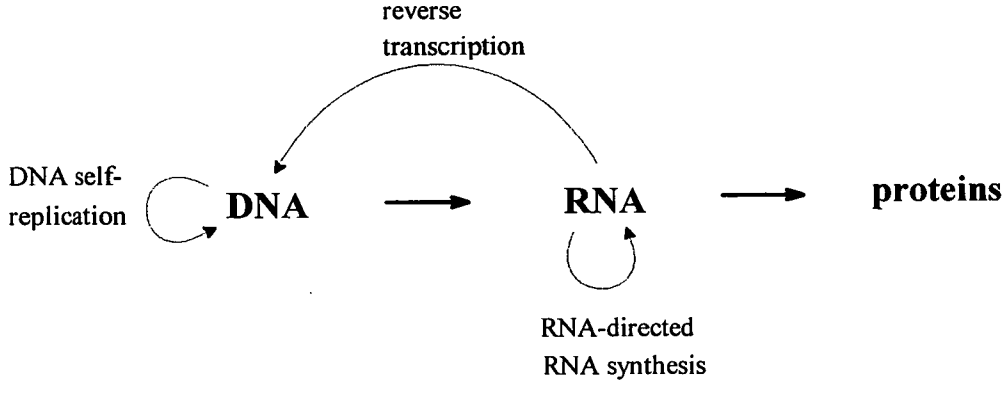


Figure 1. The central dogma of molecular biology

1.2. DNA and RNA Structures

Since the discovery of nucleic acids in the latter part of the last century, several decades passed before the structures of the constituent purines and pyrimidines, the corresponding nucleosides, and the nature of the internucleotide bonds in the polynucleotides were clarified. This was followed by elucidation of the structure of DNA in 1953, earning Watson, Crick and Wilkins the Nobel prize for chemistry in 1962.

1.2.1. Primary Structure

Nucleic acids are very long, thread like polymers made from a linear array of monomers called nucleotides. Nucleotides are the phosphate esters of nucleosides and these are the components of both DNA and RNA. The primary structures of DNA and RNA consist of these phosphodiester linked nucleotides each containing a 2'-deoxy-D-ribose or D-ribose sugar ring (in DNA and RNA respectively) and an

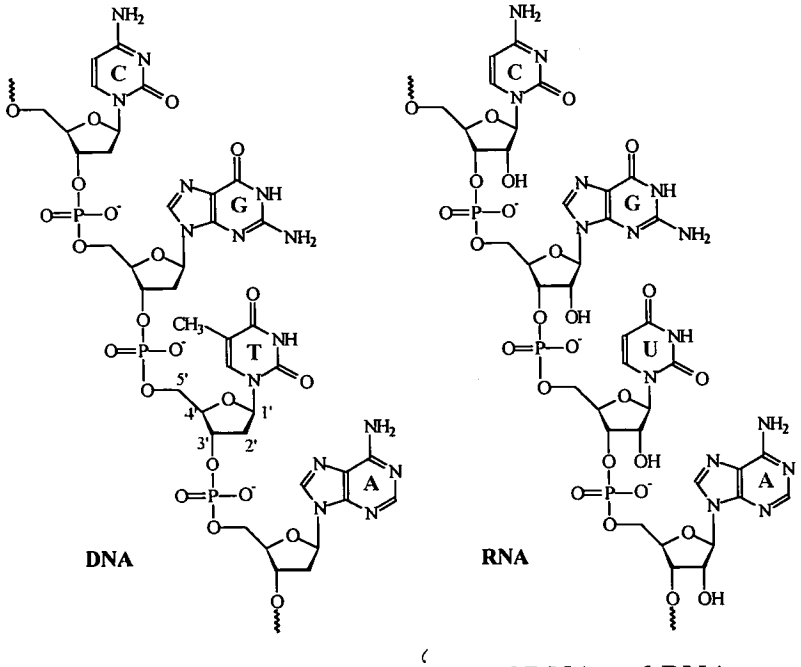


Figure 2. Primary structures of DNA and RNA

aromatic nucleobase (Figure 2). The nucleobases found in DNA include the purines adenine (A) and guanine (G), and the pyrimidines cytosine (C) and thymine (T), while RNA incorporates the pyrimidine uracil (U) in place of T. The polynucleotide chain has a consistent 5'→3' polarity with a sequence of relatively hydrophobic nucleobases and a negatively charged sugar-phosphate backbone. These attributes play a major role in the assembly and maintenance of the secondary and tertiary structures of DNA and RNA.

1.2.2. Secondary Structure

Complementarity between the individual nucleobases, through hybridization, results in an ordered structure that spirals around a central polymer axis. This is known as Watson-Crick base pairing, where A and T, or G and C, interact through hydrogen bonding (Figure 3).

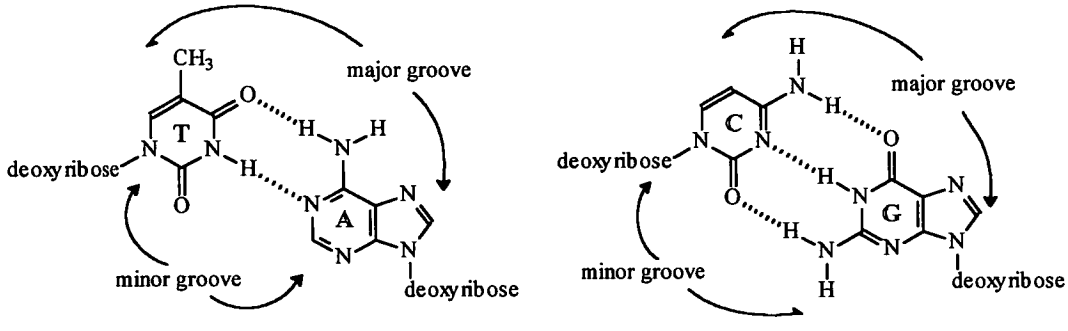


Figure 3. Watson-Crick Base Pairs

The governing DNA structure found is referred to as the *B-form,* in which the double helix is comprised of two single strands running anti-parallel to each other. In *B*-DNA the C-2' of the deoxyribose ring lies above the plane of the ring in a C-2' endo conformation (Figure 4). Two distinct helical grooves are created in *B*-DNA, called the major and minor grooves, which spiral around the surface of the double strand. Two other conformations are known: *A*-DNA, in which the C-3' of the deoxyribose ring has a C-3' endo conformation; and *Z*-DNA has a left handed helical structure

compared to right handed configurations for *B* and *A*. The phosphates in the backbone of *Z*-DNA are found to be in a zig-zag conformation around the helical structure.

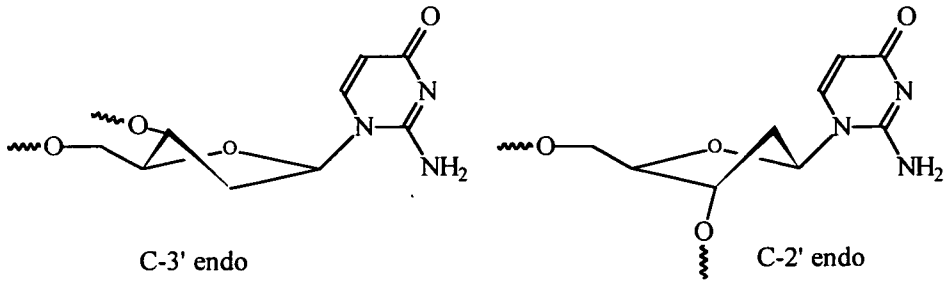


Figure 4. Conformations of A-DNA (C-3' endo) and B-DNA (C-2' endo)

RNA has the added complexity of a hydroxyl function at the 2'-position which causes this nucleic acid to adopt different conformations from those of DNA.[3] This gives RNA a greater structural versatility in the variety of its species, in its chemical reactivity and in its diversity of conformations.

RNA occurs mainly as a single polynucleotide chain but many other forms exist within the three main functional classes of RNA found in cells (Section 1.3.). Natural RNA can either form long, double stranded structures or contain short double helical regions connected by single strands. These double helical regions, known as hairpin regions, form when some complementary sequences found in different parts of the RNA chain are antiparallel to each other. Other regions in RNA contain loops and bulges which are stabilised by both Watson-Crick (G-C and A-U) and the energetically less stable non-Watson-Crick hydrogen bonding (G-U). The specific three-dimensional structure of RNA is the most important factor in the particular activity and biological function of the RNA molecule.[4]

1.3.Classifications of RNA

Protein synthesis depends on the collaboration of several classes of RNA molecules. In the synthesis of proteins, genes specify the types of proteins that are made in the cell. This involves copying certain regions of DNA into RNA, which itself is the direct template for protein synthesis. This is known as transcription. Transcription is the mechanism by which a template strand of DNA is used by specific RNA polymerases to generate one of three different types of RNA. These three RNA classes are:

1. **Messenger RNA (mRNA):** This class of RNA carries the information for protein synthesis. It determines the order of amino acids incorporated into the growing polypeptide chain.
2. **Transfer RNA (tRNA):** This class of 'small' RNA (most are between 70 and 90 nucleotides in length) allows the correct insertion of amino acids into the growing polypeptide chain by covalent bonds to individual amino acids and recognising the encoded sequences of the mRNA.
3. **Ribosomal RNA (rRNA):** This class of RNA is constructed, together with numerous ribosomal proteins, to form the ribosomes. Ribosomes take on the mRNA and form a catalytic domain into which the tRNA enters with its attached amino acid. The proteins of the ribosome catalyse all of the functions of polypeptide synthesis.

RNA polymerase starts its synthesis at a specific DNA sequence, called the promoter, which signals where RNA synthesis should begin. It then completes its synthesis at a termination point, when the RNA polymerase encounters a second specific sequence in the DNA. At this point, both are released and as the DNA-DNA helix rewinds the RNA is displaced as a single stranded molecule typically between 70 and 10,000 nucleotides long. The resultant RNA from transcription is thus complementary to the template strand of the DNA duplex and identical to the non-template strand. The non-template strand is known as the coding strand since its sequences are identical to those of the mRNA, except uridine which is substituted for thymine.

1.4. Roles of RNA

Over the last 15 years, discoveries have led to a fuller awareness of the diverse roles that RNA plays in biological systems. It has been shown that proteins are not the only catalysts of cellular reactions but also that a number of RNA molecules are capable of catalysing RNA cleavage and ligation reactions.[4,5,6] Cech[4] discussed the chemical mechanisms of RNA catalysed reactions using the self-splicing ribosomal RNA precursor of *Tetrahymena* and the enzymatic activities of its intervening RNA sequence. It has also been suggested that RNA takes a more active catalytic role during protein synthesis.[7] RNA has been found to act as an enzyme, termed a ribozyme, either by itself[8] or when complexed to a protein. This has led to an increased interest and research in these and other areas.[9]

1.5. Applications of Synthetic Oligonucleotides

The way of thinking of scientists has been transformed by the increased availability of synthetic oligonucleotides, hence, new ideas and approaches to problems have arisen. The development over the last 40 years of rapid and efficient syntheses, and simultaneously the advent of molecular cloning techniques, has widened the availability of synthetic oligonucleotides. They have found their way into many areas of biology and biochemistry. For example in the cloning and synthesis of man-made genes,[10] as primers for various polymerase chain reaction (PCR) applications[11] and for the sequencing of DNA.[12] They have also found applications as probes for examining how proteins interact with polynucleotides,[13] for studies on nucleic acid structure[14] and in the antisense approach for the treatment of viral diseases and cancer.[15] They have been used in recombinant DNA technology, which was developed in the late seventies to synthesise proteins. Genes for a specific protein are chemically synthesised before being expressed in micro-organisms to produce the protein.

Probably the major application for synthetic oligodeoxynucleotides (ODNs) is in the area of DNA based diagnostic and antisense based therapeutic approaches. In this, analogues are now slowly beginning to reach the stage of clinical trials for the treatment of infections and some cancers. The antisense approach to rational drug design has created huge interest in this area of research, and has been proposed as an important class of new pharmaceuticals. Antisense refers to the use of small synthetic oligonucleotides which inhibit gene expression through hybridization to coding (sense) sequences by Watson-Crick base pairing. This is discussed in the appendix .

The important roles tRNA, mRNA and rRNA play in biological systems have been recognised, and major advances in the understanding of these have been made. There is the potential of ribozymes being used for therapeutic purposes, using them in the cleavage of mRNA in harmful genes. As a fuller understanding of the diverse roles RNAs play in biological systems is gained, new applications for the use of synthetic RNAs will occur.

1.6. Synthesis of Oligonucleotides

1.6.1. Introduction

As previously described, an oligonucleotide is a single stranded chain consisting of a certain number of nucleoside units linked together by phosphodiester bridges. The phosphodiester bridges are usually between the 3'-hydroxyl of one nucleoside and the 5'-hydroxyl of another forming the backbone of the oligonucleotide. Oligonucleotides are very sensitive molecules which possess wide ranging functionalities so only the mildest of reaction conditions can be employed in their synthesis. In order to achieve the specific and sequential formation of internucleoside 3'-5' phosphodiester linkages, selective blocking and deblocking strategies must be employed. These strategies are used in the protection and deprotection of the primary and secondary hydroxyl groups, primary amino groups and often two of the three oxygens of the phosphate

group. The bonds between the sugar and the purine or pyrimidine bases are also sensitive, especially the $C^{1'}$-N glycosylic bond which is labile to acids. Protecting groups must therefore be removed under mild conditions without causing any side reactions or alterations to the oligonucleotide.

1.6.2. Phosphodiester Chemistry

The most convenient way to assemble an oligonucleotide is by the use of nucleoside phosphates as the building blocks, coupling them together to form a single strand. In 1958 Khorana *et al*[16] were the first to use the phosphodiester approach for the synthesis of dinucleotides (**3**) with a $C^{5'}$-$C^{3'}$ linkage, and then went on to successfully synthesise larger oligonucleotides. The phosphodiester method involves the condensation of a nucleoside (**1**), having a free 3'-hydroxyl group and a 5'-hydroxyl group protected by the acid sensitive trityl group, with a nucleoside (**2**) containing a 5'phosphate group and the 3'-hydroxyl protected with an acetyl group.

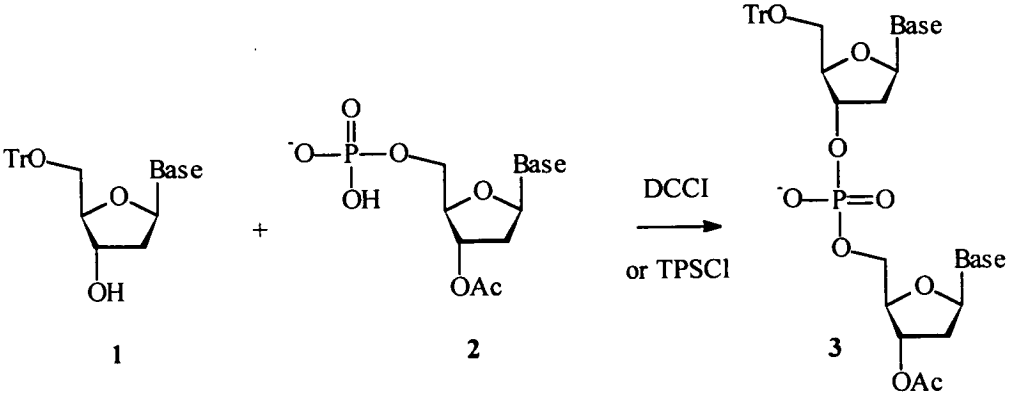


Figure 5. Phosphodiester approach to oligonucleotides

Condensing agents such as 1,3-dicyclohexylcarbodiimide (DCCI) or 2,4,6-triispropylbenzenesulfonyl chloride (TPSCl) were used in the coupling reaction of the two nucleosides. The exocyclic amino groups of the nucleobases were also suitably protected during coupling using benzoyl or isobutyryl groups, both of which are still used in modern oligonucleotide chemistry. One disadvantage of the phosphodiester

approach was the time consuming nature of separating various condensation products using ion-exchange chromatography.

1.6.3. Phosphotriester Chemistry

In 1955 that Michelson and Todd[17] reported the first chemical synthesis of a dinucleotide containing a $C^{5'}$-$C^{3'}$ linkage. Figure 6 shows the activation of 5'-O-acetylthymidine-3'-O-benzyl hydrogen phosphonate (**4**) as the corresponding phosphorochloridate (**5**) using N-chlorosuccinimide (NCS). The reaction of **5** with 3'-O-acetylthymidine (**6**) produced the dinucleotidephosphate triester (**7**) which could then be deprotected.

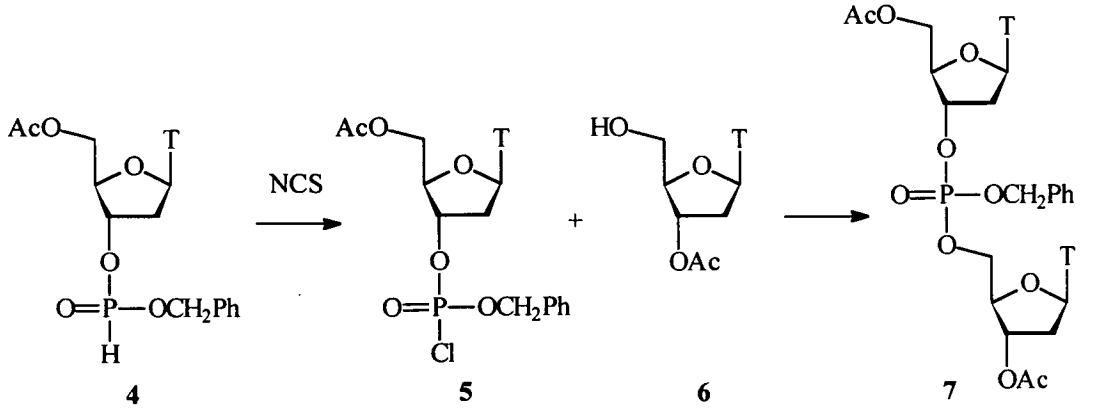


Figure 6. Michelson and Todd's phosphotriester approach

It was a decade later when Letsinger *et al*[18,19] introduced the β-cyanoethyl phosphate protecting group using the solid phase synthesis principle. They used an insoluble polymeric support for the stepwise synthesis of di-, tri-, and tetranucleotides, for small scale synthesis. They continued the use of this method in solution phase synthesis, as shown in Figure 7. The condensation of the 5'-O-protected deoxyribonucleoside (**8**) with phosphate monoester, activated with 2,4,6-trimethylbenzenesulfonyl chloride (MS-Cl), afforded the nucleoside phosphodiester (**9**).

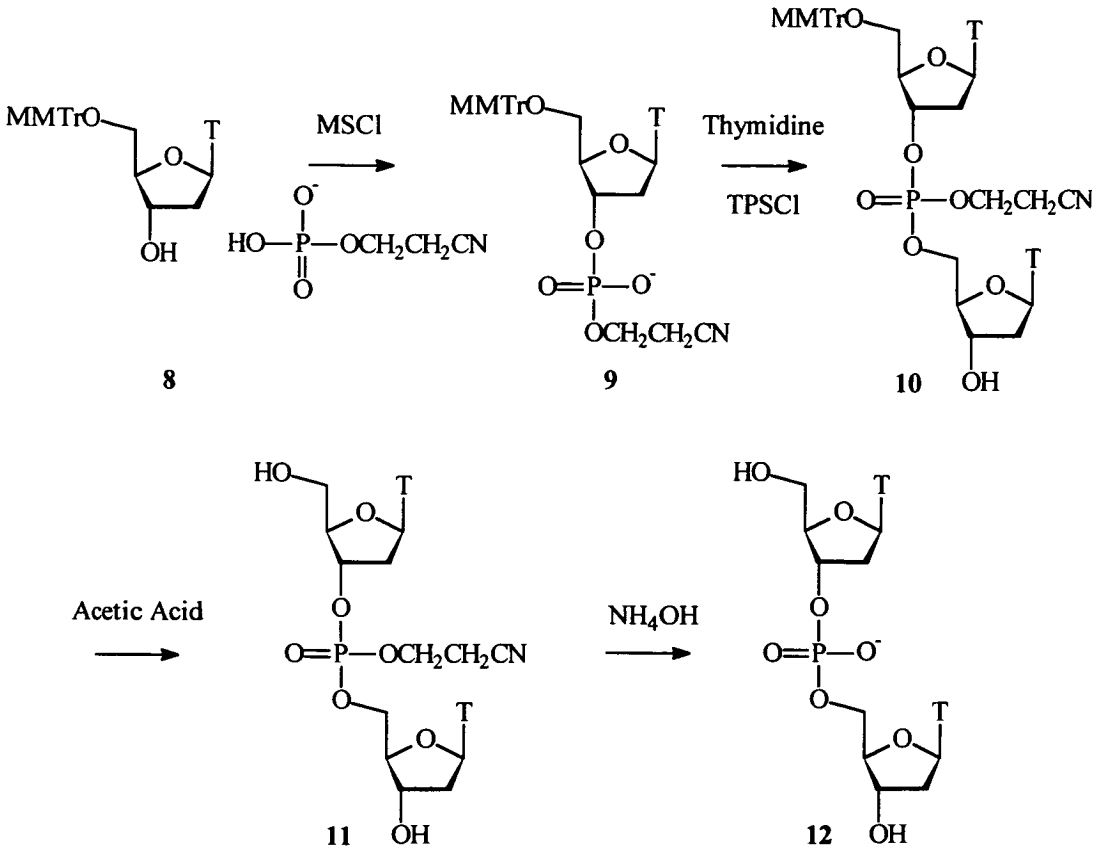
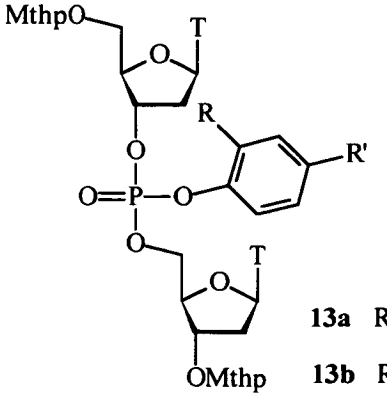


Figure 7. Letsinger's Phophotriester approach

Addition of thymidine, with its 5'-hydroxy function unprotected, in the presence of 2,4,6-triisopropylbenzenesulfonyl chloride (TPS-Cl) produced the protected dinucleoside phosphate triester (**10**). The monomethoxytrityl (MMTr) and the β-cyanoethyl protecting groups were removed by treatment with acetic acid and aqueous ammonia respectively to give the fully deprotected dimer (**12**).

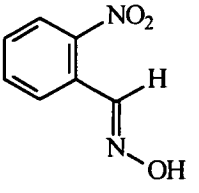
To prevent undesired 3'-3' and 5'-5' coupled products, Letsinger *et al*[20] developed the β-benzoylpropionyl 3'-hydroxy protecting group. This could be removed using hydrazine hydrate in pyridine-acetic acid, without disruption of the phosphotriester function. These developments led to a strategy for the stepwise synthesis of oligodeoxyribonucleotides containing other nucleosides as well as thymidine.[21] It was found that the 2-cyanoethyl phosphate protecting group was too labile to withstand the conditions used in work-up and purification of large ODNs. It was therefore

necessary to find a stable phosphate protecting group which was capable of being removed efficiently. Reese *et al*[22] developed the use of aryl groups containing electron withdrawing groups (**13a, 13b**), which were removed by either (E)-2-nitrobenzaldehyde oxime (**14**) or *syn*-pyridine-2-carboxaldehyde oxime (**15**), instead of cleavage during the alkaline hydrolysis step.



13a R' = Cl, R = H

13b R' = H, R = Cl



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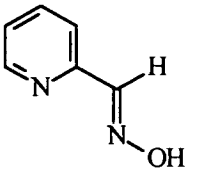

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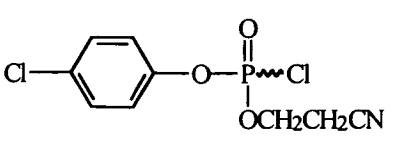
Figure 8. Reese's phosphate protecting group and deprotection reagents

Investigations into cleavage conditions[23] showed that the 2-chlorophenyl group was the most effective aryl group for phosphate protection in the phosphotriester approach to ODN synthesis. It was found to be stable during synthesis, and was removed with the minimum of cleavage to the internucleotide linkages.

The development and synthesis of nucleoside building blocks were designed so as to eliminate the formation of 3'-3' and 5'-5' internucleotide linkages. Therefore, monofunctional phosphorylating agents became the reagents of choice, with phosphorochloridates (**16, 17**) the most frequently used.[24]



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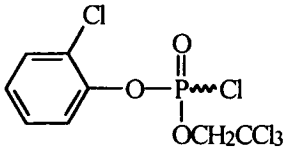

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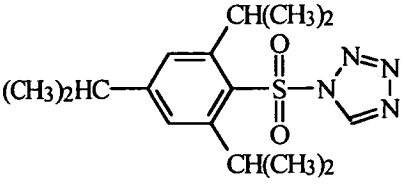
Figure 9. Phosphorochloridates

Activation of the phosphodiester building blocks is a crucial step in the phosphotriester approach to ODN synthesis. Sterically hindered arylsulfonyls (**18, 19**)[25,26] became the preferred activators of phosphodiesters due to their ability to minimise the sulfonylation of the free 5'-hydroxyl function of the incoming nucleosides.



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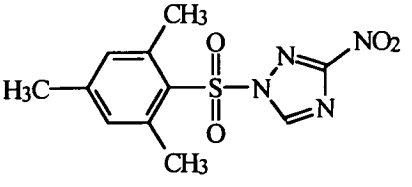


19

Figure 10. Coupling reagents

The rate of reaction can be enhanced by addition of a nucleophilic catalyst, such as *N*-methylimidazole (Figure 11). Figure 11 shows the formation of the mesitylenesulfonic acid-phosphate mixed anhydride (**21**) and the symmetrical pyrophosphate tetraester (**22**). Without the presence of a catalyst, the reaction of **21** and **22** with 3'-O-acetylthymidine to produce the dinucleoside phosphate triester (**24**) proceeds slowly. However, when *N*-methylimidazole is introduced the reaction proceeds rapidly (10 min, 22°C). The *N*-methylimidazole is a better leaving group and participates in the reaction by forming a more activated phophorylating intermediate (**23**). 1*H*-Tetrazole and 3-nitro-methylimidazole have also been used as catalysts in this reaction.

The yield of the phosphotriester coupling is an improvement to that of the phosphodiester approach, since the phosphotriester is protected from further reaction with phosphorylating agents. With the development of phosphate protecting groups and efficient condensing reagents, coupling steps could be reduced from several hours in solution to less than 5 minutes on solid phase.[27] The synthesis of a 20-residue ODN could be accomplished in 8 hours.[28]

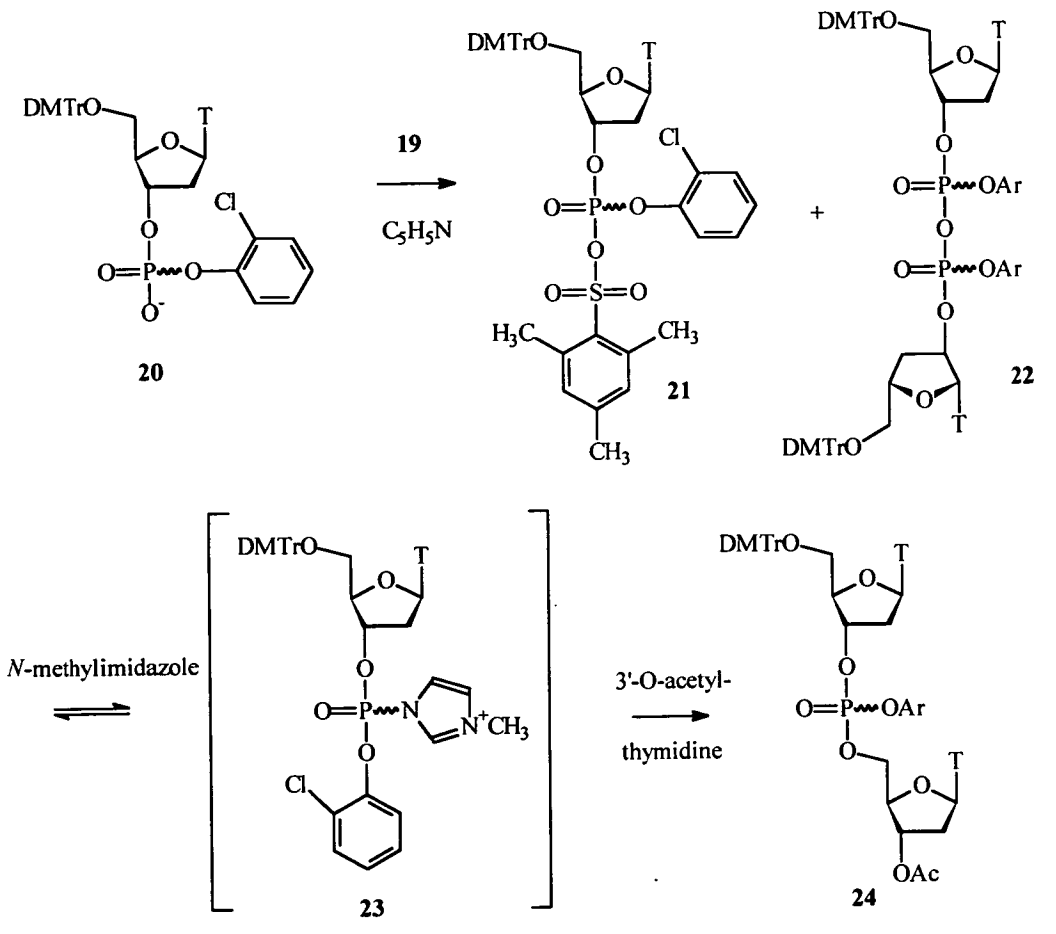


Figure 11. Formation of a dinucleoside phosphate triester

Two side reactions give rise to limitations with the phosphotriester approach. During coupling, there is a competitive reaction of sulphonylation of the 5'-hydroxyl group with the coupling reagent of about 1 %. This therefore reduces the coupling efficiency of the phosphotriester couplings to 97-99 %, and thus limits the length of the ODN attainable to about 40 residues.[29] Secondly, and more seriously, deoxyguanosine residues can be phosphorylated and substituted with nitrotriazole at the O^6-position unless another protecting group is used.

For large scale (multi-gram) synthesis of short ODNs, the phosphotriester method, usually solution phase, is especially useful. However, the phosphotriester approach does not have the speed and efficiency of the phosphite-triester method described in the next section.

1.6.4. Phosphite Triester Chemistry

The development of the phosphite triester method by Caruthers *et al*[30], is often referred to as phosphoramidite chemistry, revolutionised oligonucleotide synthesis from a manual or semi-manual operation into a commercialised process using automated instrumentation. The coupling of two nucleotide units could be achieved in minutes rather than hours and were easily oxidised to the required phosphotriester by aqueous iodine. Originally introduced in 1975 by Letsinger *et al*[31] the phosphite-triester method was based on the great reactivity of phosphorochloridite reagents upon alcohols giving phosphite-triesters. Figure 12 illustrates the reaction between a 5'-O-protected thymidine (**25**) and 2,2,2,-trichloroethylphosphorodichlorodite, generating the deoxyribonucleoside-3'-O-phosphorochloridite intermediate (**26**) within 5 min at -78°C.

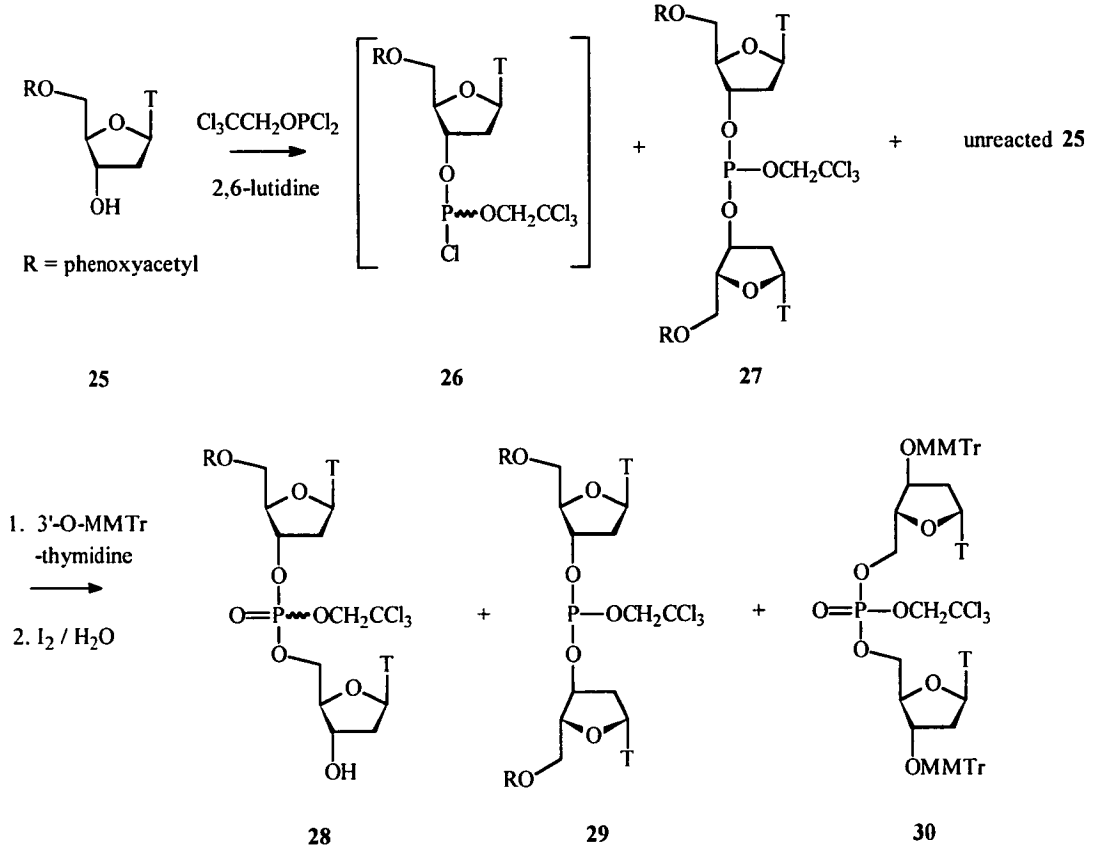
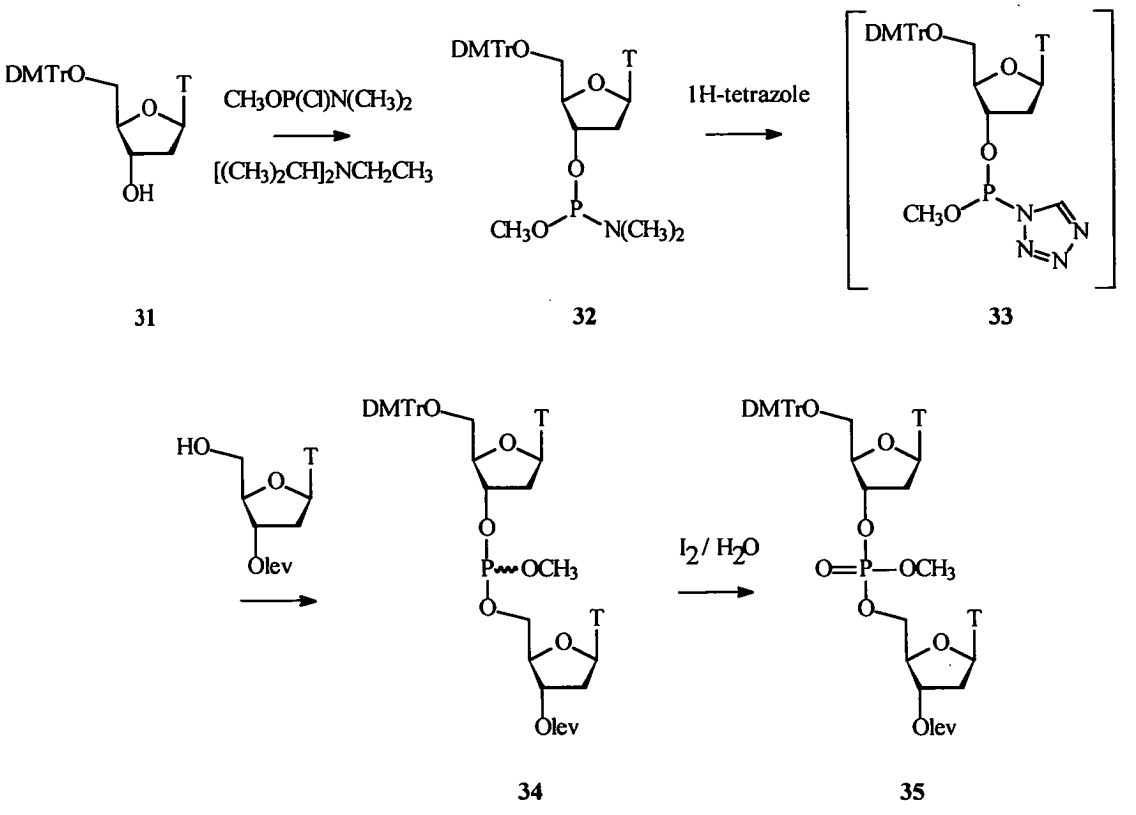


Figure 12. Letsinger's phosphite triester approach

3'-O-protected thymidine was added followed by aqueous iodine oxidation to give the rapid formation of the dinucleoside phosphate triester (**28**), and also the symmetrical 3'-3' (**29**) and 5'-5' (**30**) dinucleoside phosphate triesters.[32] The 2,2,2-trichloroethyl group was shown to be suitable for the synthesis of a thymidine tetranucleotide. Several other phosphate protecting groups were investigated by Ogilvie *et al.*[33] such as 2,2,2-tribromoethyl, *p*-chlorophenyl, 2-phenylethyl, 2-*p*-nitrophenylethyl, benzyl, methyl and β-cyanoethyl. The methyl and β-cyanoethyl groups were found to be the most useful and remain the most widely used today. Matteucci *et al*[34,35] demonstrated the solid phase synthesis of a dodecanucleotide using the methyl group for phosphate protection and 1*H*-tetrazole as the catalyst for coupling. Several serious problems accompanied the use of nucleoside chlorophosphites or tetrazolides in the solid-phase synthesis of ODNs. Low temperatures (-70°C) and dry, inert conditions were required for the preparation of these reagents immediately prior to use. During preparation amounts of 3'-3' dinucleoside phosphite triesters were formed which created purification problems and lower yields. These problems were overcome, by Beaucage *et al*[36] in 1981, with the development of deoxyribonucleoside phosphoramidites. These have the advantage of being more stable to hydrolysis and room temperature but are still readily activated to form an internucleotide linkage in essentially quantitative yield with few side products. Therefore, two of the most important criteria for solid-phase ODN synthesis, high yields and few side products, are covered by the use of deoxyribonucleoside phosphoramidites.

Figure 13 is an example of this approach consists of reacting the 5'-O-dimethoxytrityl protected deoxyribonucleoside (**31**) with chloro-*N,N*-dimethylaminomethoxyphosphine and *N,N*-diisopropylethylamine, to afford **32** which can be isolated by conventional methods and stored as a dry powder. **32** is activated with 1H-tetrazole and the reaction with 3'-O-levulinylthymidine gives the corresponding phosphite triester (**34**) in quantitative yields in a few minutes. This is then oxidised using aqueous iodine to give the protected phosphate dimer (**35**).

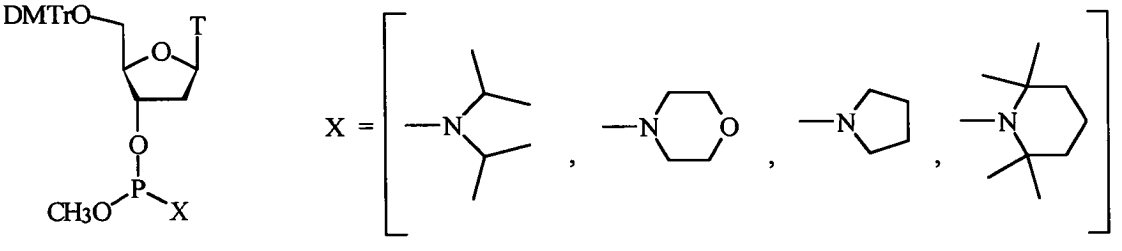


31 32 33

34 35

Figure 13. Caruthers phosphite triester approach

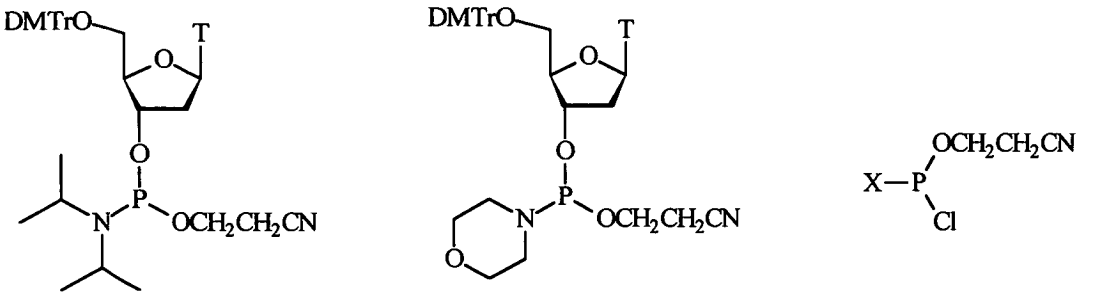
Due to the varied instability of **32** in acetonitrile, they were unreliable for automated systems. This brought about the study of other possible *N,N*-dialkylaminomethoxyphophines[37] including the diisopropylamino (**a**), morpholino (**b**), pyrrolidino (**c**) and the 2,2,6,6-tetramethylpiperidino (**d**) substituents (figure 14).



36 (a) (b) (c) (d)

Figure 14. N,N-dialkylaminomethoxyphosphines.

It was found that the diisopropylamino (**a**) and morpholino (**b**) phosphoramidites could be purified by silica gel chromatography and were stable to acetonitrile for at least a month without significant decomposition. However, it was seen as a disadvantage having the deprotection step for the removal of the methyl phosphate protecting groups. In order to eliminate this step, the β-cyanoethyl group was introduced by Sinha *et al*[38] in 1983.

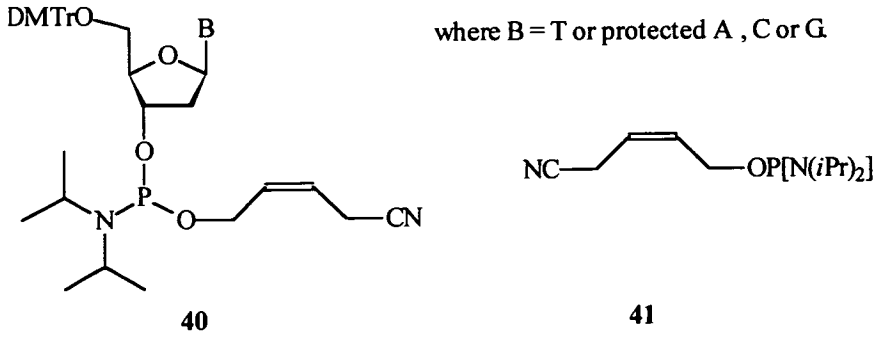


37 **38** **39**

Figure 15. Diisopropyl and morpholino phosphoramidites

The phosporamidites (**37, 38**) were prepared from the protected deoxyribonucleosides using the monofunctional phosphitylating reagent **39** (Figure 15). This allowed the elimination of thiophenol from the deprotection procedures since the β-cyanoethyl group could be cleaved under the basic conditions required for the deprotection of nucleobase protecting groups.

Among the other reported phosphate protecting groups such as 2,2,2-trichloro-1,1-dimethylethyl,[39] *p*-nitrophenylethyl,[40] and allyl,[41] only the β-cyanoethyl group meets most of the demands and remains the most widely used today. It is capable of protecting the phosphorus group during automated synthesis, allowing fast and efficient synthesis, and is removed under conditions which eliminate the possibility of chain cleavage. However, it is still thought that the β-cyanoethyl protected deoxyribonucleoside phosphoramidites are expensive to produce even on kilogram scale.[42] Ravikumar *et al*[42] reported the use of cyanobutenyl group for the protection of internucleotide linkages (Figure 16).



40 41

Figure 16. Cyanobutenyl as a phosphate protecting group.

The bis[*N,N*-diisopropylamino]-4-cyano-2-butenyloxy-phosphine is easily accessible, has excellent phosphitylating properties and the cyanobutenyl group can be removed under mild conditions using aqueous ammonium hydroxide. The calculated cost of manufacturing the cyanobutenyl phosphoramidites on kilogram scale was found to be approximately 60% cheaper than the cost of cyanoethyl phosphoramidites.

1.6.5. H-Phosphonate Chemistry

The H-phosphonate method, which was first described be Todd *et al*[43] in 1957, has recently been re-examined in the last few years for both DNA[44] and RNA[45] synthesis, and has emerged with high potential. The renewed interest is due to the increased stability of nucleotides and the speed of reaction, avoiding the need for a phosphorous protecting group. An example of this approach is shown in Figure 17.

Treatment of the nucleoside H-phosphonate (**42**) with pivaloyl chloride rapidly generates the mixed anhydride **43**. This intermediate is then coupled to a 5'-unprotected nucleoside (**44**) which is attached to a solid support. The resulting phosphite diester (**45**) can then be capped, detritylated with acid and condensed with the next 5'-dimethoxytrityldeoxynucleoside 3'-H-phosphonate. The oxidation of all the internucleotide linkages is carried out after the assembly of the oligonucleotide by treatment with aqueous iodine to give the natural oligomer (**46**).

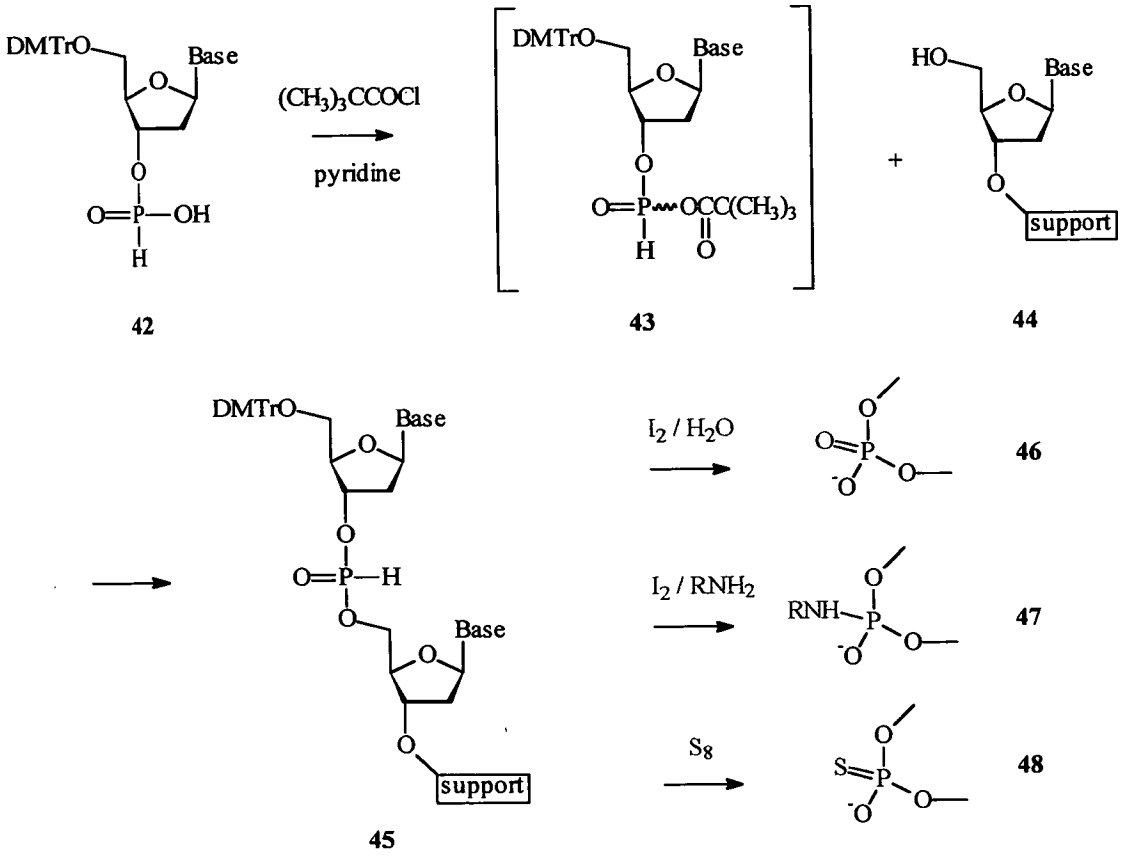


Figure 17. H-phosphonate approach

Alternatively, by choosing appropriate oxidants, other analogs are possible. For example, the use of a primary or secondary amine produces the corresponding phosphoramidites **47** and the oxidation with elemental sulphur in carbon disulphide and triethylamine generates phosphorothioates (**48**).[46] Although the *H*-phosphonate approach is not as efficient as the phosphoramidite approach, it is still effective in the synthesis of selected analogues.

1.7. Solid-Phase Synthesis

Solid-phase synthesis is fundamentally a heterogenous coupling reaction between a nucleotide derivative and another residue attached to an insoluble support. Originally developed by Merrifield[47] for the synthesis of proteins, Letsinger[18] was the first to

apply this principle for the synthesis of di- and trinucleotides in 1965. The solid-phase approach to the synthesis of oligonucleotides has become the accepted and standard technique worldwide.

The chemical synthesis of oligonucleotides is generally carried out using phosphoramidite chemistry in the 3' to 5' direction. This takes advantage of the high chemical reactivity of the primary 5'-hydroxyl function. Generally, solid-phase synthesis has several advantages over the solution phase method. The first residue is attached *via* a linker to an insoluble support giving the advantage of being able to use a large excess of the soluble nucleotide derivative, forcing the coupling reaction to high yield. The excess reactant nucleotide and reagents can then be simply removed by filtration and washing, without loss of the desired support bound dinucleotide. Other coupling reactions can be carried out and the reagents similarly removed, aiding purification. All the reactions occur in one vessel, allowing for automation of the reaction processes resulting, in reduction of labour and synthesis times.

1.7.1 Solid Support

The two main solid supports used in oligonucleotide synthesis are the controlled pore glass (CPG) and polystyrene supports.[48] The supports have to be inert towards, and insoluble in all the solvents and reagents that are used in synthesis. The CPG support was found to be ideal due to its rigidity and inability to swell. The supports are manufactured with different particle sizes, generally between 500 and 1000Å in pore size, with the latter favoured for syntheses over 45 residues. Automated syntheses using these supports is limited to scales of between 10 and 50 $\mu mol\ g^{-1}$, below which the reactions become irreproducible, and above which are subject to steric crowding between growing chains.

The initial residue is loaded onto the support at a functionalised reaction site (Figure 18). In order to extend these sites away from the surface of the support and to ensure accessibility of the reagents, the growing oligonucleotide is attached to a spacer arm.

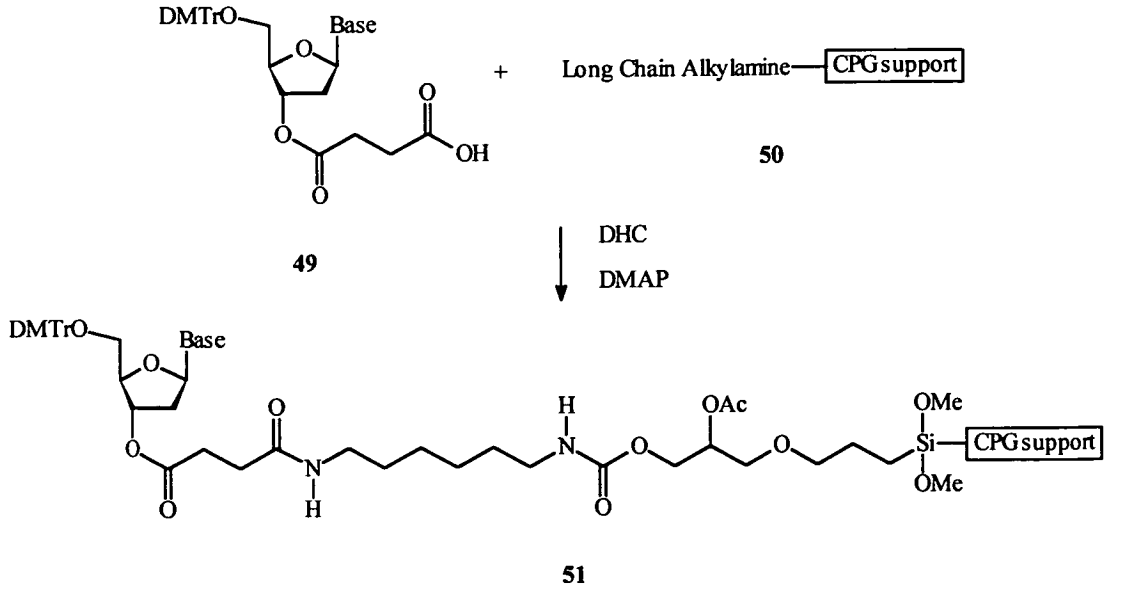


Figure 18. Attachment of a nucleoside to controlled pore glass (CPG)

In the synthesis of DNA, the first deoxynucleotide is attached at its 3'-position *via* a 3'-succinate derivative (**49**) to an amino group on the spacer arm using dicyclohexyldicarboidiimide (DHC) and DMAP. The spacer arm shown in Figure 18 is a long chain alkylamine attached, to the CPG.

1.7.2. Assembly of an Oligonucleotide Chain

A small column is packed with the deoxynucleoside loaded support which can be used with a DNA synthesiser. These are commercially available along with the solvents and reagents needed for the synthesis of oligonucleotides. The synthesiser allows the solvents and reagents to flow through the column in a predetermined order allowing the assembly of the chain.

Figure 19 shows the basic steps involved in one cycle of solid-phase synthesis using the phosphoramidite approach.

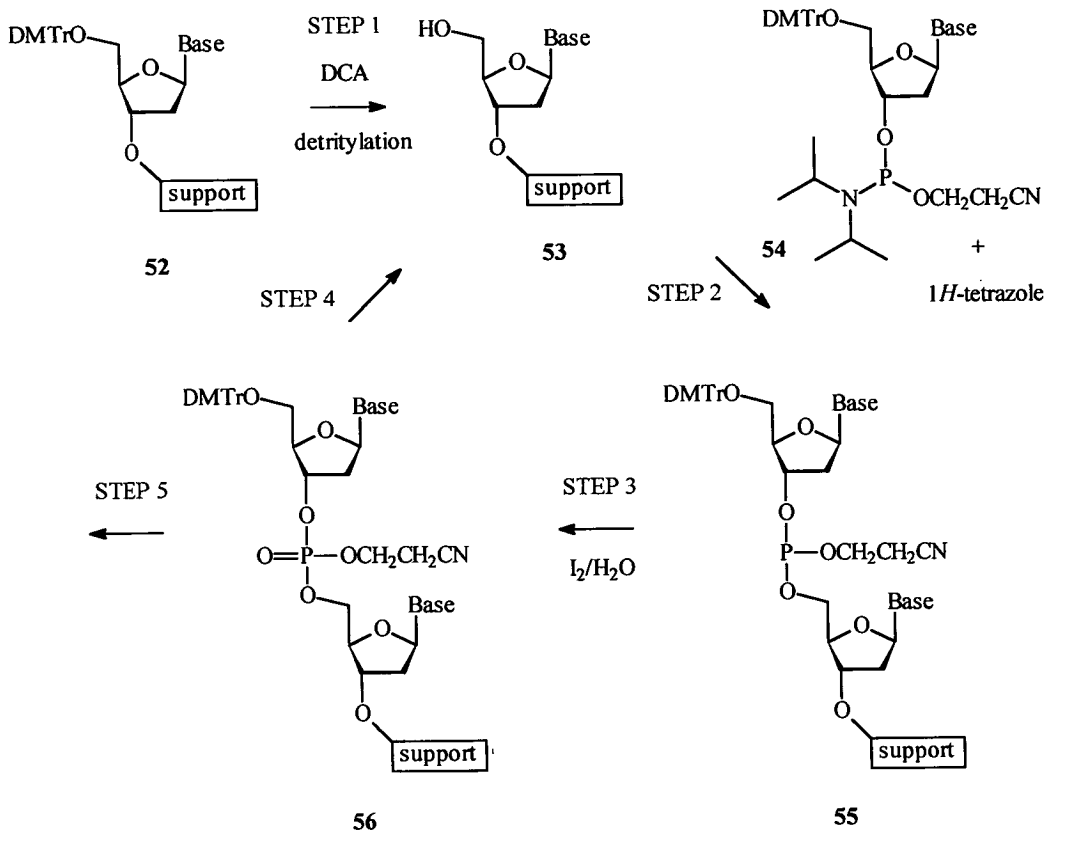


Figure 19. Solid-phase synthesis cycle using the phosphoramidite approach.

STEP 1. The initial step is the removal of the dimethoxytrityl group from the polymer attached nucleoside (**52**) with dichloroacetic or trichloroacetic acid (TCA), to give the free 5'-hydoxyl (**53**).

STEP 2. The phosphoramidite nucleotide (**54**) is activated with 1H-tetrazole and added to the polymer bound nucleoside (**53**) to form the phosphite-triester dimer (**55**).

This is followed by the capping of all of the unreacted free 5'-hydroxyl functions using acetic anhydride. This also has the benefit of reversing any phosphitylation of the O-6 position of guanine.

STEP 3. The intermediate phospite-triester dimer (**55**) is then oxidised to the phosphotriester dimer (**56**) with aqueous iodine with the hydrogen iodide released during oxidation being neutralised with pyridine or 2,6-lutidine.

STEP 4. The cycle is then repeated by detritylation of the dimer and the addition of a further nucleotide to the chain. This is then repeated until the desired length and sequence of 3'→5' oligonucleotide is obtained.

STEP 5. Once the synthesis is complete, the oligonucleotide is cleaved form the solid support and deprotected with ammonium hydroxide.

In the use of the phosphoramidite approach, either 2-cyanoethyl (shown) or methyl groups are used as the phosphate protecting groups. The 2-cyanoethyl group is removed by β-elimination using ammonium hydroxide as the oligonucleotide is cleaved from the solid support, whereas the methyl group is removed by treatment with thiophenate ion generated by thiophenol and triethylamine.

In the phosphotriester approach, aryl groups are used as the phosphate protecting groups, which are selectively displaced using *syn*-pyridine-2-carboxaldehyde or (E)-2-nitrobenzaldehyde oxime (Figure 8).

The average coupling efficiency in the phosphoramidite approach to DNA synthesis, when carried out on a machine, is approx. 99 %. The coupling efficiencies are calculated by spectroscopic measurement of the intensity of orange colour (dimethoxytrityl cation) liberated during detritylation.

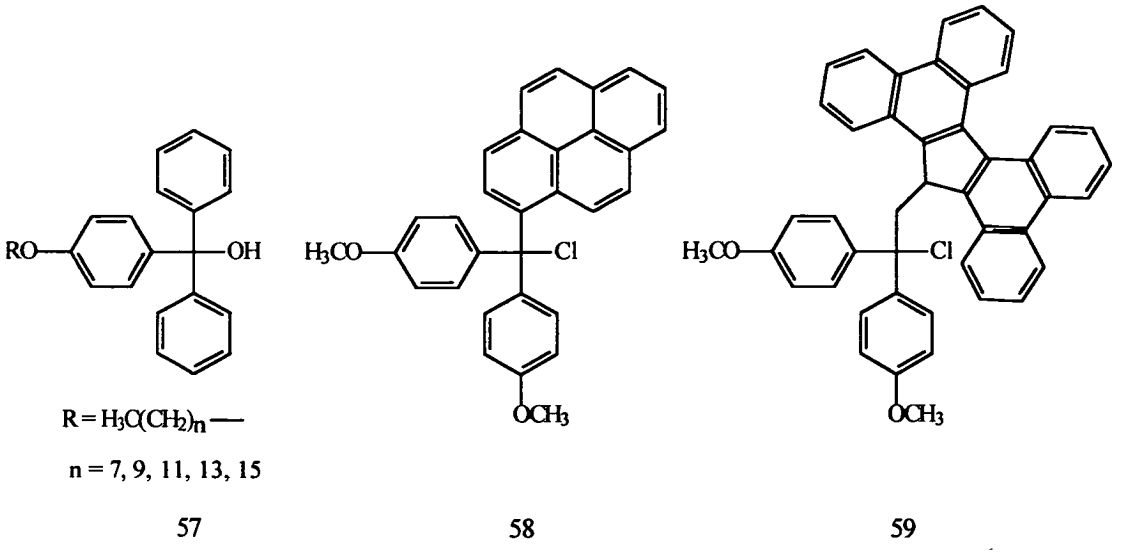
All of the above steps can be performed on a commercially available automated DNA synthesiser. The purification of the crude oligonucleotides is the only remaining step which requires manual manipulation. Due to the large number of reactions that take place during oligonucleotide synthesis, a number of impurities are formed on the

support. These are cleaved as the desired oligonucleotide is cleaved from the support, therefore purification is necessary.

1.7.3. Purification of Oligonucleotides

The phosphoramidite approach to oligonucleotide synthesis employs a capping step that acetylates the 5'-hydroxyl functions that have failed to undergo coupling. This prevents the build-up of large amounts of incorrectly sequenced and shortened chains. The truncated sequences that have been capped remain so until the end of the synthesis when they are uncapped during the ammonia deprotection procedure.

Two powerful separation methods have become popular for the purification of oligonucleotides. Polyacrylamide gel electrophoresis uses the charge difference of a oligonucleotide, with it capable of separating fragments with only one charge difference. The other is high performance liquid chromatography (HPLC), in particular reverse phase chromatography, which uses the hydophobic nature of the product to aid purification. The DMTr group, in the final nucleotide in the sequence, can either be removed or retained. Retaining the group aids purification through its highly lipophilic character, allowing the oligonucleotide to be well resolved from the shorter, non-DMTr protected impurities. After purification, the terminal 5'-O-DMTr group can then be simply removed by treatment with aqueous 80% acetic acid. However, the change in the total polarity, introduced by the addition of a single DMTr group, is greatly reduced when synthesising longer oligonucleotides (> 30-mer). Consequently when a long oligonucleotide is purified by reverse-phase HPLC, the retention times of the failure sequences and the desired product are similar. This creates separation and purification difficulties. In an attempt to increase the hydrophobic character of the 5'-hydroxyl protecting group, several researchers modified the DMTr group (Figure 20). Seliger and Gortz[49] introduced para-alkoxy groups on triphenylmethylcarbinol (**57**). Fourrey *et al*[50] introduced the fluorescent 1,1-bis-(4-methoxyphenyl)-1'-phenylmethyl chloride (**58**). However, in both cases, only the 5'-protected phosphoramidite thymidine had been successfully prepared.



57 58 59

Figure 20. Hydrophobic 5'-hydroxyl protecting groups

Ramage and Wahl[51] introduced 4-(17-tetrabenzo[*a,c,g,i*]Fluoromethyl)-4',4"-dimethoxytrityl chloride (Tbf-DMTr-Cl) (**59**) as a new hydrophobic protecting group. Ramage *et al*[52] previously demonstrated the advantages of using peptidic derivatives of Tbf for the purification of peptides by reverse phase HPLC. The Tbf moiety possessed strong hydrophobic and fluorescent properties, ideal for purification purposes. The DMTr group was modified by substituting position 4 of one of the phenyl ring with Tbf. This preserved the regioselectivity of the trityl group to primary hydroxyls, while forming a new hydrophobic 5'-protecting group. In comparison with DMTr-on purification, Tbf-DMTr showed increased separation from truncated sequences and demonstrated the usefulness of Tbf-DMTr in the purification of short (12-mer) and longer (>100-mer) sequences.[53] The Tbf-DMTr group was then removed by treatment with 2% TFA.

1.8. Synthesis of Oligoribonucleotides

Discoveries during the last 15 years have transformed the understanding of the diverse roles that RNA plays in biological systems (Sections 1.4. and 1.5.). Therefore, there has been an increasing interest in the synthesis, especially chemical synthesis, of

biologically active RNA for biomedical, biochemical and physical studies. The developments made in the methodology of the chemical synthesis of DNA sequences has had an enormous effect on biological research. Methodologies for the chemical synthesis of oligoribonucleotides on solid phase supports have not kept pace with advances in DNA synthesis. The lack of efficiency in chemical RNA synthesis compared to DNA synthesis is due to the additional hydroxyl group at the 2' position and the necessity for its protection during synthesis. There is a lack of wholly satisfactory protecting group strategies. However, improved techniques have been developed for solid-phase oligoribonucletide synthesis, as well effective enzymatic synthesis procedures.

1.8.1. Enzymatic Synthesis of Oligoribonucleotides

As described in Section 1.1, the flow of genetic information in living systems is from DNA being transcribed to RNA and then translated to protein. The cell uses enzymes called RNA polymerases to make RNA from a DNA template. Milligram quantities of RNA can be produced by a researcher using RNA polymerase from bacteriophage T7, template DNA and nucleoside triphosphates.[54] The template used consists of two chemically synthesised oligodeoxynucleotides annealed together.

The T7 polymerase system has proved useful as a simple means of producing specific RNA sequences. However, it is highly dependent on length and sequence. Shorter oligoribonucleotides of 12 bases or less give poor yields and are difficult to purify to homogeneity. It has been observed that the T7 polymerase undergoes abortive initiations, and may add an undesired base, thus complicating purification.[54] Sequences which do not begin with a guanosine at the 5'-end have proven extremely troublesome to make. For each particular sequence, conditions have to be customised to optimise yields. Finally, T7 polymerase incorporates only the four natural RNA bases into an oligoribonucleotide, and therefore does not allow for the incorporation of modified or non-standard nucleoside bases or even deoxyribonucleosides.[55]

1.8.2. Chemical Synthesis of Oligoribonucleotides

An alternative to the T7 method is the chemical synthesis of RNA, which has, to a certain degree, paralleled the methodology of DNA synthesis previously described in this chapter. The development of practical methods to synthesise RNA has been hindered by the requirement to protect the additional 2'-hydroxyl group. Although the assembly of oligoribonucleotides is analogous to that of oligodeoxyribonucleotides, the rate of the coupling reactions are slower. This is due to the use of bulky 2'-hydroxyl protecting groups. The choice of protecting group is extremely important, as it must remain stable during chain assembly, and be removed only at the very end of the deprotection cycle. This applies in particular to the phosphate protecting groups, since triester phosphates are highly unstable in the presence of a neighbouring hydroxyl group. It must also be removed quantitatively under conditions that will not lead to cleavage or migration of the internucleotide linkages (Figure 21).

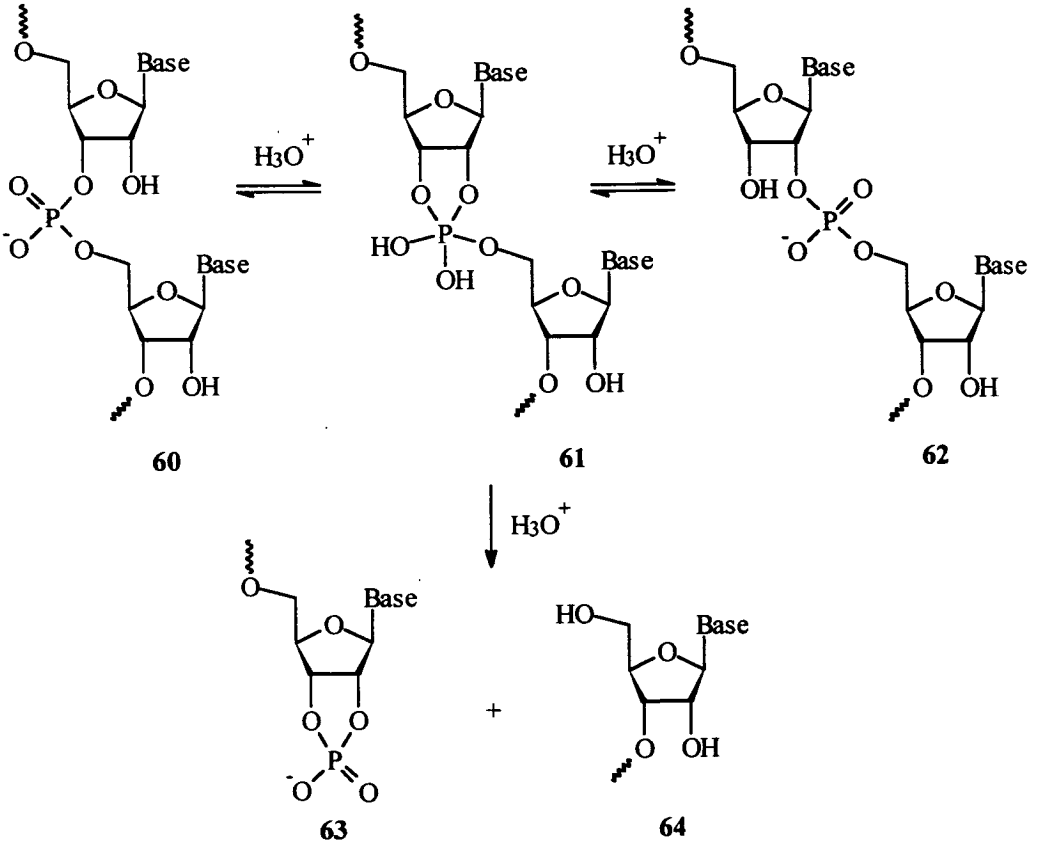
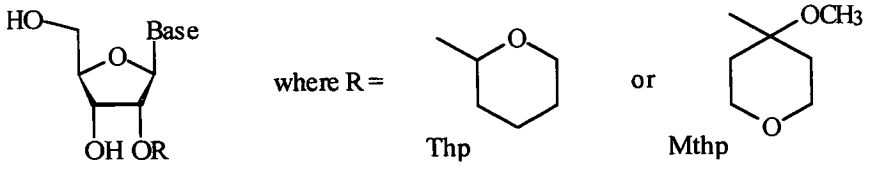


Figure 21. Acid-catalysed cleavage and migration of inter-ribonucleotide linkages

It has long been realised that the internucleotide linkages of RNA sequences can both migrate (as in **60→61→62**) and undergo cleavage (as in **60→61→63 + 64**) under acidic or basic conditions.[55] The mechanism of acid-catalysed migration and cleavage is believed to be that suggested in Figure 21.[56,56] The cleavage and migration of RNA takes place via a cyclic pentacoordinate trigonal bipyrimidal oxyphosphorane intermediate/transition state (**61**).[57]

In 1956, Brown *et al*[58] described an experiment showing the partial hydrolysis of cytidine 3'-O-benzylphosphate under basic and acidic conditions. This experiment indicated that the conversion of a 3'→5' to a 2'→5' internucleotide linkage can occur with phosphodiester and protected phosphotriester RNA oligomers under these conditions.[59] This is a major problem, since it is impossible, with current technologies, to separate oligoribonucleotides containing one or multiple 2'→5' internucleotide linkages from correctly connected oligoribonucleotides. It is, therefore, important to avoid phosphoryl migration or chain cleavage during deprotection of the 2'-hydroxyl function.

The development of the 2'-hydroxyl protecting group began when Griffin *et al*[60,61] tested the acid labile tetrahydropyran-2-yl group (Thp) (**65**) for this purpose in 1964 (Figure 21).

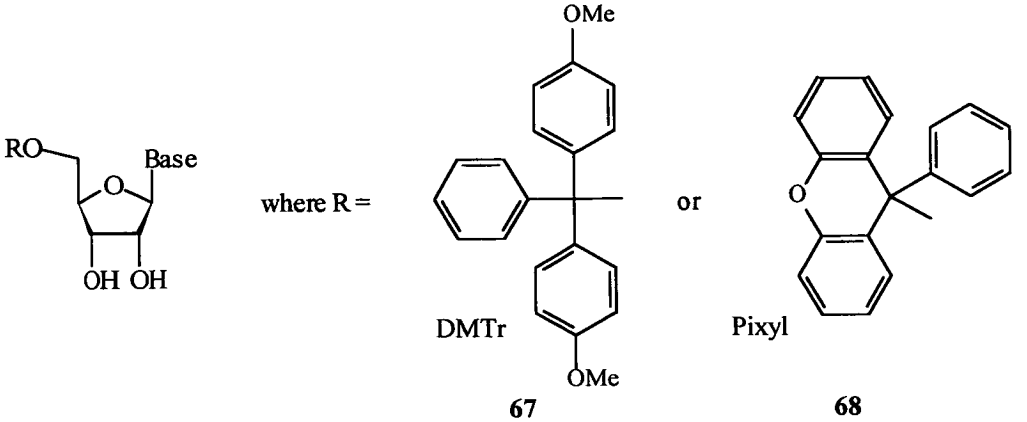


65 **66**

Figure 22. 2'-Hydroxyl Protecting Groups

Complete removal of the Thp group was achieved in 3 - 4 hours using 0.01M HCl at 20 °C. These relatively mild conditions did not induce significant phosphoryl migration. The Thp group has been successfully used in the phosphotriester approach to oligoribonucleotide synthesis.[62] However, only relatively short sequences of 20

units or less were capable of being made using this method due to the protecting group strategies involved for the 5'-hydroxyl function. Current strategies use 9-phenylxanthen-9-yl (pixyl) (**68**) or 4,4'-dimethoxytrityl (**67**) groups for the protection of the 5'-hydroxyl function (Figure 23).



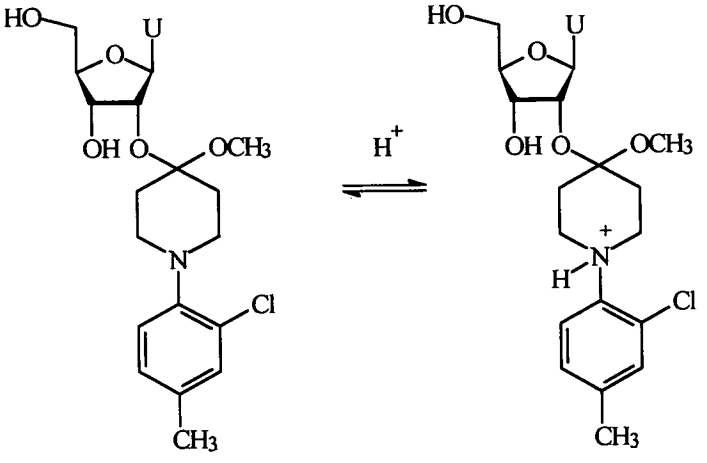
67 **68**

Figure 23. 5'-Hydroxyl Protecting Groups

These groups are removed under acidic conditions prior to chain elongation during oligoribonucleotide synthesis. During the acid deprotection step, the 2'-Thp group undergoes partial hydrolysis.[63] Following partial deprotection, acid-catalysed internucleotide bond cleavage and gives rise to the accumulation of various side products which limits this approach. Another disadvantage of the Thp is its inherent chirality. This complicates the purification of the ribonucleoside building blocks, due to the formation of diastereoisomers. This led to its replacement with an achiral alternative, 4-methoxytetrahydropyran-4-yl (Mthp) (**66**), for protection of the 2'-hydroxyl function during oligoribonucleotide synthesis in solution.[64] This solved the problem of chirality, but, due to its acid lability, the 2'-O-Mthp group is also incompatible with the pixyl and dimethoxytrityl groups used for the protection of the 5'-hydroxyl functions.

Interest in developing methods for the rapid synthesis of oligoribonucleotides on solid supports using the 5'-O-pixyl and dimethoxytrityl group strategies, led Reese *et al*[65] to introduce the 1-[(2-chloro-4-methyl)phenyl]4-methoxypiperidin-4-yl (Ctmp) (**69**) group for protection of the 2'-hydroxyl function.

The Ctmp group was designed to be unaffected by the acid conditions required for removal of the 5'-terminal protecting group, yet it is easily removed under mildly acidic conditions (eg. pH 2.0-2.5. R.T.) in the final deprotection step. This is achieved by the tertiary anilino function of the acetal, which is protonated at pH < 2 (**70**), thus producing a strong inductive effect and enhanced stability under the conditions used for 5'-O-pixyl and dimethoxytrityl deprotection (Figure 24).



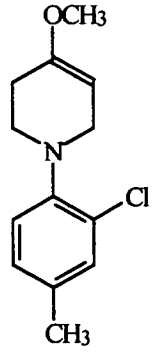
69 **70**

Figure 24. 1-[(2-chloro-4-methyl)phenyl]4-methoxypiperidin-4-yl (Ctmp)

Typically, the rate of hydrolysis of the 2'-O-Mthp uridine was 140 times faster at pH 1.0 than at pH 3.0. This was found to be considerably quicker than the rate of hydrolysis of 2'-O-Ctmp uridine which was only approximately 2.25 times faster at pH 1.0 than at pH 3.0. Furthermore, the Ctmp and Mthp protecting groups both undergo hydrolysis at pH 2 - 2.5 and at room temperature, and are predicted to be completely removed under conditions which RNA is completely stable. It was soon established that the Ctmp group was suitable for the protection of the 2'-hydroxyl functions in the solid phase synthesis of oligoribonucleotides, using either the *H*-phosphonate[66] or the phosphoramidite approach.[67]

Although the Ctmp group proved satisfactory for the protection of the 2'-hydroxyl functions in oligoribonucleotide synthesis, the preparation of the 1-[(2-chloro-4-methyl)phenyl]4-methoxypiperidin-4-yl reagent (**71**) is rather long and inefficient. This

led to the development of a new synthetic route, thus making a number of similar halogenated enol ethers available.[68] It was found that 1-(2-fluorophenyl)-4-methoxy-1,2,5,6-tetra-hydropyridine (**72**) was easier to prepare than the Ctmp reagent and that the 1-(2-fluorophenyl)-4-methoxypiperidin-4-yl (Fpmp) protecting group had enhanced properties compared to those of the Ctmp protecting group.



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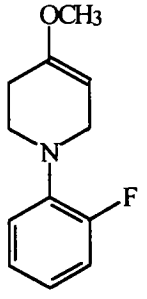

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Figure 25. Enol Ether reagents for the introduction of the Ctmp and Fpmp

The Fpmp group was found to be approximately 1.3 times more stable to hydrolysis than the Ctmp group in the pH range 0.5 - 1.5.[69] Therefore, the removal of the 5'-O-DMTr or pixyl protecting group during oligoribonucleotide synthesis would be more selective when Fpmp rather than Ctmp groups are used to protect the 2'-hydroxyl function. The 5'-O-DMTr-2'-O-Fpmp protected phosphoramidite building blocks are now commercially available and have been widely used in oligoribonucleotide synthesis.[70]

The introduction of Fpmp, and other similar acetals, has been simplified by use of a bifunctional silylating reagent, 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane, which was introduced by Markiewicz[70] in 1978. Markiewiczs' reagent is used to simultaneously protect both the 5'- and 3'-hydroxyl functions (**74**), thus leaving the 2'-hydroxyl free for introduction of the Fpmp group (**75**). Removal of the cyclic silyl group is achieved with fluoride ions to give a 2'-O-Fpmp nucleoside (**76**), which can be then be converted to its 5'-O-DMTr-2'-O-Fpmp protected ribonucleoside 3'-O-phosphoramidite (**78**) in a 2 step procedure (**76**→**77**→**78,**) as shown in Figure 26.

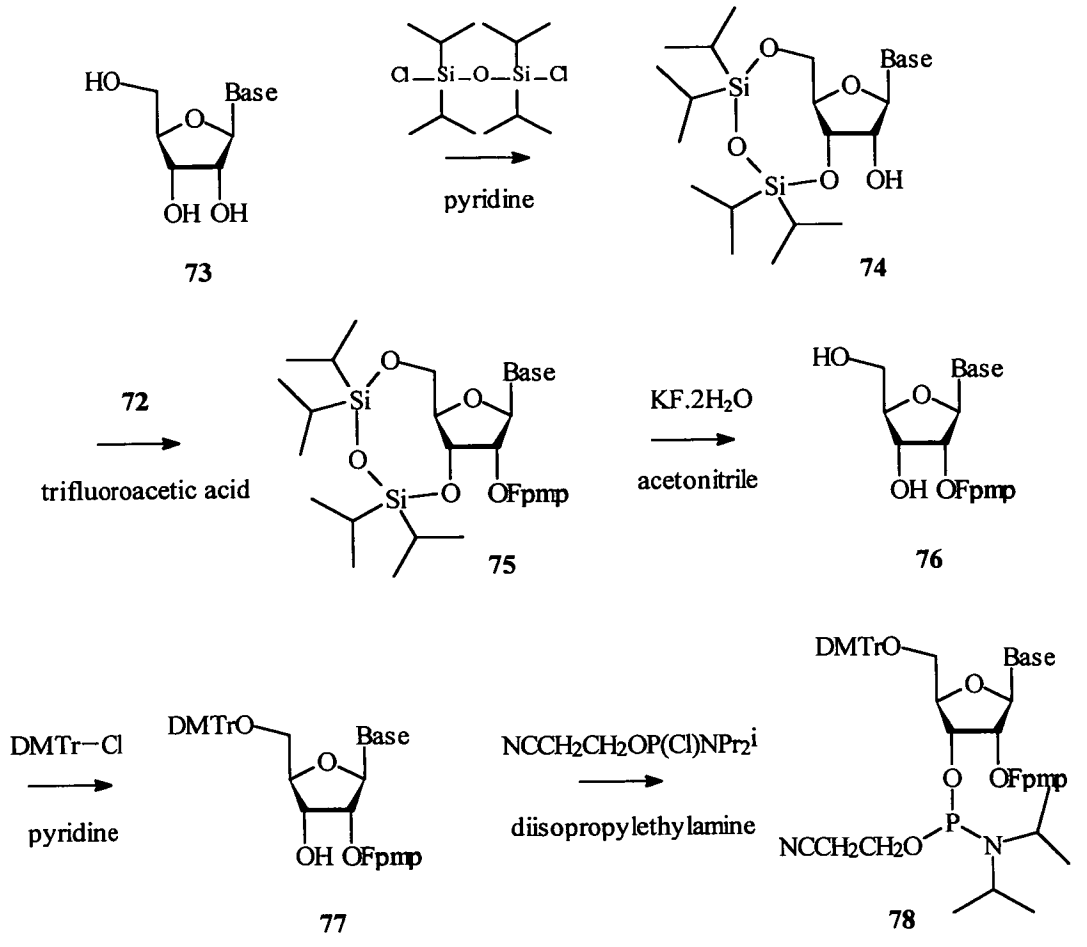
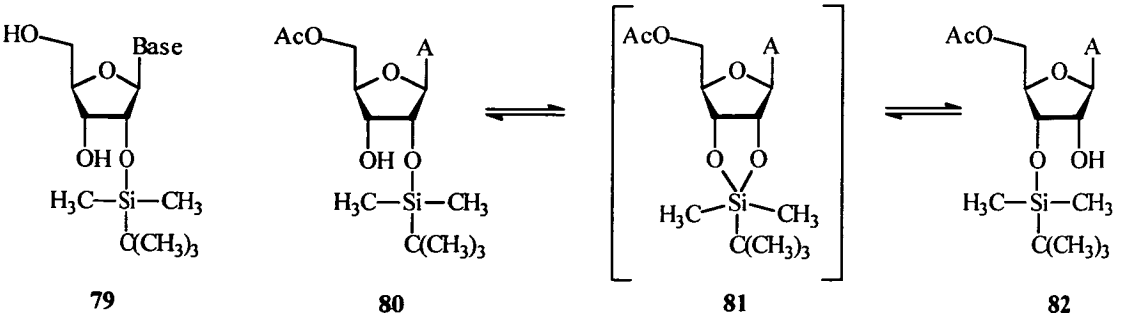


Figure 26. Synthesis of 2'-O-Fpmp protected ribonucleoside 3'-O-phosphoramidite

Recent observations have indicated that certain RNA sequences can undergo internucleotide cleavage and migration under the conditions suggested for the removal of the Fpmp groups (pH 2.0 - 2.5).[71] Improvements to the RNA synthetic cycle protocol has shown that a solution of sodium acetate (pH 3.25, 30°C, 36 hours) is suitable for the removal of all the 2'-O-Fpmp protecting groups without any disruption to the internucleotide bonds.[72]

Another group which has been successfully used in the protection of the 2'-hydroxyl function is the t-butyldimethylsilyl (TBDMS) (**79**) group. First developed in 1973 by Ogilvie,[73] the group is reasonably stable to either basic or acidic conditions, and is

cleaved rapidly by fluoride ion within 30 minutes at 20 °C.[74] The usefulness of the TBDMS group as a 2'-O-hydroxyl protecting group for ribonucleosides in the synthesis of oligoribonucleotides was questioned by Jones and Reese.[75] They reported the migration of the 2' and 3'-O-TBDMS group (**80** and **82**) of the ribonucleosides when in methanolic solutions at 36 °C (Figure 27).



79 **80** **81** **82**

Figure 27. 2'-O-tert-Butyldimethylsilyl protecting group

Under these conditions, the interconversion half-life is approximately 1 hour. In anhydrous pyridine, the $T_{1/2}$ conversion of **80** to **82** was 1140 minutes at 36 °C, considerably slower than that of the methanolic conditions. However, under identical conditions, conversion of **82** to **80** was seen to have a half-life of 380 minutes. Under the conditions of 80% acetic acid, 0.1M methanolic hydrochloric acid or in pure solvents such as chloroform and anhydrous DMF, no detectable isomerisation occurred within 25 hours at 20 °C.[76] Further investigations have been carried out into the possibility of interconversion during the synthesis of 2'-O-TBDMS phosphoramidites (Figure 28).[76] Generally, the introduction of TBDMS on to the 2'-O-hydroxyl function of a 5'-O-DMTr protected nucleoside is achieved by use of TBDMS chloride in the presence of imidazole (**83**→**84** and **85**). This produces a mixture of 2'-O and 3'-O-silyl isomers (**84** and **85** respectively), which require seperation by careful silica gel chromatography. Phosphitylation of the 2'-O-silyl isomer is usually carried out by use of (2-cyanoethoxy)-N,N-diisopropylaminochlorophosphine in the presence of N,N-diisopropylethylamine.

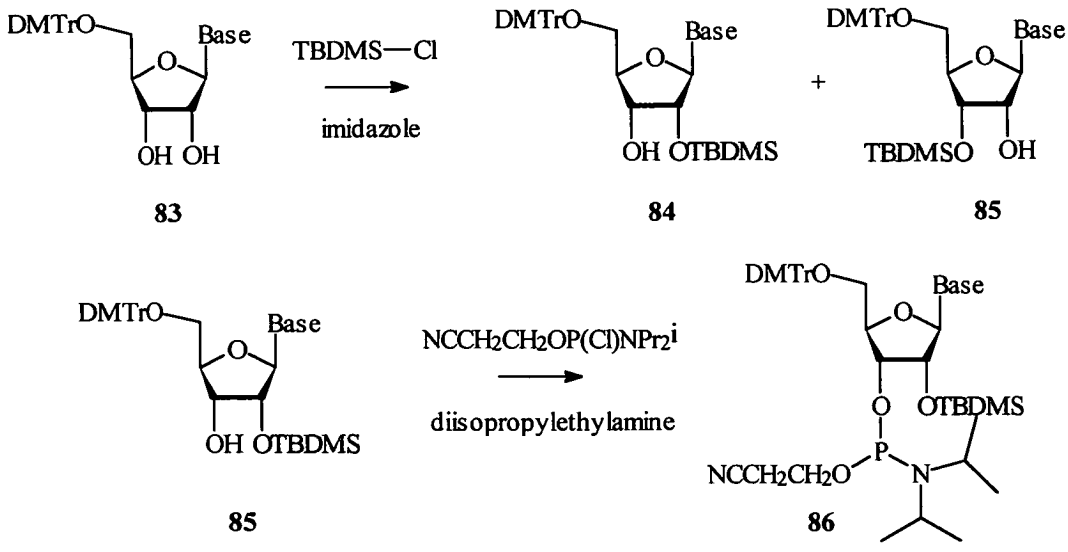


Figure 28. Synthesis of 2'-O-TBDMS protected ribonucleoside 3'-O-phosphoramidite

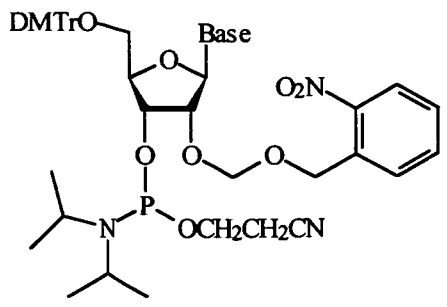
Usman *et al*[77] carried out the automated chemical synthesis and purification of an oligoribonucleotide similar to the 3'-half molecule of an *E.coli* formylmethionine tRNA. The oligoribonucleotide, 43 nucleotides in length, was successfully prepared using 2'-O-TBDMS protected phosphoramidites with a 15 minute coupling time. Stawinski *et al*[78] found that treatment with concentrated ammonium hydroxide at 55°C, used for the deprotection of nucleobase and removal from the solid support, led to the partial removal of the 2'-O-TBDMS group (27%, 8h) and cleavage of the internucleotide linkages (3% after 8h). The 2'-O-TBDMS cleavage rate could be greatly reduced by the addition of ethanol to the conc. ammonium hydroxide in a 1:3 mixture and performing the cleavage at room temperature for 2 to 3 days.[79] The final deprotection step involves the removal of the 2'-O-TBDMS group from the oligoribonucleotide. This is generally achieved using tetra-*n*-butylammonium fluoride (TBAF) within 4 hours, without cleavage or migration of the phosphodiester function. However, removal of the TBDMS groups from larger oligoribonucleotides requires the use of 50 equivalents of TBAF per TBDMS group for 16 hours at ambient temperature.[80]

The commercially available 2'-O-TBDMS protected phosphoramidites have been extremely popular in the chemical synthesis of oligoribonucleotides.[78,79]This is due to the ease of preparation of 2'-O-TBDMS ribonucleoside derivatives and facile removal by the action of fluoride ion sources such as TBAF and $(Et_3N)_3.3HF$.[80] However, some serious inconveniences have been described[81] using the TBDMS group, due to the hindrance of the internucleotide coupling reaction and incomplete elimination of fluoride ions.

Steric hindrance has resulted in the synthesis of oligoribonucleotides being slow. Coupling times of up to 15 minutes were required in each nucleotide addition cycle, compared with the two minute reactions commonly required for oligo-deoxynucleosides. The relatively long coupling times were thought to be a consequence of steric interfering by the bulky, lipophilic TBDMS group.[82] Thus, the steric bulk of the 2'-hydroxyl protecting group became an additional factor which needed to be considered in the design of a suitable protecting group.

Schwartz *et al*[83] developed a novel 2'-hydroxyl protecting group, *o*-nitrobenzyloxymethyl, that was designed to be compatible with oligo-deoxyribonucleotide synthesis (Figure 29). The 2'-O-(*o*-nitrobenzyloxymethyl) ribonucleoside phosphoramidites (**87**) gave average coupling yields greater than 98%, using condensation times of 2 minutes, in the synthesis of two 16-mers and a 31-mer.[83] The *o*-nitrobenzyloxymethyl group is then photochemically cleaved by exposure to long-wave UV light for 4.5 hours at pH 3.7.The improved efficiency was thought to be due to the relief of steric crowding in the vicinity of the phosphoramidite function. However, care was needed during synthesis to prevent the loss of *o*-nitrobenzyloxymethyl protection due to UV emission from overhead lighting.

In the course of studies aimed at developing less hindered systems, Gough *et al*[83] discovered the *p*-nitrobenzyloxymethyl group (**88**) for the protection of the 2'-hydroxyl function. As in the case of *o*-nitrobenzyloxymethyl, it led to 2 minute



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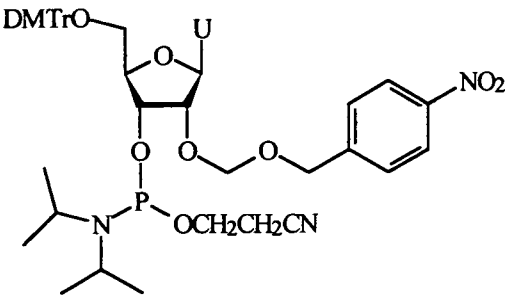

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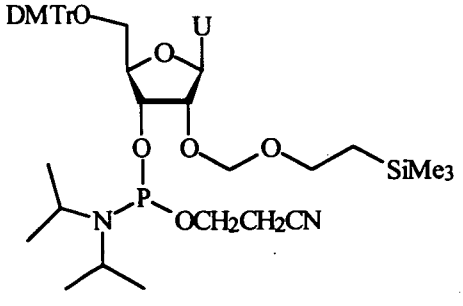
Figure 29. Ortho and para nitrobenzyloxymethyl protecting groups

coupling times with high efficiencies, thus allowing RNA to be synthesised as rapidly as DNA. Similar to TBDMS, the *p*-nitrobenzyloxymethyl group is cleaved by treatment with 1.0M tetrabutylammonium fluoride in THF for 24 hours.

In the search for the ideal 2'-hydroxyl protecting group, other systems have been studied for their suitability in oligoribonucleotide synthesis. The (trimethylsilyl)ethoxymethyl ether (SEM) (**89**) was reported[84] to possess many of the qualities required in a 2'-hydroxyl protecting group (Figure 30).



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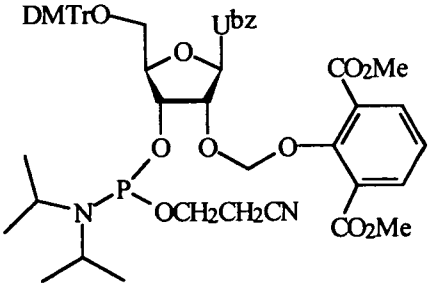

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Figure 30. 2'-O-(SEM) and 2'-O-(HIFA) ribonucleoside phosphoramidites

The SEM group is stable to base and all but the harshest acidic conditions. It can be readily introduced and the oxygen-carbon bond rather than the oxygen-silicon bond,

as in the TBDMS system, renders it unable to migrate. Finally, this group can be readily removed by treatment with $BF_3.OEt_2$ in acetonitrile for 15-30 minutes.

Rastogi and Usher[85] have proposed a new 2'-hydroxyl protecting group, the 2-hydroxyisophthalate formaldehyde (HIFA) (**90**). This group had the unique feature of requiring a two stage deprotection. As the bis-ester, it was relatively stable to acidic conditions and was found to be 112 times more stable than the Fpmp group at pH 1. During the basic conditions required for the removal of the base and phosphate protecting groups and cleavage from the solid support, the bis-ester was converted to the bis-carboxylate. It was reported that the conversion increases the rate of acid-catalysed hydrolysis of the acetal by 42-fold at pH 1, and possibly, by 1320-fold at pH 3. It was found to be within a factor of 2.35 of the rate of hydrolysis of Fpmp at pH 3. However, long coupling times of 5 minutes and lengthy deprotection conditions were required for the synthesis.

In a recent publication, Klosel *et al*[86] described the potential of the 1,1-dianisyl-2,2,2-trichloroethyl group (DATE) (**91**) for the protection of the 2'-hydroxyl function. Although DATE was found to be compatible with established protection techniques for oligoribonucleotide synthesis, its obvious bulk required longer coupling times (5 minutes) and produced poorer yields.

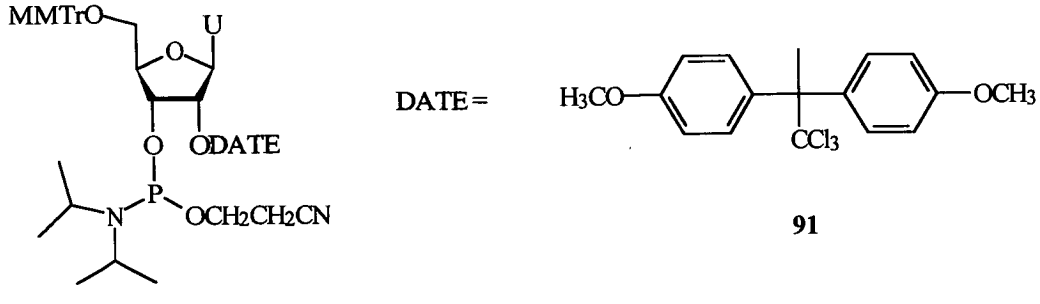


Figure 31. 1,1-Dianisyl-2,2,2-trichloroethyl group.

As an alternative to the phosphoramidite approach to oligoribonucleotide synthesis, interest in the *H*-phosphonate approach has increased (see Section 1.6.5.). The first report of oligoribonucleotide synthesis using *H*-phosphonates, used the TBDMS

group as protection for the 2'-hydroxyl function.[87] Later reports have included the use of the photolabile *o*-nitrobenzyl group[88] and the acid sensitive Thp[89] and Ctmp[90] groups. Evaluation of 2'-hydroxyl protection using the *H*-phosphonate approach was undertaken by Rozners *et al*[90], using a variety of 2'-hydroxyl protecting groups (TBDMS, o-chlorobenzoyl, Thp, Ctmp, Fpmp and the 1-(2-chloroethoxy)ethyl groups). Out of those compared, the most successful syntheses were clearly those where the TBDMS group was used for the protection of the 2'-hydroxyl function. They also concluded that the use of 2'-acetal protection in combination with H-phosphonates was of limited value in oligoribonucleotide synthesis.

1.9. Project Aims

Due to the progress made in the understanding of the diverse roles RNA molecules play in biological systems, an increased demand for synthetic RNA has evolved. However, methodologies for the chemical synthesis of RNA has not kept pace with advances in DNA synthesis. As previously discussed, the difficulty arises mainly from the presence of the additional 2'-hydroxyl function. Suitable selective protection and deprotection of the 2'-hydroxyl function is the key to successful RNA synthesis.

Thus, the main aim of this project was to develop a novel 2'-hydroxyl protecting group for the automated synthesis of oligoribonucleotides. An effective synthesis and introduction of the proposed group was to be obtained, and its potential examined. This will be discussed in the following chapters.

2.0. Results and Discussion

2.1. Proposed 2'-Hydroxyl Protecting Group for RNA Synthesis

The selection of a suitable protecting group for the 2'-hydroxyl function for RNA synthesis is a non-trivial matter and must satisfy several requirements:

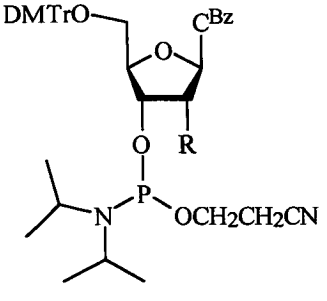
- It must be stable to the acidic conditions employed for the repeated removal of the dimethoxytrityl group (the most common 5'-hydroxyl protecting group used in DNA and RNA synthesis).
- It should be stable to the alkaline conditions that are used in the concerted removal of the heterocyclic base and phosphate protecting groups and cleavage of the chain from the solid support.
- It should be removable after completion of the synthesis, under sufficiently mild conditions such that there is no modification of the bases, or cleavage or migration of the internucleotide linkages.
- Steric bulk should be minimised so that extended coupling times are not required.
- The reagent employed in the removal of the 2'-hydroxyl protecting group should not make purification and isolation of the fully deprotected RNA too cumbersome or time consuming.

The removal of the 2'-hydroxyl protecting group should involve as little manipulation as possible, as RNA readily undergoes digestion in the presence of contaminating traces of endonucleases, such as ribonuclease. It would therefore be advantageous if the 2'-hydroxyl protecting group remained intact during purification. This would facilitate prolonged storage until the RNA was required, at which point the 2'-hydroxyl functions could be deprotected.

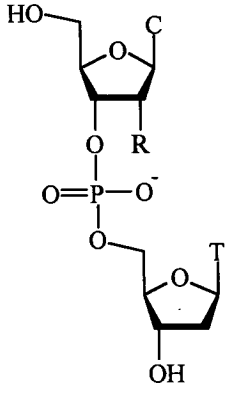
A variety of protecting group strategies have been studied. These were discussed in the previous chapter, along with the difficulties and problems which arose in their application. The complexity of the protecting group strategy can best be seen from the fact that only two types of ribonucleoside-3'-phosphoramidites, namely the 5'-O-(DMTr)-2'-O-(Fpmp) and the 5'-O-(DMTr)-2'-O-(TBDMS) combinations, are commercially available and have been applied with some success (Section 1.8.2.).

However, coupling times of ≥ 10 minutes are required for both the Fpmp and TBDMS group strategies, compared with 2 minute reactions commonly used for DNA synthesis. The relatively long coupling times are thought to be a consequence of steric interference by the 2'-hydroxyl protecting group. Such steric crowding in the vicinity of the phosphoramidite function could be relieved by incorporation of a flexible arm into the structure of the 2'-hydroxyl protecting group.

Kierzek *et al*[91] discussed the coupling efficiency of the condensation reactions between the ribonucleoside phosphoramidites **92 (a-d)** and deoxythymidine bound to a solid support. Analysis and quantitation of the dimers after treatment with ammonium hydroxide observed the coupling efficiencies of **93 (a-d)** decrease in order of **93a > 93b > 93c > 93d.**



where R = H **92 (a)**
R = OCH_3 **(b)**
R = OThp **(c)**
R = OTBDMS **(d)**



93 (a-d)

Figure 32. Steric hindrance of 2'-O-protecting group

Therefore, in the design of a least hindered system, it would be advantageous if it contained sterically undemanding groups, α and β to the 2'-O-position, thus reducing the steric interference around the activated phosphoramidite moiety. In order to satisfy these requirements, a functionalised propargyl system was proposed (Figure 33).

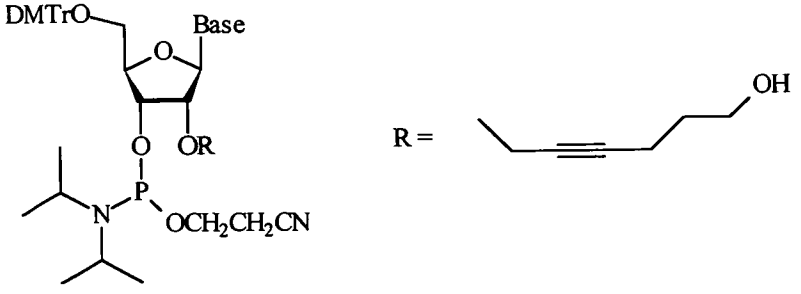
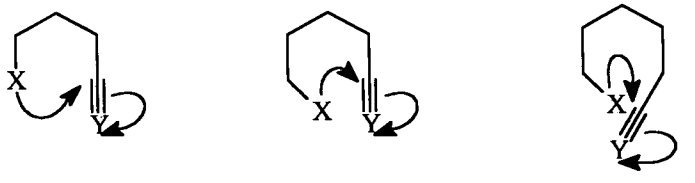


Figure 33. Functionalised propargyl based system

Baldwin[92] proposed a set of 'rules' for ring forming reactions which gave guidelines on the prediction of intramolecular cyclisation processes. He proposed 5 to 7-*Exo-Dig* favoured cyclisations (Figure 34).[93,94] According to the guidelines that Baldwin suggested, the proposed functionalised propargyl system, hex-4-yne-1-ol, can undergo a 5-*Exo-Dig* ring formation.



5-*Exo-Dig* 6-*Exo-Dig* 7-*Exo-Dig*

Figure 34. Baldwin's 'rules' for ring closure

The cyclisation of acetylenic amines and alcohols have been well documented. [95,96] It has been shown that a hydroxy group can undergo intramolecular addition to a triple bond in the transformation of internal enynols to furans.[97,98,99] Figure 35 shows the cycloisomerisation of (Z)-2-en-4-yn-1-ol to its corresponding furan.[100,101]

Similar intramolecular cyclisations of acetylenic carbonyl compounds, *via* their silyl enol ether derivatives, are precedent in the literature (Figure 36).[102,103,104]

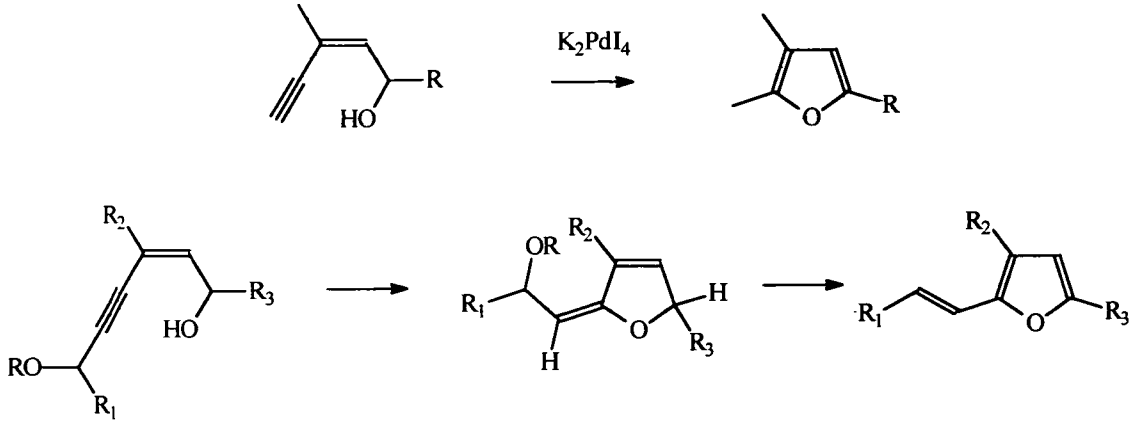


Figure 35. Synthesis of furans via enynols

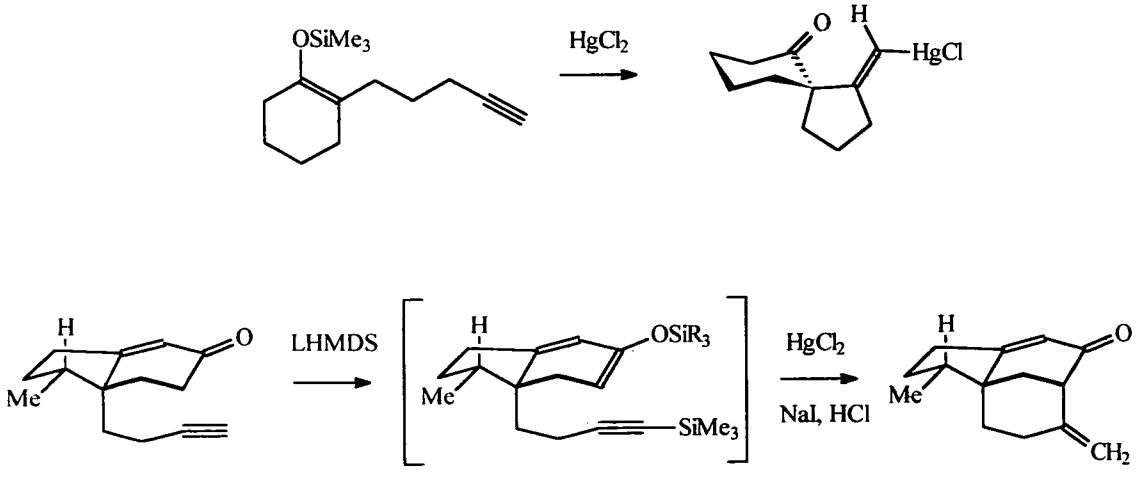


Figure 36. Intramolecular cyclisations via silyl enol ether derivatives

The proposed functionalised propargyl system is required to be stable during synthesis; therefore, the hydroxyl function of the ring system precursor is protected with a benzoyl group (Figure 37, **94**). The benzoyl group is commonly used in the protection of the heterocyclic bases, and can be removed under the alkaline conditions required for the removal of the phosphate protecting groups and cleavage of the completed chain from the solid support. This ensures complete stability during

synthesis and should generate the proposed 5-*Exo-Dig* ring system precursor during the cleavage from the support (Figure 37).

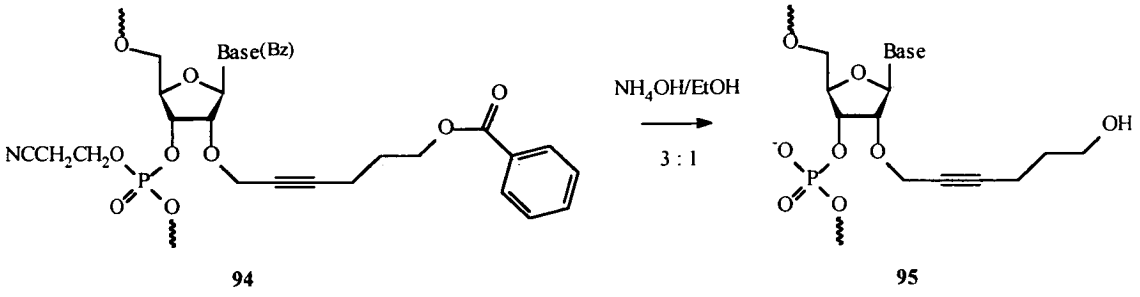


Figure 37. Alkaline deprotection conditions

The partially deprotected 2'-hydroxyl protecting group could then be cleaved under mild acidic conditions, without interference to the internucleotide linkages or modification to the bases, by the proposed mechanism (Figure 38). Similar mechanistic pathways have been proposed in the synthesis of furans using deuterium labelled species.[96]

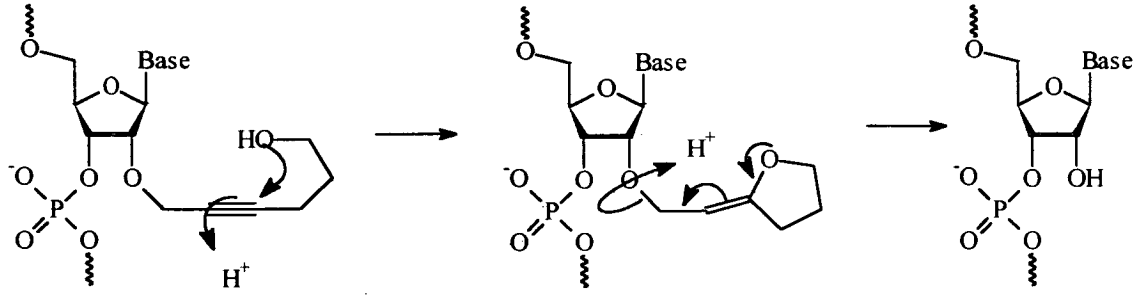


Figure 38. Mechanism of cleavage of 2'-hydroxyl protecting group

Standard protocols for the introduction of a 2'-hydroxyl protecting group involves the protection of the exocyclic amino functions and simultaneous protection of the 5'- and 3'-hydroxyls thus, leaving the 2'-hydroxyl function free for manipulation. Incorporation of an alkyl group onto the 2'-hydroxyl position is generally achieved using standard Williamson type procedures. This employs the use of sodium hydride[105] or silver(1) oxide[106,107,108] with an excess of alkyl halide to form the ether.

To allow incorporation of the protecting group onto the 2'-hydroxyl function, 6-bromo-hex-4-ynyl benzoate (**104**) was synthesised (Figure 39).

2.2. Synthesis of proposed 2'-hydroxyl protecting group

Figure 39 describes the initial synthetic route considered for the synthesis of 1-bromo-2-hexynbenzoate, commencing from the commercially available 1-pentyne-4-ol (**96**).

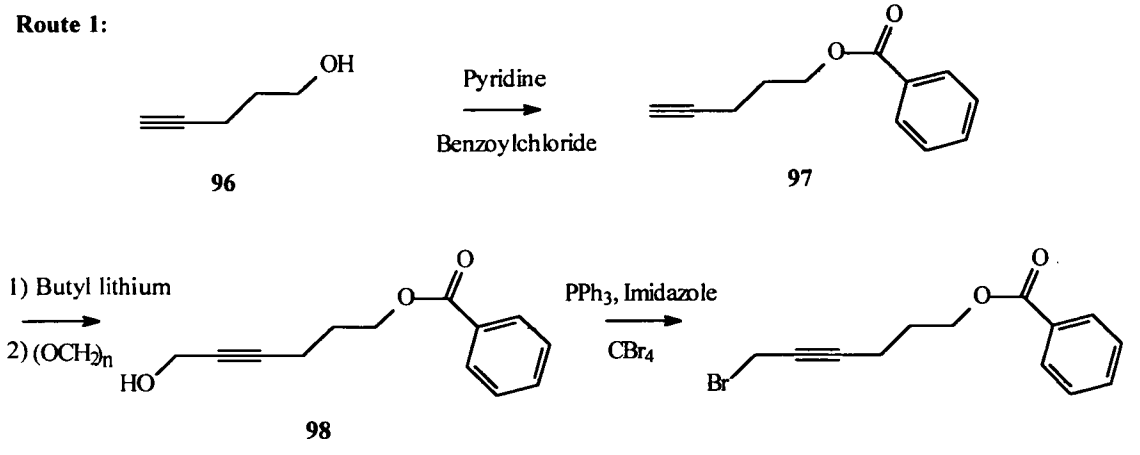


Figure 39. Route 1: Synthesis of 6-Bromo-hex-4-ynyl benzoate

Formation of pent-1-ynyl benzoate (**97**) was achieved in 87% yield by nucleophilic displacement of chlorine from benzoyl chloride in the presence of pyridine. This was then treated with one equivalent *n*-butyllithium in THF at -78°C followed by paraformaldehyde, and allowed to stir overnight at room temperature. After aqueous work up and purification by wet flash chromatography, 6-hydroxy-hex-4-ynyl benzoate was obtained in 10% yield. After several attempts, the yield could not be enhanced and, therefore, a second route was proposed (Figure 40).

The initial step in this second route involved alkylation of propargyl tetrahydropyranyl ether (**100**). This was achieved by treatment of the ether with one equivalent of *n*-butyllithium in THF at -78°C, followed by 3-bromo-1-chloropropane. After heating at reflux overnight, 6-(tetrahydropyranyloxy)-hex-4-ynyl chloride (**101**) was obtained in 84% yield after purification by distillation. The benzoate ester, 6-

(tetrahydopyranyloxy)-hex-4-ynyl benzoate (**102**), was obtained in 81% yield by heating **101** in DMF at reflux in the presence of sodium benzoate. The tetrahydropyranyl ether was simply cleaved by treatment with 10% aqueous oxalic acid in MeOH (48 hours, room temperature) to give 6-hydroxy-hex-4-ynyl benzoate (**103**) in 86% yield (48% overall).

Route 2:

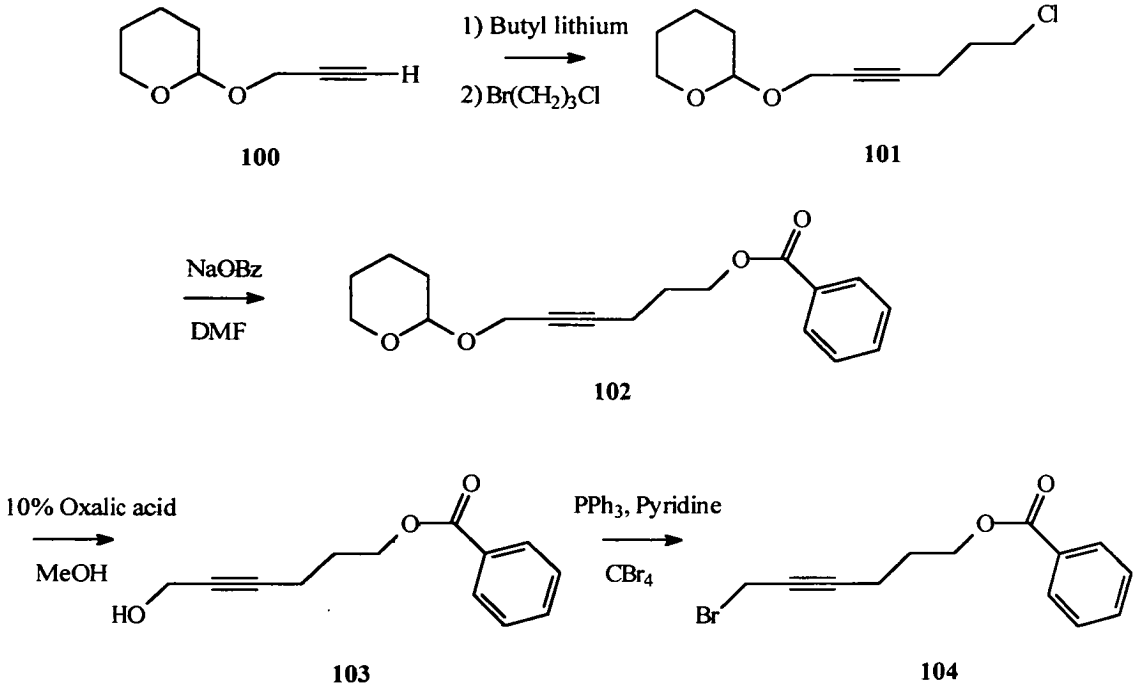


Figure 40. Route 2: Synthesis of 6-Bromo-hex-4-ynyl benzoate

Conversion of **103** to 6-Bromo-hex-4-ynyl benzoate (**104**) was achieved by treatment with triphenylphosphine, carbon tetrabromide and pyridine to give a yield of 82%.

2.3. Incorporation of the 2'-hydroxyl protecting group

As previously discussed, the alkylation of the 2'-hydroxyl function is generally achieved *via* simultaneous protection of the 5'- and 3'-hydroxyl functions, thus leaving the 2'-hydroxyl free for manipulation. Using the Markiewicz[70] reagent, 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane, with pyridine, the 5'- and 3'-hydroxyl functions of uridine (**105**) were simultaneously protected, giving 3',5'-O-

(tetraisopropyl-disiloxane-1,3-diyl)uridine (**106**) in a yield of 72% (Figure 41). Previously, Wagner *et al.*[105] discussed the necessity to protect the N^3 position of uridine to prevent alkylation. This was achieved through the use of three different groups. Introduction of the benzoyl group to the N^3-position of uridine was performed by the addition of benzoyl chloride to a mixture of **106**, Na_2CO_3 and 5% tetrabutylammonium bromide in DCM:H_2O (1:1), producing the N^3-benzoyl protected uridine (**107**) in 62% yield.[109]

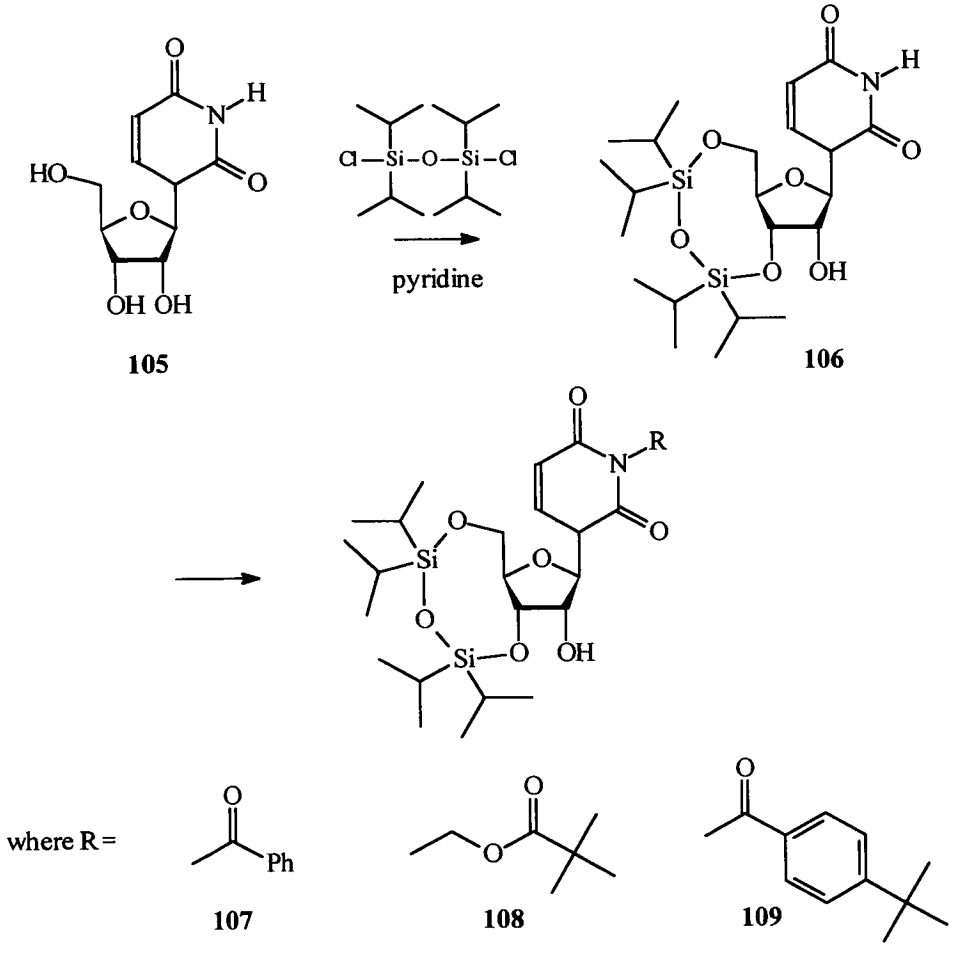


Figure 41. 3',5'-O-(Tetraisopropyldisiloxane-1,3-diyl)-N^3-(protected)uridine

The N^3-pivaloyl protected uridine (**108**) was obtained in 79% yield by treatment of **106** with chlormethyl pivalate in the presence of base.[110] The 2'-hydroxyl function of **106** was transiently protected by reaction with chlorotrimethylsilane before isobutyrylation at the N^3-position. After treatment with 4-toluenesulphonic acid to

remove the trimethylsilyl group, 3',5'-O-(tetraisopropyl-disiloxane-1,3-diyl)-N^3-(4-*tert*-butylbenzoyl) uridine (**109**) was obtained in 49% yield.[111]

Various forms of N^3 protection were required due to the different procedures required for the incorporation of alkyl groups to the 2'-hydroxyl function. Attempts to incorporate the proposed protecting group failed using excess 1-bromo-2-hexynbenzoate with sodium hydride or silver(1)oxide. The leaving group was then improved by replacement of bromide with iodine to give 6-iodo-hex-4-ynyl benzoate (**110**). Synthesis of **110** was achieved similarly to the bromide, by the addition of iodine in one portion to a solution of **103**, triphenylphosphine and imidazole to give **110** in a yield of 98%.[112] However, reactions under Williamson-type conditions were still unsuccessful.

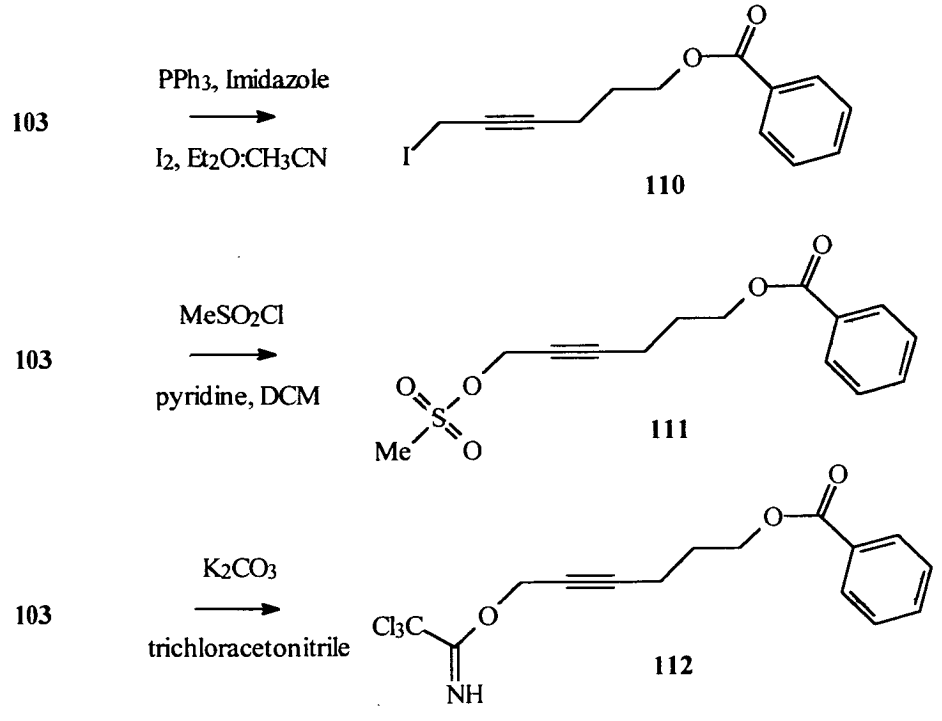


Figure 42. Synthesis of functionalised systems

In order to further improve the leaving group for ether formation, 6-mesyl (**111**) and 6-trichloroacetimidate-hex-4-ynyl benzoate (**112**) were synthesised (Figure 42).[113] Although ether formation had been achieved on a variety of other systems,[113,114]

application of these leaving groups to the proposed system with fully protected uridine did not yield any of the desired product.

Sproat *et al*[115] used the strong and sterically hindered base, 2-*tert*.-butylimino-2-diethyl-amino-1,3-dimethylperhydro-1,3,2-diazaphosphorin (BDDDP), in the synthesis of 2'-O-methyl and 2'-O-allyl analogues.[116,117,111] However, use of this procedure, substituting methyl iodide with 6-iodo-hex-4-ynyl benzoate, failed to incorporate the proposed 2'-hydroxyl protecting group. Difficulty in the formation of 2'-O-(hex-4-ynyl benzoate) was thought to be due to steric effects created by the bulk of the uridine protecting group strategies.

The activation of hydroxyl groups using organotin derivatives in the field of carbohydrates, nucleosides, polyols and selected natural products has been reviewed.[118,119] In 1974, Moffatt *et al*[120] described the synthesis and isolation of some organotin derivatives of nucleosides. The preparation of 2',3'-O-(dibutylstannylene) nucleosides (**113**) was achieved by heating a methanolic suspension of the nucleoside with dibutyltin oxide (Figure 43). Upon treatment of 2',3'-O-(dibutylstannylene) nucleosides, Moffatt was able to acylate the 2'- and 3'-hydroxyls using various acylating agents, producing mixtures of 2'-O- and 3'-O-acyl nucleosides (**114a** and **114b** respectively) in approximately 1:3 ratios.

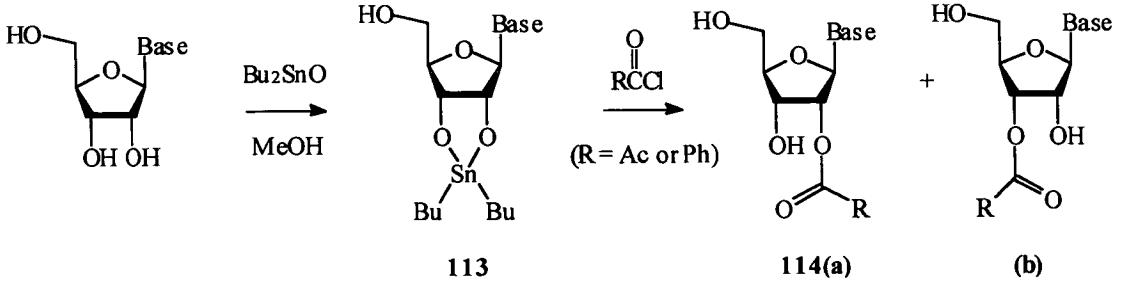


Figure 43. 2',3'-O-(dibutylstannylene) nucleosides

Schwartz *et al*[82] incorporated the *o*-nitrobenzyloxyethyl group onto the 2'- and 3'-hydroxyls of uridine using 2',3'-O-(dibutylstannylene) uridine and tetra-*n*-butylammonium bromide in DMF. A slight modification of this procedure was

employed for the incorporation of the proposed protecting group, 2'-O-(hex-4-ynyl benzoate). 6-Iodo-hex-4-ynyl benzoate was added to a solution of uridine, dibutyltin oxide and tetra-*n*-butylammonium bromide in DMF. After heating for 24 hours at 60°C, a mixture of the 2'- and 3'-O-(hex-4-ynyl benzoate) uridine isomers (**115a** and **115b** respectively) were obtained in an approximate ratio of 3:1 (Figure 44).

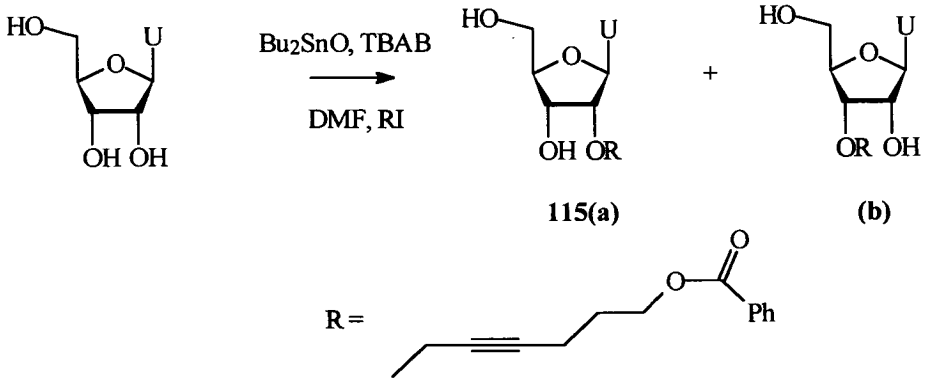


Figure 44. Synthesis of 2'- and 3'-O-(hex-4-ynyl benzoate) uridine

Chromatographic separation of the two isomers at this stage was not possible; however, two separation procedures were developed.

In the first, treatment with Markiewicz reagent, followed by deprotection using potassium fluoride dihydrate, produced solely the 2'-O-(hex-4-ynyl benzoate) uridine isomer in an overall yield of 31%.

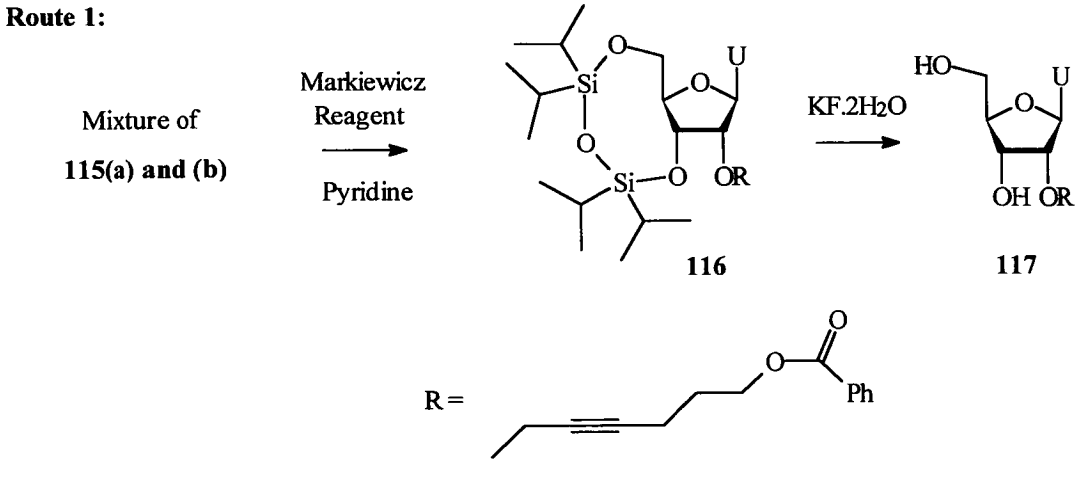


Figure 45. Route 1: Isolation of 2'-O-(hex-4-ynyl benzoate) uridine

The second, and preferred method, was achieved by dimethoxytritylation of the mixture (**115**), which enabled the chromatographic separation of the two isomers (Figure 46). The mixture of isomers were dried by coevaporation with pyridine, then treated with dimethoxytrityl chloride, producing the 2'-O-(hex-4-ynyl benzoate) uridine (**119**) and 3'-O-(hex-4-ynyl benzoate) uridine (**118**) in yields of 53% and 18%, respectively.

Route 2:

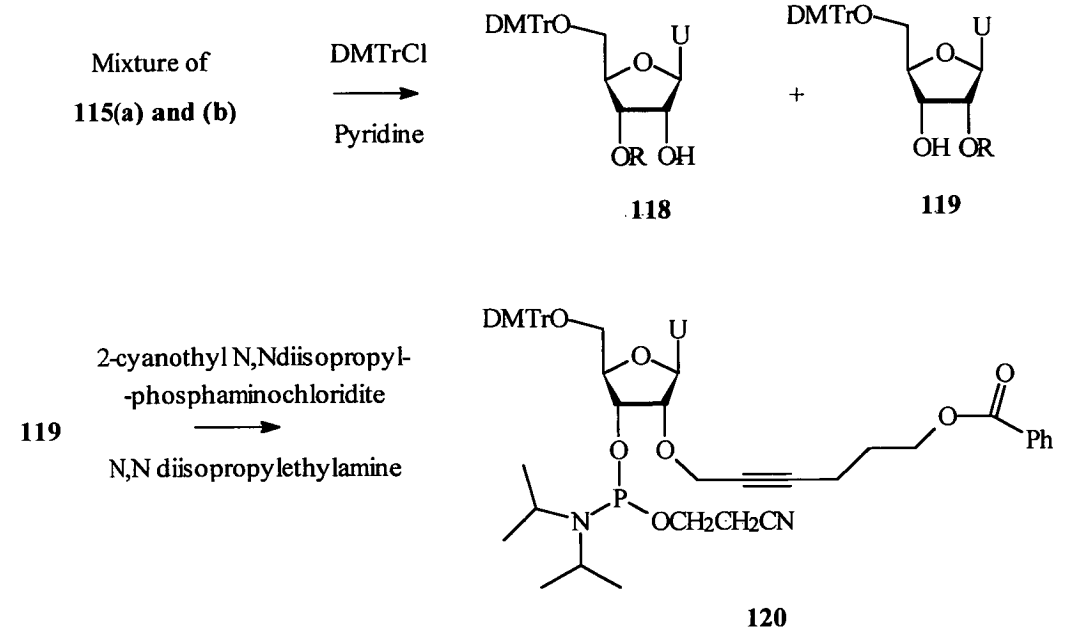
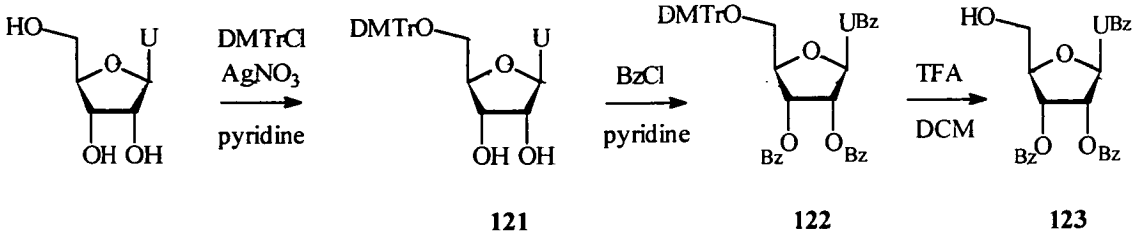


Figure 46. Synthesis of 5'-O-(dimethoxytrityl)-2'-O-(hex-4-ynyl benzoate)-uridine-3'-O-(2-cyanoethyl-N,N-diisopropylphosphoramidite)

The 2'-O-(hex-4-ynyl benzoate) isomer was then phosphitylated using standard phosphoramidite synthesis procedures.[121] Purification by both chromatography and precipitation were required to give the desired phosphoramidite (**120**) in a yield of 72%. Before evaluating the coupling efficiency of the phosporamidite derivative (**120**) in solid phase synthesis, a solution synthesis of a dinucleoside was performed.

Preparation of the 3'-terminal uridine unit was carried out by a simple three step synthesis (Figure 47). Dimethoxytritylation of the 5'-hydroxyl of uridine (**121**),

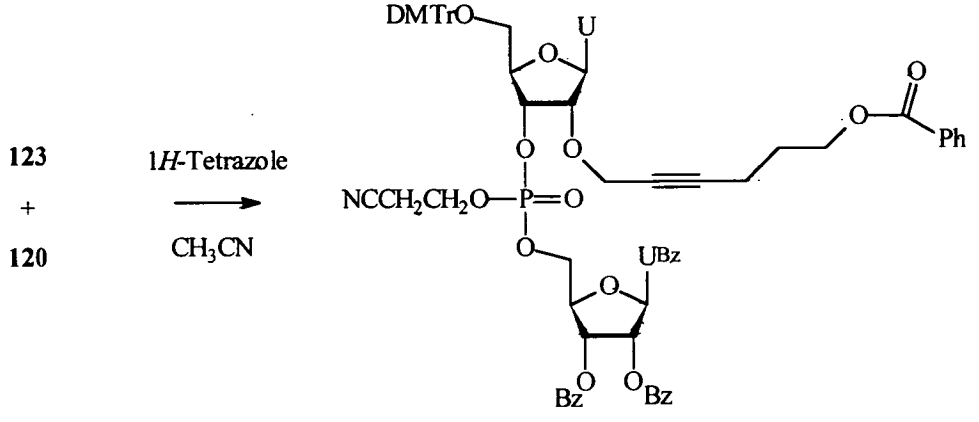
followed by benzoylation of the N^3 position and 2'- and 3'-hydroxyls gave the fully protected uridine (**122**). Removal of the dimethoxytrityl group by treatment with trifluoroacetic acid in DCM gave the desired 3',2',N^3-tri(benzoyl)-uridine (**123**).



121 **122** **123**

Figure 47. Synthesis of 3',2',N^3-tri(benzoyl)-uridine

Condensation of 5'-O-(dimethoxytrityl)-2'-O-(hex-4-ynyl benzoate)-uridine-3'-O-(2-cyanoethyl-N,N-diisopropylphosphoramidite) (**120**) with the 3'-terminal uridine unit was carried out in acetonitrile in the presence of 1*H*-tetrazole, to give the fully protected dimer in a 66% yield.[105]



124

Figure 48. Solution synthesis of diribonucleotide derivative

2.4. Solid Phase Synthesis of PolyUridine

The internucleotide linkages of oligouridylic acids [such as $(U'p)_9U$] have been shown to be considerably more susceptible to acid-promoted hydrolysis, and presumably also to migration, than other oligoribonucleotides of similar or greater length containing

different base sequences. Capaldi *et al*[71] has proposed that the increased acid lability of r[(U'p)$_9$U] compared with that of uridylyl-(3'→5')-uridine is due to distant nucleotide, or even uracil residues in r[(U'p)$_9$U], promoting the interaction between specific 2'-hydroxyl functions and internucleotide linkages.

It was, therefore, decided to synthesise oligouridylic acids using the 2'-O-(2-hexynbenzoate) group. If the protecting group strategy for the 2'-hydroxyl function proved successful for the syntheses of oligouridylic acids, it should then be possible to transfer the protocols to the syntheses of other sequences.

Solid phase synthesis using the phosphoramidite derivative (**120**) was carried out. First it was necessary to attach the terminally protected ribonucleoside to the commercially available long-chain alkylamine silica support. This was achieved by conversion of 3'-O-(hex-4-ynyl benzoate) uridine to the corresponding 2'-O-succinate derivative which was subsequently reacted with the amino group of the support. The 3'-O-protected nucleoside was used, since it is produced during the protection of the 2'-O-hydroxyl and is not required for the synthesis of RNA containing normal 3'-5' phosphodiester linkages. The support was dried overnight and the loading of the bound ribonucleoside was determined by detritylation of 2 mg portions of the support with perchloric acid and measurement of the absorbance at 498nm (16 μmol g^{-1}).[122]

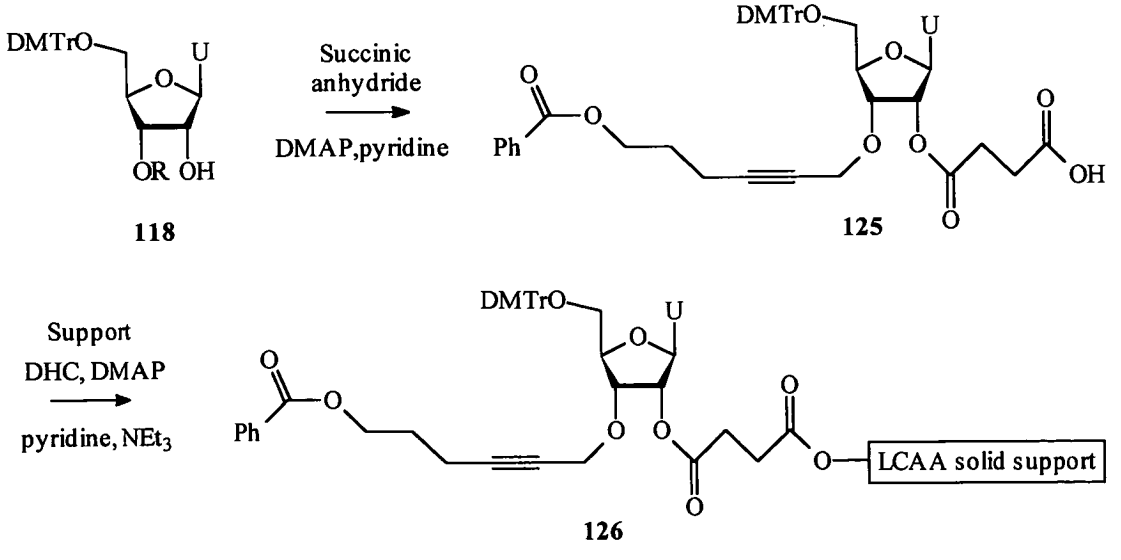


Figure 49. Derivatization of LCAA solid support

The derivatized silica support was then used for the synthesis of polyuridine, 10 nucleotides in length [DMTr-(U'p)$_9$U]. The phosphoramidite derivative (**120**) was dissolved in acetonitrile to a concentration of 0.1M, and was used in the synthesis of oligouridylic acid [DMTr-(U'p)$_9$U] using three different conditions. Coupling times of 2, 5 and 10 minutes were employed for the condensation reactions, hence enabling the evaluation of the coupling efficiency and, if any, the steric interference of the phosporamidite derivative (**120**). No other variables were altered during the syntheses, as shown in the synthetic cycle protocols below (Figure 50).

Synthetic Cycle Steps	**Reagent and/or Solvent**	**Duration (t/sec) Synthesis A**	**Duration (t/sec) Synthesis B**	**Duration (t/sec) Synthesis C**
Coupling reaction	0.5M Tetrazole in CH_3CN	120	300	600
Capping	Ac_2O, THF, Lutidine	60	60	60
Oxidation	0.1M I_2 in THF, Pyridine, Water	30	30	30
Detritylation	3% TCA in DCM	30	30	30
Overall Coupling Efficiency		**81%**	**83%**	**84%**

Figure 50. Synthetic cycle protocols for DMTr-(U'p)$_9$U

The coupling efficiencies obtained from the three syntheses showed little variation from the 2 minute (81%) to 10 minute (84%) coupling times, thus supporting the idea that the 2'-O-(hex-4-ynyl benzoate) group did not introduce steric interference in the vicinity of the phosphoramidite moiety.

2.5. Deprotection and Stability Studies

Before subjecting the synthesised oligonucleotide to deprotection conditions, the 2'-O-(hex-4ynyl benzoate) deprotection was first tested at the nucleoside level (Figure 51). The 2'-protected nucleoside (**119**) was detritylated, as before, to produce 2'-O-(hex-4-ynyl benzoate)uridine (**127**) in a yield of 98%. The 2'-O-(hex-4-ynyl benzoate) group was found to be stable to the acidic conditions employed in the removal of the 5'-DMTr group. To further test its stability to acidic conditions, (**127**) in 0.1 mol dm^{-3} hydrochloric acid (pH 1.1) at 25°C was monitored using reverse-phase HPLC over 7 days. At the end of this period, it was found to be completely intact: therefore, due to its stability to acidic conditions it would remain intact during the repeated detritylation steps employed in solid phase synthesis.

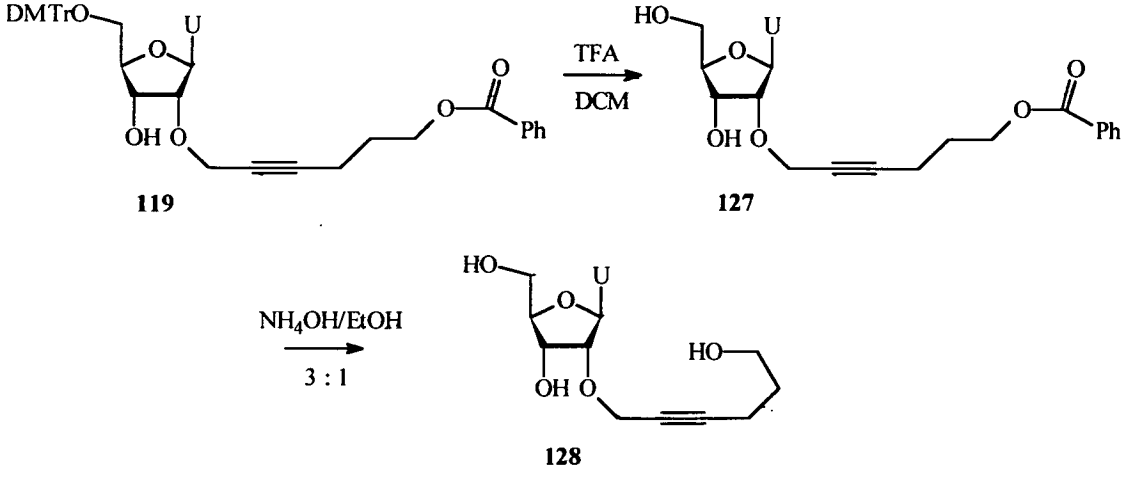


Figure 51. Synthesis of 2'-O-(hex-4-yn-1-ol) uridine

The conditions required for the removal of the phosphate protecting groups, and cleavage of the completed chain from the solid support, were used in the removal of the benzoyl group. Complete removal of the group required treatment for 4 days with 33% aqueous ammonia solution and ethanol (3 : 1) at room temperature, yielding 2'-O-(hex-4-yn-1-ol) uridine (**128**) quantitatively. Analysis of the product by reverse-phase HPLC firmly established that complete deprotection of the 2'-hydroxyl funtion, to give uridine, did not occur. This indicates that the 2'-O-(hex-4-yn-1-ol) group is completely stable to the alkaline deprotection conditions, thus preventing the

formation of a free 2'-hydroxyl group, which would of led to either hydrolytic cleavage or migration of the internucleotide linkages of RNA. It would also allow the synthetic oligoriboncleotides to be purified, without any risk of degradation, before the 2'-O-protecting groups are required to be removed.

Capaldi and Reese[71] studied the stability of internucleotide linkages of oligoribonucleotides in the aqueous acid conditions required during cleavage of Ctmp and Fpmp acetal protecting groups. From their studies, it was concluded that the ideal final deblocking process of the 2'-hydroxyl function should be carried out above pH 3 within 24 hours, if hydrolytic cleavage and migration were to be avoided.

Deprotection of the 2'-O-(hex-4-yn-1-ol) group was studied over the pH range 1 to 3 using 0.1 dm^{-1} (pH 1.1), 0.01 dm^{-1} (pH 2.0) hydrochloric acid, and a buffer solution of 50mM ammonium formate (pH 3.1). The 2'-O-(hex-4-yn-1-ol) uridine monomer (1 mg) was dissolved in appropriate aqueous acid or buffer solution, and monitored by reverse-phase HPLC.

Deprotection Conditions (room temp.)	**2'-O-(hex-4-yn-1-ol) uridine → Uridine (hours)**
pH 1.1	**9.5 h**
pH 2.0	**24.3h**
pH 3.1	**96.5h**

Figure 52. Deprotection Studies of 2'-O-(hex-4-yn-1-ol) uridine

The tabulated data of the time taken for the deprotection of 2'-O-(hex-4-yn-1-ol) to the fully deprotected uridine is shown in Figure 52. It can be seen from the results that complete deprotection was obtained, however, the conditions required for the cleavage were harsh and would therefore cause cleavage and migration of the

internucleotide linkages of RNA. Addition of an equimolar quantity of $HgCl_2$ to the deprotection conditions was found to increase the rate of removal; however, reproducibility of results proved extremely difficult.

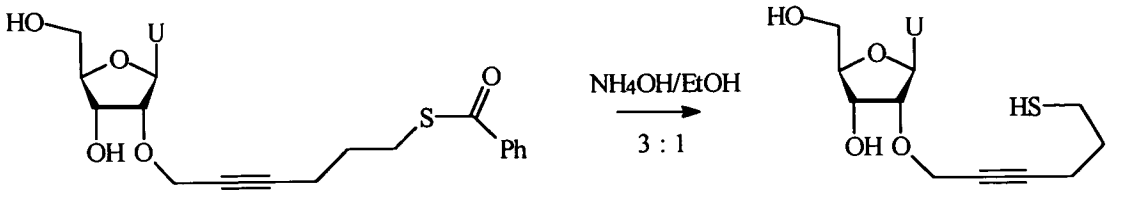
In conclusion, it was found that the two cleavage steps, in the deprotection of 2'-O-(hex-4-ynyl benzoate) uridine to uridine, required extended reaction times and harsher conditions than those which are suitable for the handling of RNA. Therefore, modification of the original 2'-O-(hex-4-ynyl benzoate) system was required to improve the efficiency of both cleavage steps.

2.6. Modification of 2'-Hydroxyl Protecting Group

The conditions required for the clevage of the 2'-hydroxyl protecting group from the completed RNA molecule, is by far the most important factor to be now considered in the design a protecting group. Increase in the rate of base hydrolysis of the benzoyl group can be achieved by the incorporation of a *para* or *ortho* electron withdrawing group, such as a nitro substituent, onto the phenyl ring. However, on base hydrolysis, the alcohol 2'-O-(hex-4-yn-1-ol) is still formed, yielding the acid cleavage system that has already been studied. Therefore, by replacement of the O-benzoate by S-benzoate would create 2'-O-(hex-4-yn-1-thiol) system (**138**) for the final cleavage step (Figure 52). The interaction of the thiol with the acetylene may be favoured due to the 'softness' of the nucleophile.

2.6.1. Synthesis of 2'-O-(Hex-4-ynyl thiobenzoate) uridine

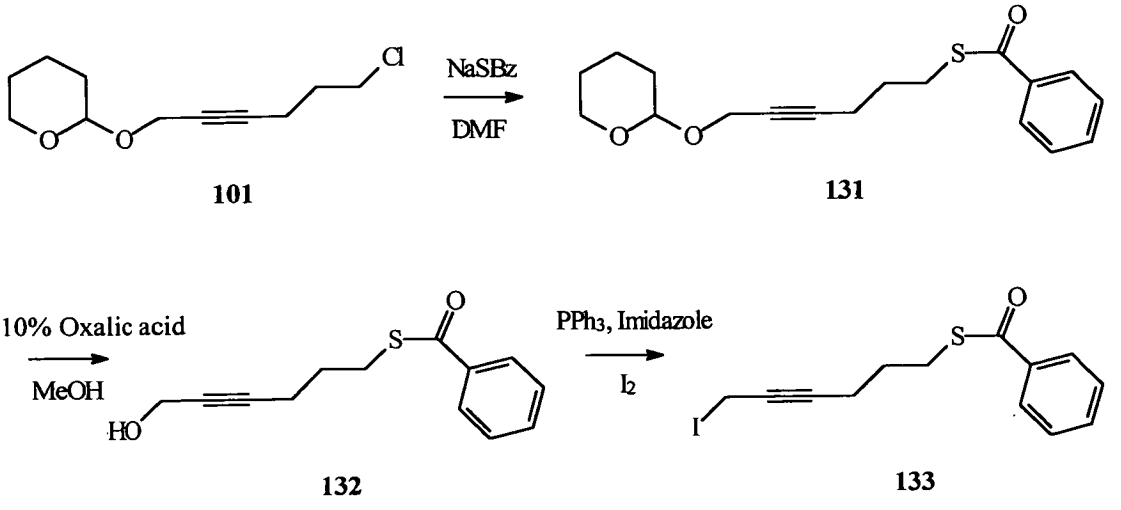
To study the feasibility of a thiol-based system for the final deprotection step, it was necessary to synthesise 2'-O-(hex-4-ynyl thiobenzoate) uridine (**137**). (Figure 52).



137 138

Figure 52. 2'-O-(hex-4-ynyl thiobenzoate) uridine

The synthetic procedures used in the synthesis and incorporation of the 2'-O-(hex-4-ynyl benzoate) group were applied to the synthesis of **137**. The ester, 6-(tetrahydopyranyloxy)-hex-4-ynyl thiobenzoate (**131**), was obtained in 71% yield by heating **101** in DMF at reflux, in the presence of sodium thiobenzoate. The tetrahydropyranyl ether was cleaved by treatment with 10% oxalic acid to give 6-hydroxy-hex-4-ynyl thiobenzoate (**132**) in 50% yield. Formation of 6-Iodo-hex-4-ynyl thiobenzoate by the addition of iodine in one portion to a solution of **132**, triphenylphosphine and imidazole gave **133** in a yield of 76%.[112]



101 131

132 133

Figure 53. Synthesis of 6-Iodo-hex-4-ynyl thiobenzoate

Incorporation of the hex-4-ynyl thiobenzoate group to the 2'-hydroxyl function of uridine was achieved using identical protocols to those used in the synthesis of 2'-O-(hex-4-ynyl benzoate) uridine (Figure 51). **133** was added to a solution of uridine, dibutyltin oxide and tetra-*n*-butylammonium bromide in DMF. After heating for 24 hours at 60°C, a mixture of the 2'- and 3'-O-(hex-4-ynyl thiobenzoate) uridine

isomers (**134a** and **134b** respectively) were obtained in an approximate ratio of 3:2 (Figure 54). Chromatographic separation of the two isomers was achieved by dimethoxytritylation of the mixture (**134**), producing 2'-O-(hex-4-ynyl thiobenzoate) uridine (**136**) and 3'-O-(hex-4-ynyl thiobenzoate) uridine (**135**) in yields of 30% and 20%, respectively. Dimethoxytritylation of 2'-O-(hex-4-ynyl thiobenzoate)-5'-O-(dimethoxtrityl) uridine in DCM with TFA gave **137** in a yield of 76%.

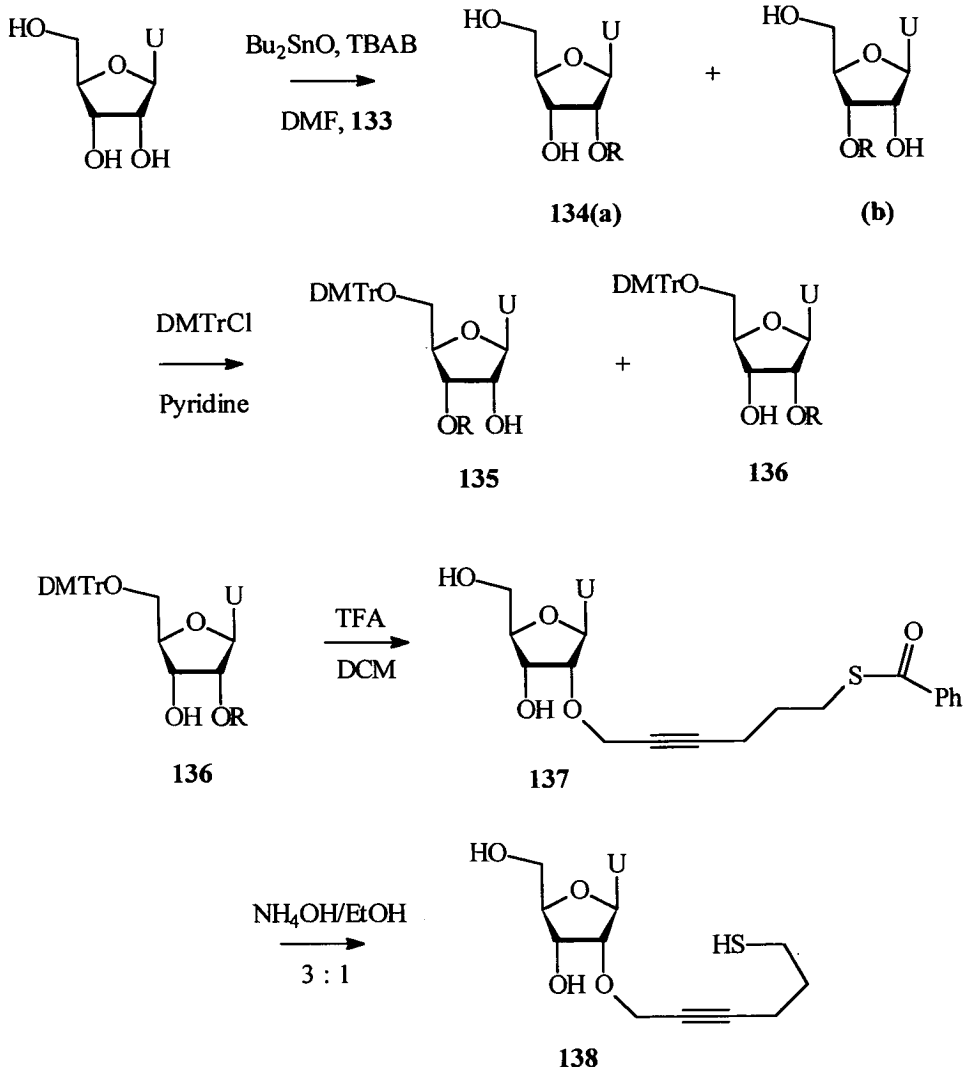


Figure 54. Synthesis of 2'-O-(hex-4-yn-1-thiol) uridine

2.6.2. Deprotection and Stability Studies of 2'-O-(Hex-4-ynyl thiobenzoate) uridine

The 2'-O-(hex-4-ynyl thiobenzoate) group showed similar characteristics to 2'-O-(hex-4-ynyl benzoate) uridine. Using reverse-phase HPLC, 2'-O-(hex-4-ynyl thiobenzoate) uridine (**137**) was monitored over a 7 day period under acidic conditions (pH 1.1). It was found to be still intact at the end of this period, thereby indicating its potential stability during repeated acid cleavage of the trityl group in the synthesis cycle.

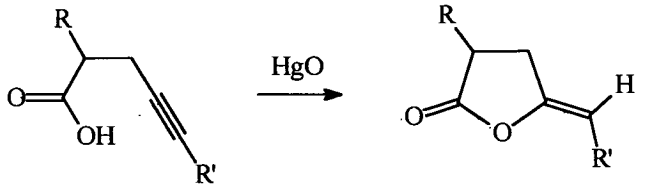
Cleavage of the benzoyl group (**137→138**) was achieved using the standard conditions required for the removal of the phosphate protecting groups, along with cleavage of the completed chain from the solid support. Complete removal of the group required treatment for 72 hours with 33% aqueous ammonia solution and ethanol (3: 1) at room temperature. Analysis of the product by HPLC established that the complete deprotection of the 2'-hydroxyl function to give uridine did not occur. As with the 2'-O-(hex-4-yn-1-ol) group, 2'-O-(hex-4-yn-1-thiol) uridine was found to be stable to alkaline conditions, thus preventing formation of the free 2'-hydroxyl group which would have led to either hydrolytic cleavage and/or migration of the internucleotide linkages of RNA. It would also allow the synthetic oligoribonucleotides to be purified, without any risk of degradation, before the 2'-O-protecting groups are required to be removed.

The conditions used in the study of the deprotection of 2'-O-(hex-4-yn-1-ol) uridine were employed in the study of 2'-O-(hex-4-yn-1-thiol) uridine (**138**). Treatment of **138** with acidic solutions in the range pH 1 to pH 3 were monitored using HPLC. However, formation of the unprotected uridine monomer was not observed under any of the conditions employed. It was found that it was necessary for the thiol based system to be stored and studied under argon, due to susceptibility of the system to oxidation.

It was, therefore, necessary to further investigate alternative systems based on the proposed propargyl system.

2.7. Acetylenecarboxylic acids as potential 2'-Hydroxyl Protecting Groups

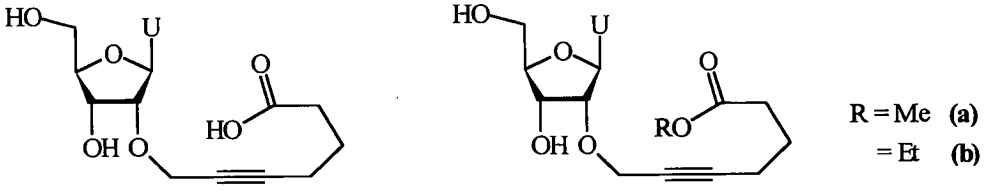
As well as intramolecular additions of alcohols to acetylenes, cyclisation of acetylenecarboxylic acids (**139**) to lactones (**140**) has also been observed.[123,124,125] Yamamoto reported the synthesis of γ-methylenebuyrolactones using this procedure (Figure 55).[126]



139 **140**

Figure 55. Cyclisation of Acetylenecarboxylic acids

Therefore, the study of a propargyl based system incorporating a carboxylic acid based system was proposed (Figure 56).



141 **142**

Figure 56. Carboxylic acid based system

Protection of the carboxylic acid functionality would be required during RNA synthesis, hence protection as the methyl or ethyl esters were suggested (**142**). On treatment with the standard alkaline cleavage conditions formation of the carboxylic

acid based system would be achieved. To offer an indication of the products formed during base hydrolysis of the methyl or ethyl ester, the methyl and ethyl esters of phenyl acetic acid (**143** and **145**) were treated with 33% ammonia solution and ethanol (3 : 1). Formation of the phenyl acetamide (**144**) and phenyl acetic acid (**146**) were observed, respectively (Figure 57).

In order to determine the feasibility of both groups in the final deprotection step of the 2'-hydroxyl function, the methyl and ethyl esters of 2'-O-(hept-5-ynoic acid) uridine were synthesised.

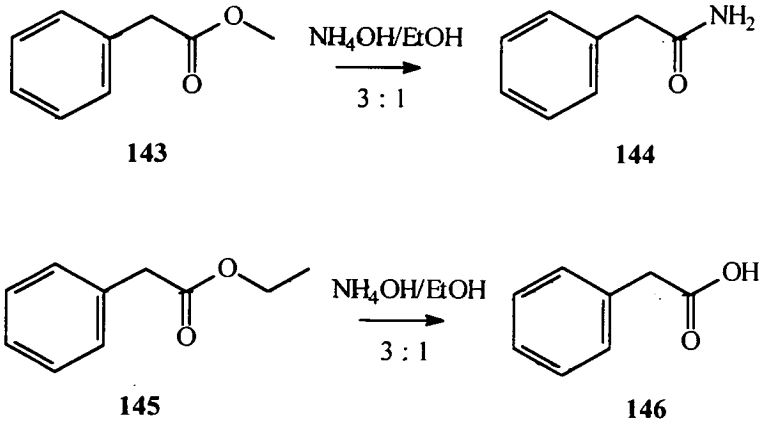


Figure 57. Base hydrolysis of methyl and ethyl esters

2.7.1. Synthesis of Methyl and Ethyl-7-iodo-hept-5-ynoate

The synthetic procedures used in the incorporation of the two previous systems were applied to the synthesis of 2'-O-(methyl-hept-5-ynoate) uridine and 2'-O-(ethyl-hept-5-ynoate) uridine.

Synthesis of methyl and ethyl-7-iodo-hept-5-ynoate were achieved, each in a 5 step synthesis from 6-(tetrahydropyranyloxy)-hex-4-yne chloride (**101**) in overall yields of 27% and 41%, respectively (Figure 58). **101** in the presence of sodium cyanide gave the formation of 6-(tetrahydropyranyloxy)-hex-4-yne cyanide (**147**), in a yield of

96%.[127] Heating **147** to reflux in 25% NaOH solution and methanol gave 7-(tetrahydropyranyloxy)-hept-5-ynoic acid (**148**) in a 69% yield.[128]

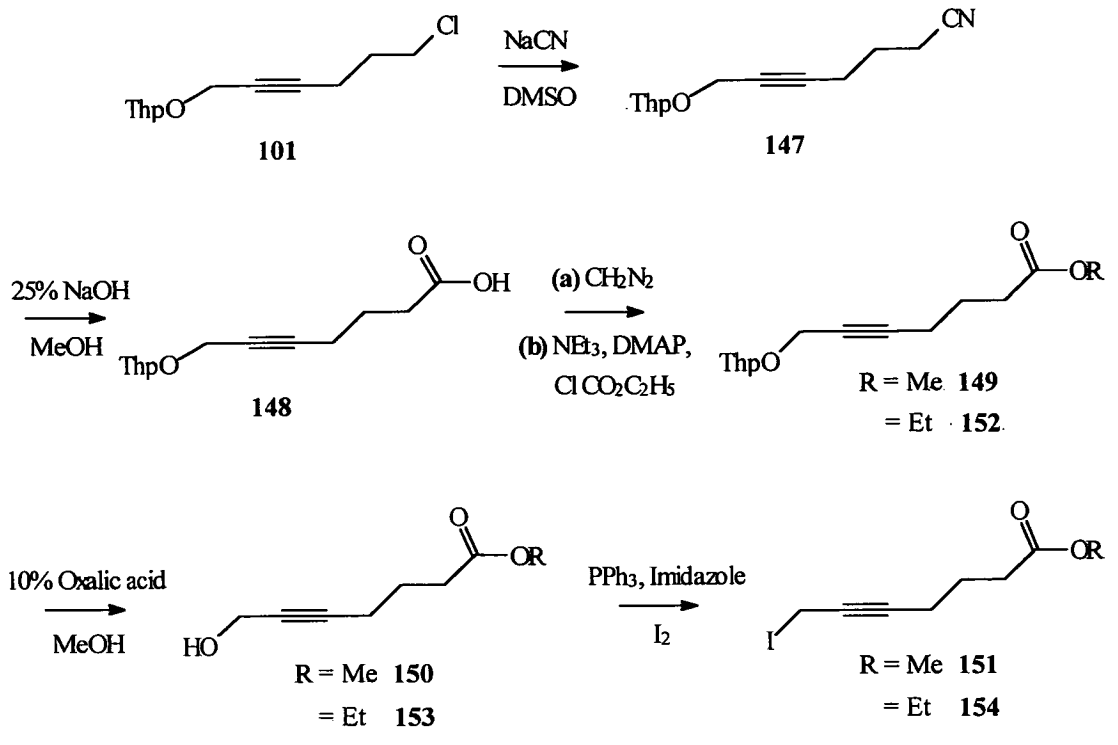


Figure 58. Synthesis of Methyl and Ethyl-7-iodo-hept-5-ynoate

The formation of the methyl ester (**149**) was achieved in a quantitative yield by treatment of **148** with diazomethane. **148** in the presence of DMAP, NEt_3 and ethylchloroformate gave the ethyl ester (**152**) in a yield of 85%.[129] This was followed by the removal of the tetrahydropyranyl ether (**149**→**150** and **152**→**153**), and the formation of the iodide (**150**→**151** and **153**→**154**).

2.7.2. Synthesis of 2'-O-(Methyl- and 2'-O-(Ethyl-hept-5-ynoate) uridine

Incorporation of both groups was accomplished by the treatment of uridine, in the presence of DBTO, TBAB in DMF, with the previously prepared methyl and ethyl-7-iodo-hept-5-ynoate (**151** and **154**), in yields of 81% and 80% respectively (Figure 59). Dimethoxytritylation enabled the chromatographic separation of both sets of isomers,

of which the 2'-O-protected isomers were detritylated to give 2'-O-(methyl-hept-5-ynoate) uridine (**158**) and 2'-O-(ethyl-hept-5-ynoate) uridine (**162**) both in overall yields of 17%.

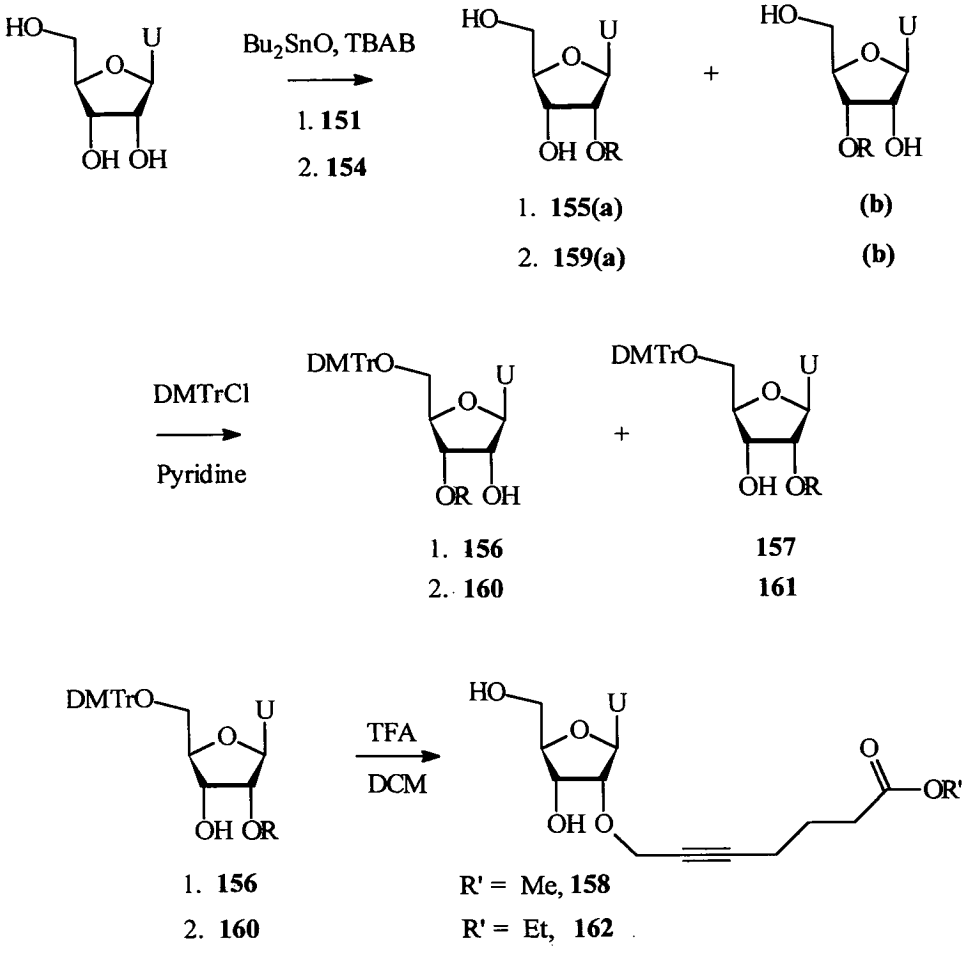
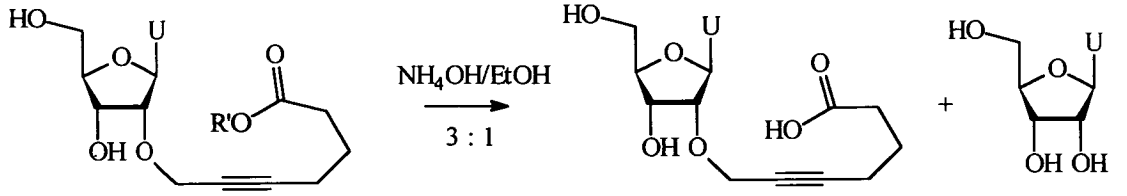


Figure 59. Synthesis of 2'-O-(Methyl- and 2'-O-(Ethyl-hept-5-ynoate) uridine

The alkaline cleavage conditions required for the removal of the phosphate protecting groups, and cleavage of the completed chain from the solid support, were employed in the base hydrolysis of the methyl and ethyl esters. Treatment with 33% aqueous ammonia solution and ethanol (3 : 1) at room temperature over 3 days gave complete hydrolysis of the methyl and ethyl esters. However, on analysis of the products by reverse-phase HPLC, it was established that deprotection of the 2'-hydroxyl function, to give uridine had also occurred. Therefore, the instability of the proposed propargyl

systems to the required ammonia deprotection conditions would lead to cleavage and migration of the internucleotide linkages of a completed RNA chain. Thus, the methyl and ethyl-hept-5-ynoate systems are not suitable for the protection of the 2'-hydroxyl function for the synthesis of RNA.



R' = Me, **158**

R' = Et, **162**

163

Figure 60. Base hydrolysis

2.8. Conclusions

An original series of 2'-hydroxyl protecting groups for the synthesis of oligoribonucleotides have been designed and synthesised. The design of these groups was based on a functionalised propargyl system, that involved cleavage *via* a ring system precursor. The precursor was protected during synthesis, using compatible protecting group strategies, and was activated during the conditions required for removal of the phosphate protecting groups and cleavage from the solid support.

Three functionalised propargyl based systems were proposed; 2'-O-(Hex-4-yn-1-ol) (**128**), 2'-O-(hex-4yn-1-thiol) (**137**) and 2'-O-(hept-5-ynoic acid) (**142**) groups were studied for their cleavage conditions and their stability to the parameters required during the synthesis of RNA. Efficient syntheses and incorporation procedures of the proposed protecting groups were found.

The 2'-O-(hex-4-ynyl benzoate) group (**127**) satisfied most characteristics of an ideal 2'-hydroxyl protecting group. Synthesis of three 10mers of polyuridine were achieved using the phosphoramidite approach to solid-phase synthesis. The coupling efficiencies from the three syntheses showed little variation for the 2, 5 and 10 minute

coupling times employed for the condensation reactions of the phosphoramidite monomers (**120**) (81%, 83% and 84%, respectively). This indicated minimal steric interference in the vicinity of the phosphoramidite moiety, which is a major drawback in the commercial systems presently available.

The 2'-O-(hex-4-ynylbenzoate) group was found to be completely stable to the parameters required during synthesis, and was hydrolysed to the hydroxyl bearing ring system precursor (**128**) under basic ammonia conditions (Figure 51). Cleavage of the group was achieved at pH 3.1 in 96.5 hours (Figure 52).

In an attempt to improve the rate of cleavage, thiol (**137**) and carboxylic acid (**142**) based systems were studied. Once hydrolysed to the thiol based system (**138**), cleavage of the group from the 2'-hydroxyl function was not obtained. This may be ascribed to the thiols susceptibility to oxidation. The carboxylic acid based system was found to be unstable to the aqueous ammonia conditions. This caused partial full deprotection of the 2'-hydroxyl function to give uridine, which would have led to the cleavage and migration of the internucletide linkages. Thus, the thiol and carboxylic acid systems were observed to be unsuitable for the protection of the 2'-hydroxyl function during RNA synthesis.

The immense difficulty in finding suitable protection for the 2'-hydroxyl function for RNA synthesis has been highlighted through the study of the three proposed systems described in this chapter. This is due to the stringent criteria that have to be fulfilled. The 2'-hydroxyl protecting group has to be stable to the repeated acid conditions applied during synthesis, but is required to be stable to the basic ammonia conditions used in the cleavage of the completed chain from the solid support. Therefore, it would be advantageous to design a system that is stable to the acid conditions employed during synthesis, but is activated to an acid labile system during the ammonia cleavage step. The results obtained will be extremely useful when designing other propargyl based systems.

2.9. Future Work

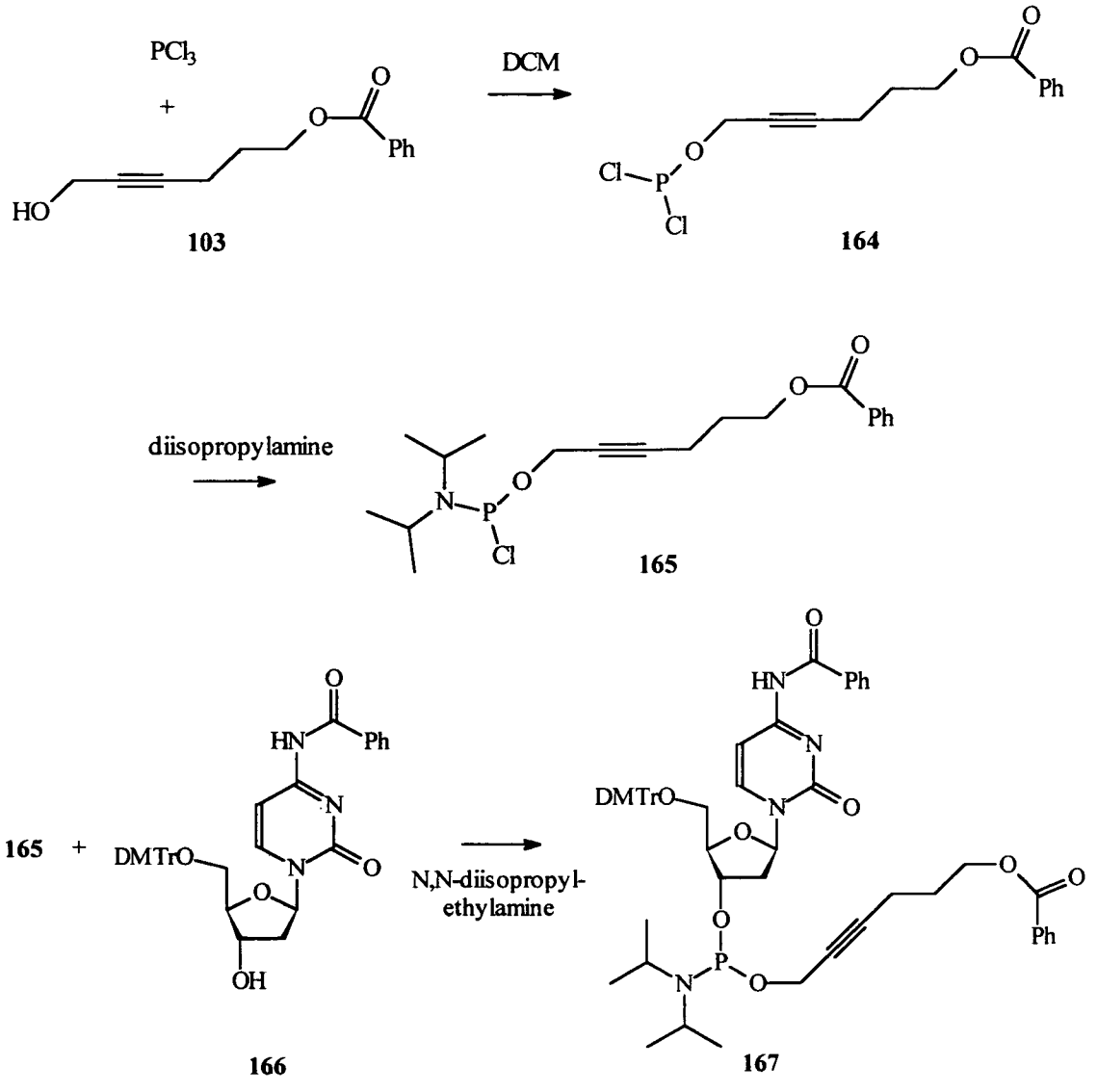
2.9.1. 2'-Hydroxyl Protecting Groups

Due to time constraints it has not been possible to fully investigate the potential of other propargyl based systems. Ideally, the system would maintain all the characteristics of the 2'-O-(hex-4-ynyl benzoate) group, but have an improved rate of cleavage in the final deprotection step, within the conditions required for the stability of RNA. Work is still required in finding a group that fulfils all the required criteria for a 2'-hydroxyl protecting group. Modifications to the alkyl chain of the 2'-O-(hex-4-ynyl benzoate) group, by addition of various substituents, would be of interest. In particular, the addition of two methyl groups at the C-3 position of hex-4-ynyl benzoate.

2.9.2. Phosphate Protecting Group

The β-cyanoethyl group is currently the most widely used phosphate protecting group for the internucleotidic bonds in the synthesis of oligonucleotides by the phosphoramidite approach (Section 1.6.4.). The feasibility of combining the 2'-hydroxyl protecting group for the protection of the phosphate group has been considered. Preliminary studies have highlighted the possibility of incorporating the propargyl based system, 6-hydroxy-hex-4-ynyl benzoate, into a deoxynucleoside *N,N*-dialkylamino phosphoramidite, *via* the chloro-*N,N*-dialkylaminoalkoxyphosphine **165** (Figure 61). Preparation of *N,N*-diisopropyl-(hex-4-ynyl benzoate)-1-phosphonamidyl chloride (**165**) was achieved by addition of *N,N*-diisopropylamine to a freshly prepared solution of (hex-4-ynyl benzoate)-1-phosphodichloridite (**164**). The phosphoramidite, 5'-O-4,4'-dimethoxytrityl-(N^4-Benzoyl)-cytidine-3'-O-[N.N-diisopropyl-(hex-4-ynyl benzoate)-1-phosphoramidite] (**167**), was prepared by

addition to **165** in the presence of diisopropylethylamine. The phosphoramidite was isolated by chromatography, and precipitated from dry toluene, in a yield of 72%.



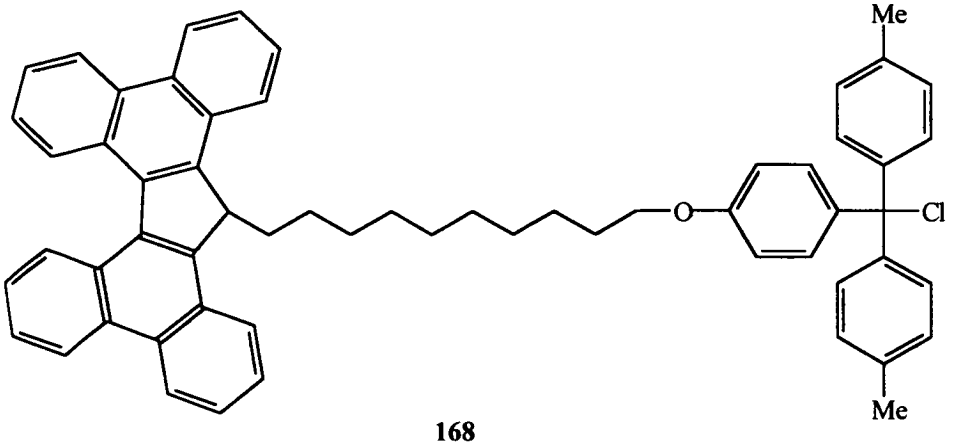
103 164 165 166 167

Figure 61. Synthesis of 5'-O-4,4'-dimethoxytrityl-(N^4-Benzoyl)-cytidine-3'-O-[N,N-diisopropyl-(hex-4-ynyl benzoate)-1-phosphoramidite]

The applicability of the phosphoramidite (**167**) was not demonstrated; however improvements to the propargyl based system would lead to evaluation of this type of phosphate protection.

2.9.3. Hydrophobic 5'-O-Protecting Group

The purification of oligonucleotides using modified DMTr groups has previously been discussed (Section 1.7.3.). Ramage and Wahl[51] demonstrated the potential of 4-(17-tetrabenzo[a,c,g,i]fluoromethyl)-4',4''-dimethoxytrityl chloride (Tbf-DMTr-Cl) (**59**) as a new hydrophobic 5'-hydroxyl protecting group. The Tbf moiety has been proven to be ideal for purification purposes due to its strong hydrophobic and fluorescent properties. It was, thus, desirable to sufficiently separate the Tbf moiety from the synthesised chain, to avoid steric bulk from the bulky Tbf moiety. Hay[130] and Hager[131] achieved this through connection of the Tbf moiety *via* a linker to a trityl compound. The linker consisted of a ten carbon alkyl chain with one alkoxy group to allow acid cleavage following purification of the oligomer (Figure 62).



168

Figure 62. Bis-(4-methylphenyl)-4-(10-(17-tetrabenzo[a,c,g,i]fluorenyl)decyloxy) phenyl methyl chloride

2.9.3.1. Synthesis of 5'-O-Hydrophobic Protecting Group

Work by Hay[130] and Hager[131], within the group, discussed the synthesis of ethyl-4-[10-(17-tetrabenzo[a,c,g,I]fluorenyl)-decyloxy] benzoate (**171**) from the terabutyl-ammonium salt of 8*bH*-Tbf (**170**) and the bromo ester, ethyl-4-(10-bromodecyloxy)-benzoate (**169**). Bis-(4-methylphenyl)-4-(10-(17-tetrabenzo[a,c,g,I]fluorenyl)-

decyloxy) phenyl methanol (**173**) was formed *via* a Grignard reaction of 4-bromotoluene with **171** in a yield of 52% (Figure 63).

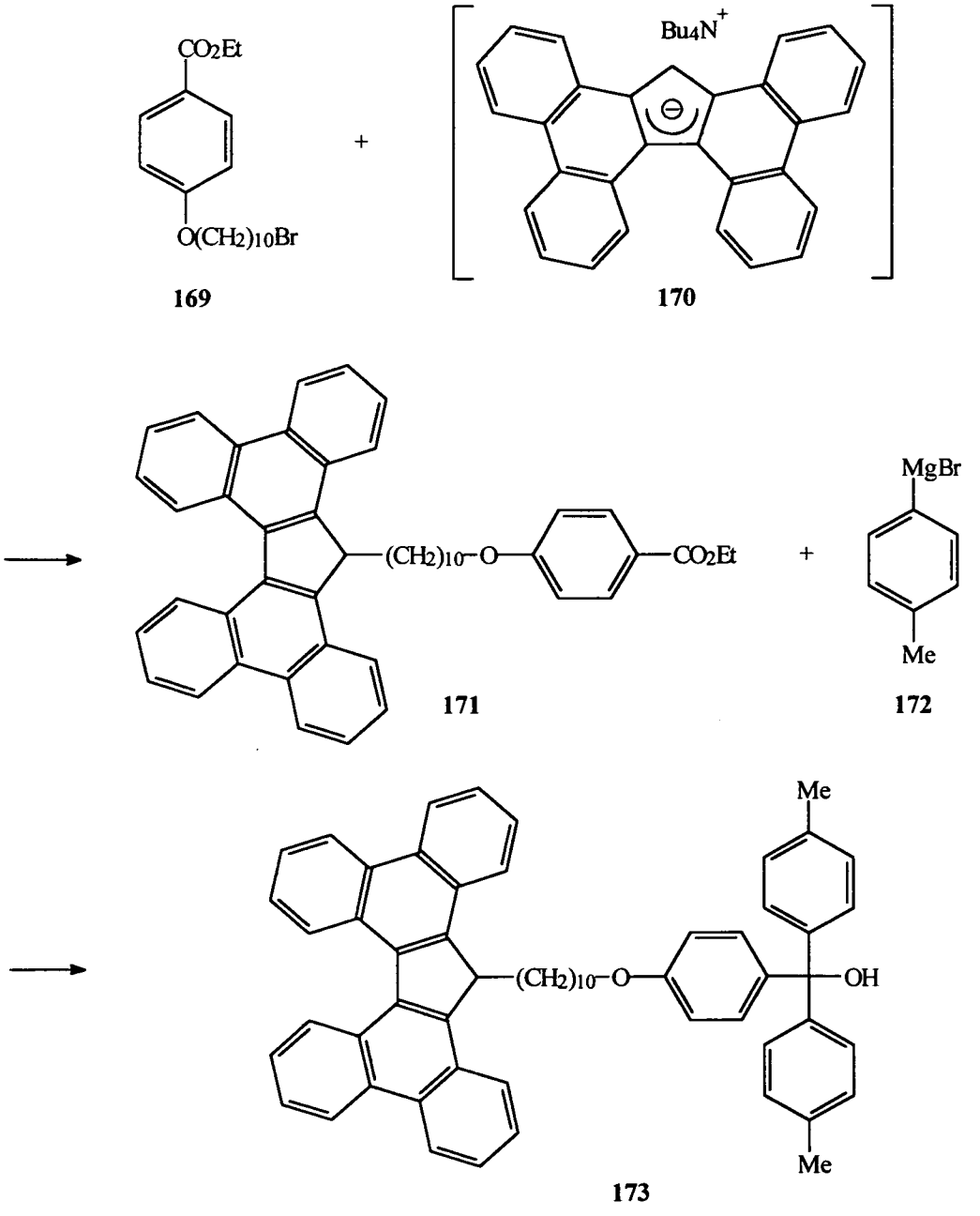


Figure 63. Synthesis of Bis-(4-methylphenyl)-4-(10-(17-tetrabenzo[a,c,g,I]fluorenyl)decyloxy) phenyl methanol

Study of the chlorination of **173** to **168,** and incorporation to the 5'-hydroxyl function of a nucleoside, is on going within the research group.

3.0. Experimental

3.1. Notes

Chemicals were purchased from commercial sources such as Aldrich, Lancaster, and Fluka, checked and used without further purification. Melting points were determined in open capillaries using a Buchi 510 melting point apparatus and are uncorrected. Thin layer chromatography (tlc) was performed on aluminium sheets precoated with silica gel (Kieselgel 60 F254) in the solvent system indicated. Wet flash chromatography was performed using silica gel 60 (230-400 mesh). Compounds were visualised using suitable combinations of ultra violet absorption at 254 and 365nm, iodine vapour and methanolic sulphuric acid. Infrared spectra were recorded on a Perkin Elmer Lamda 11 single beam spectrophotometer. Fast atom bombardment mass spectra (FAB MS) were recorded on a Kratos MS50TC and electron impact mass spectra (EI MS) on a 4/500 Finnigan. Electospray and APCI on a VG Platform. Elemental analyses were carried out on a Perkin Elmer 2400 instrument. Proton NMR were recorded on a Varian Gemini 200 and a Bruker AC250 (250MHz) in the solvent indicated relative to tetramethylsilane (TMS) as the external standard. Carbon-13 NMR spectra were recorded on a AC250 (60MHz) instrument in the solvents indicated relative to TMS. Phosphorus-31 NMR spectra were recorded on a AC 250 (60MHz) instrument in the solvents indicated relative to phosphoric acid as the internal standard. A Bruker WH360 was used to record NMR spectra at 300MHz. The chemical shifts (δ) were reported in parts per million. All solvents were of analytical grade or were distilled before use. The following were dried when required using the regents indicated; dichloromethane (calcium hydride), diethyl ether (sodium wire), tetrahydofuran (sodium/benzophenone indicator), pyridine (calcium hydride), acetonitrile (calcium hydride).

3.1.1. Oligoribonucleotide Synthesis

The synthesis of oligoribonucleotides was performed on an Applied Biosystems 381A DNA synthesiser courtesy of Link Technologies. Tetrazole coupling catalyst, acetic anhydride and lutidine capping reagents, trichloroacetic acid deprotection solution, iodine oxidation mixture, acetonitrile and solid support were supplied by Link Technologies. Syntheses were carried out using the protocols as described in Section 2.4.

3.1.2. HPLC

Deprotection and stability studies were monitored using reverse-phase high performance chromatography (RP-HPLC). This was performed using a Gilson system comprising of two 306 solvent delivery systems, an 811C dynamic mixer, an 805 manmetric module, a 119 UV/VIS detector and a Gilson software-driven gradient controller. Gradients were eluted from a Vydac reverse phase silica column (5μl particle size) C_{18}, 250 x 2.6mm using a linear grade of acetonitrile (A) in Milli-Q grade water (B) with a flow rate 1ml/min. Gradient used; 0-2min 0%A, 2-32min 0-90% A.

3.2.Synthetic Procedures

Pent-4-ynyl-benzoate (97).

Anhydrous pyridine (1.88g, 22.4mmol) and benzoyl chloride (3.35g, 23.8mmol) were added dropwise to a solution of 1-pentyne-4-ol (**96**) (2.0g, 23.8mmol) in anhydrous CH_2Cl_2 (15ml) at room temperature. The reaction mixture was heated at reflux for 6 hours, cooled to room temperature and the pyridine hydrochloride filtered off. The filtrate was washed with 2M NaOH solution (2x25ml), separated, dried ($MgSO_4$),

filtered and reduced *in vacuo*. The residue was purified by wet flash chromatography (DCM) to give the *title compound* as a clear oil (3.91g, 87%). **tlc** $\mathbf{R_f}$ (30% Hexane/DCM) 0.68; $\mathbf{v_{max.}}$ 3297 (C-H alkyne), 2117 (carbon-carbon alkyne), 1718 (C=O stretch); **δH** (200MHz, $CDCl_3$) 1.95-2.04 (m, 3H, H-C, CH_2), 2.33-2.42 (m, 2H, CH_2), 4.42 (t, 2H, CH_2, J = 6.2), 7.38-7.70 (m, 5H, aromatic)ppm; **δC** (63MHz, $CDCl_3$) 15.14 (CH_2), 27.45 (CH_2), 63.25 (CH_2), 68.95 (alkyne), 82.82 (C-H alkyne), 128.16, 128.69, 129.34, 130.35, 132.77 (aromatic), 166.31 (C=O) ppm; **m/z** (FAB) 189 (MH^+); **HRMS** (FAB) Found 189.09197, $C_{12}H_{13}O_2$ Requires 189.08977.

6-Hydroxy-hex-4-ynyl benzoate (98).

Compound (**97**) (1.0g, 5.3mmol) in anhydrous THF (20ml) was cooled to -30°C under an atmosphere of argon. 1.6M nButyl lithium (3.36ml, 5.3mmol) was added dropwise and the reaction allowed to stir for 30 minutes, at -30°C. Previously dried paraformaldehyde (0.48g, 16mmol) was added and the reaction allowed to warm to room temperature and stirred overnight. Diethyl ether (30ml) and water (30ml) were added to the reaction mixture and K_2CO_3 was added until the aqueous layer became pasty. The organic layer was then separated, dried ($MgSO_4$) and reduced *in vacuo*. The residue was purified by wet flash chromatography (DCM) to give the *title compound* as a clear oil (0.12g, 10%). **tlc** $\mathbf{R_f}$ (5% MeOH/DCM) 0.63; $\mathbf{v_{max.}}$ 3425 (OH), 2959 (CH, aromatic), 2226 and 2286 (carbon-carbon alkyne), 1719 (C=O stretch); **δH** (200MHz, $CDCl_3$) 1.78 (s, 1H, OH) 1.90-2.03 (m, 2H, CH_2), 2.36-2.45 (m, 2H, CH_2), 4.22 (t, 2H, ThpO-CH_2, J = 2.1Hz), 4.41 (t, 2H, CH_2-benzoate, J = 6.2Hz), 7.25-8.16 (m, 5H, aromatic)ppm; **δC** (63MHz, $CDCl_3$) 15.56 (CH_2), 27.60 (CH_2), 51.18 (CH_2), 63.46 (CH_2), 79.13 (alkyne), 84.74 (alkyne), 128.26, 129.44, 130.08, 132.86 (aromatic), 166.46 (C=O) ppm; **m/z** (FAB) 219 (MH^+); **HRMS** (FAB) Found 219.10284, $C_{13}H_{15}O_3$ Requires 219.09954.

6-(tetrahydropyranyloxy)-hex-4-yne chloride (101).

Propargyl tetrahydropyranol (**100**) (1.0g, 7.1mmol) was dissolved in anhydrous THF (10ml) and cooled to -78°C under an atmosphere of nitrogen. 2.5M nButyllithium (2.85ml, 7.1mmol) was added dropwise to the reaction mixture. After 30 minutes 3-bromo-1-chloropropane (1.12g, 7.1mmol) in THF was added dropwise over a 10 min. period. The reaction mixture was allowed to warm to room temperature and heated at reflux for 20 hours. After cooling diethyl ether (15ml) and water (15ml) were added and extracted (2x15ml). The combined organic layers were dried ($MgSO_4$), filtered and reduced *in vacuo*. The residue was taken up in ether and passed through neutral grade alumina. The alumina was washed with ether and run dry. The ether was reduced *in vacuo* and the residue was purified by Kugelrohr distillation under vacuum (94°C/0.04mmHg) to give the *title compound* as a clear oil (1.30g, 84%). **tlc** $\mathbf{R_f}$ (DCM) 0.57; **C.H.N.** Found C: 60.22%, H: 7.73, $C_{11}H_{17}O_2Cl$ Requires C: 60.97%, H: 7.91, $\nu_{max.}$ 2959 (CH), 2222 and 2286 (carbon-carbon alkyne); **δH** (360MHz, $CDCl_3$) 1.42-1.85 (m, 6H, CH_2), 1.87-2.00 (m, 2H, CH_2), 2.35-2.42 (m, 2H, CH_2), 3.64 (t, 2H, CH_2-Cl, J = 6.2Hz), 3.48-3.56 and 3.77-3.88 (m, 2H, CH_2-O), 4.12-4.32 (m, 2H, O-CH_2-alkyne), 4.79 (t, 1H, CH, J = 2.5Hz) ppm; **δC** (360MHz, $CDCl_3$) 16.01 (alkyne-CH_2), 18.85 (CH_2), 25.23 (CH_2), 30.03 (CH_2), 31.01 (CH_2), 43.39 (CH_2-Cl), 54.24 (O-CH_2-alkyne), 61.73 (CH_2-O), 77.42 (alkyne), 84.25 (alkyne), 96.46 (CH) ppm; **m/z** (FAB) 217 (M^+); **HRMS** (FAB) Found 217.10021, $C_{11}H_{17}O_2Cl$ Requires 217.09601.

6-(Tetrahydropyranyloxy)-hex-4-ynyl benzoate (102).

Compound (**101**) (0.31g, 1.43mmol) was suspended in DMF (15ml) to which dried sodium benzoate (0.34g, 2.36mmol) was added. The reaction mixture was heated at reflux for 6 hours under an atmosphere of nitrogen and then left to cool. Ether (30ml) was added before filtering and washing with ether. The ether and DMF were removed *in vacuo*. The residue was purified by wet flash chromatography (DCM) to give the

title compound as a clear oil (0.35g, 81%). **tlc R_f** (5% MeOH/DCM) 0.63; **$v_{max.}$** 2959 (CH, aromatic), 2222 and 2286 (carbon-carbon alkyne), 1718 (C=O stretch); **δH** (200MHz, $CDCl_3$) 1.48-1.84 (m, 6H, CH_2), 1.92-2.2.02 (m, 2H, CH_2), 2.37-2.45 (m, 2H, CH_2), 3.46-3.54 and 3.77-3.86 (m, 2H, CH_2-O), 4.12-4.31 (m, 2H, O-CH_2-alkyne), 4.39 (t, 2H, CH_2-benzoate, J = 6.2Hz), 4.77 (t, 1H, CH, J = 3.1Hz)), 7.24-8.03 (m, 5H, aromatic) ppm; **δC** (63MHz, $CDCl_3$) 15.43 (alkyne-CH_2), 18.76 (CH_2), 25.02 (CH_2), 27.44 (CH_2), 29.92 (CH_2), 54.15 (O-CH_2-alkyne), 61.59 (CH_2-O), 63.56 (CH_2-benzoate) 76.38 (alkyne), 84.65 (alkyne), 96.33 (CH), 128.00, 129.19, 129.88, 132.58 (aromatic), 166.06 (C=O) ppm; **m/z** (FAB) 302 (M^+); **HRMS** (FAB) (MH^+) Found 303.16015, $C_{18}H_{22}O_4$ Requires 303.15844.

6-Hydroxy-hex-4-ynyl benzoate (103).

Compound (**102**) (1.4g, 4.64mmol) was dissolved in MeOH (45ml) to which 5% Oxalic acid (15ml) was added. This was allowed to stir at room temperature for 48 hours before the MeOH was removed *in vacuo*. Water (100ml) and EtOAc (100ml) were added and the layers separated, extracting with EtOAc (2x50ml). The combined extracts were dried ($MgSO_4$), filtered and reduced *in vacuo*. The residue was purified by wet flash chromatography (DCM) to give the *title compound* as a clear oil (0.87g, 86%). **tlc R_f** (5% MeOH/DCM) 0.63; **$v_{max.}$** 3425 (OH), 2959 (CH, aromatic), 2226 and 2286 (carbon-carbon alkyne), 1719 (C=O stretch); **δH** (200MHz, $CDCl_3$) 1.78 (s,1H, OH) 1.91-2.02 (m, 2H, CH_2), 2.36-2.43 (m, 2H, alkyne-CH_2), 4.22 (t, 2H, HO-CH_2, J = 2.1Hz), 4.41 (t, 2H, CH_2-Benzoate, J = 6.2Hz), 7.25-8.05 (m, 5H, aromatic) ppm; **δC** (63MHz, $CDCl_3$) 15.54 (alkyne-CH_2), 27.57 (CH_2), 51.15 (HO-CH_2), 63.44 (CH_2-Benzoate), 79.13 (alkyne), 84.71 (alkyne), 128.24, 129.43, 130.04, 132.87 (aromatic), 166.46 (C=O) ppm; **m/z** (FAB) 219 (MH^+); **HRMS** (FAB) Found 219.10016, $C_{13}H_{15}O_3$ Requires 219.09954.

6-Bromo-hex-4ynyl benzoate (104).

A solution of triphenylphosphine (0.48g, 1.84mmol) in anhydrous ether was added dropwise to a solution of **103** (0.20g, 0.92mmol), CBr_4 (0.61g, 1.84mmol) and pyridine (0.075ml, 9.17mmol) under an atmosphere of nitrogen at 0°C. The reaction was stirred at 0°C for 2 hours and then at room temperature overnight. The ether was removed *in vacuo* at 0°C and the residue dissolved in n-pentane. This was filtered and reduced *in vacuo*. The residue was purified by wet flash chromatography (DCM) to give the *title compound* as an oil (0.21, 82%). **tlc R_f** (DCM) 0.45; **$\nu_{max.}$** 2957 (CH, aromatic), 2233 (carbon-carbon alkyne), 1717 (C=O stretch); **δH** (200MHz, $CDCl_3$) 1.92-2.02 (m, 2H, CH_2), 2.39-2.47 (m, 2H, alkyne-CH_2), 3.88 (t, 2H, Br-CH_2, J = 2.3Hz), 4.39 (t, 2H, CH_2-Benzoate, J = 6.2Hz), 7.25-8.05 (m, 5H, aromatic) ppm; **δC** (63MHz, $CDCl_3$) 15.17 (alkyne-CH_2), 15.81 (Br-CH_2), 27.44 (CH_2), 63.39 (CH_2-Benzoate), 75.97 (alkyne), 86.34 (alkyne), 128.22, 129.42, 130.04, 132.84 (aromatic), 166.33 (C=O) ppm; **m/z** (FAB) 281 (M^+); **HRMS** (FAB) Found 280.01002, $C_{13}H_{13}O_2Br$ Requires 280.00989.

3', 5'-O-(Tetraisopropyldisiloxane-1,3-diyl)-uridine (106).[70]

1,3-Dichloro-1,1,3,3-tetraisopropyldisiloxane (0.75ml, 2.2mmol) was added dropwise over 5 minutes to a suspension of uridine (**105**) (0.5g, 2.1mmol) in anhydrous pyridine cooled to 0°C under an atmosphere of argon.. The reaction was allowed to warm to room temperature and stirred for 3 hours. The reaction was quenched with MeOH (5ml) and reduced *in vacuo*. The residue was taken up in DCM and washed with 5% $NaHCO_3$ solution and extracted with DCM (2x50ml). The combined extracts were dried (Na_2SO_4), filtered and reduced *in vacuo*. The residue was purified by wet flash chromatography (1-5% MeOH/DCM) to give the *title compound* as a white foam (0.72g, 72%). **m.p.** 77-78°C; **tlc R_f** (5% MeOH/DCM) 0.34; **C.H.N.** Found C: 52.11%, H: 7.67%, N: 5.40, $C_{21}H_{38}N_2O_7Si_2$ Requires C: 51.82%, H: 7.87%, N: 5.76%; **δH** (200MHz, DMSO) 0.93-1.04 (m, 28H, $CH(CH_3)_2$), 3.85-4.17 (m, 5H,

2',3',4',5'-H), 5.49-5.54 (m, 1H, 5-H), 5.62 (d, 1H, 1'-H, J = 4.0Hz), 7.68 (d, 1H, 6-H, J = 8.1Hz), 11.38 (s, 1H, 3-H) ppm; δC (63MHz, DMSO) 12.06, 12.44, 12.84 (4xCH), 16.87-17.46 (8xCH_3), 60.33 (C-5'), 68.90 (C-3'), 73.65 (C-2'), 81.01 (C-4'), 90.64 (C-1'), 101.07 (C-5), 139.93 (C-6), 150.24 (C-2), 163.33 (C-4) ppm; **m/z** (FAB) 487 (MH^+); **HRMS** (FAB) Found 487.23117, $C_{21}H_{39}N_2O_7Si_2$ Requires 487.22958.

3', 5'-O-(Tetraisopropyldisiloxane-1,3-diyl)-N^3-(benzoyl)uridine (107).[109]

Benzoyl chloride (0.08g, 0.47mmol) was added to a vigorously stirred suspension of **106** (0.21g, 0.42mmol), Na_2CO_3 (0.36g, 3.4mmol) and 5% tetrabutyl ammonium bromide (0.007g) in DCM:H_2O (20ml, 1:1). After 72 hours the organic phase was separated and the aqueous phase extracted with DCM (2x30ml). The combined extracts were dried (Na_2SO_4), filtered and reduced *in vacuo*. The residue was purified by wet flash chromatography (1-5% MeOH/DCM) to give the *title compound* as a white foam (0.15g, 62%). **tlc R_f** (5% MeOH/DCM) 0.71; **C.H.N.** Found C: 56.66%, H: 7.31%, N: 4.65%, $C_{28}H_{42}N_2O_8Si_2$ Requires C: 56.92%, H: 7.17%, N: 4.74%; **δH** (200MHz, $CDCl_3$) 0.98-1.08 (m, 28H, $CH(CH_3)_2$), 2.95 (s, br, 1H, OH), 3.96-4.37 (m, 5H, 2',3',4',5'-H), 5.73 (s, 1H, 1'-H), 5.79 (d, 1H, 5-H, J = 8.2Hz), 7.25-7.94 (m, 6H, 6-H, aromatic) ppm; δC (63MHz, $CDCl_3$) 12.32, 12.76, 12.85, 13.23 (4xCH), 16.66-17.29 (8xCH_3), 60.02 (C-5'), 68.75 (C-3'), 75.06 (C-2'), 81.95 (C-4'), 90.66 (C-1'), 101.67 (C-5), 129.03, 130.35, 131.24, 135.03 (aromatic), 139.45 (C-6), 148.24 (C-2), 161.99 (C-4), 168.44 (C=O) ppm; **m/z** (FAB) 591 (MH^+); **HRMS** (FAB) Found 591.25685, $C_{28}H_{43}N_2O_8Si_2$ Requires 591.25503.

3', 5'-O-(Tetraisopropyldisiloxane-1,3-diyl)-N^3-(pivaloyl)uridine (108).[110]

Compound (**106**) (0.10g, 0.21mmol) was dissolved in anhydrous DMF (10ml). Anhydrous potassium carbonate (0.14g, 1.1mmol) and chloromethyl pivalate (0.03g,

0.08ml, 0.2mmol) were added and the mixture stirred overnight under an atmosphere of argon. The reaction mixture was filtered and the filtrate reduced *in vacuo*. The residue was purified by wet flash chromatography (1-5% MeOH/DCM) to give the *title compound* as an off white foam (0.099g, 79%). **tlc** $\mathbf{R_f}$ (5% MeOH/DCM) 0.69; **C.H.N.** Found C: 54.32%, H: 8.37%, N: 4.99%, $C_{27}H_{48}N_2O_9Si_2$ Requires C: 53.97%, H: 8.05%, N: 4.66%; **δH** (200MHz, $CDCl_3$) 0.97-1.07 (m, 28H, $CH(CH_3)_2$), 1.14-1.18 (m, 9H, $C(CH_3)_3$), 3.95-4.39 (m, 5H, 2',3',4',5'-H), 5.70 (m, 2H, 1'-H, 5-H), 5.71-5.73 (m, 2H, CH_2 pivalate), 7.66 (d, 1H, 6-H, J = 8.2Hz) ppm; **δC** (63MHz, $CDCl_3$) 12.36-13.21 (isopropyl CH), 16.67-17.29 (isopropyl CH_3), 26.86 (*tert*-butyl CH_3s), 38.69 (quaternary C of *tert*-butyl) 60.15 (C-5'), 64.44 (CH_2 of pivaloyloxymethyl), 68.75 (C-3'), 75.06 (C-2'), 81.95 (C-4'), 90.66 (C-1'), 101.24 (C-5), 138.70 (C-6), 149.84 (C-2), 162.43 (C-4), 177.34 (pivaloyl C=O) ppm; **m/z** (FAB) 601 (MH^+); **HRMS** (FAB) Found 600.29122, $C_{27}H_{48}N_2O_9Si_2$ Requires 600.28984

3',5'-O-(Tetraisopropyldisiloxane-1,3-diyl)-N^3-(4-tert-butylbenzoyl) uridine (109).[111]

Triethylamine (0.42ml, 3mmol) and chlorotrimethylsilane (0.24ml, 1.85mmol) were added to a suspension of **106** (0.30g, 0.62mmol) in anhydrous DCM, cooled to 0°C in an ice bath temperature under an atmosphere of argon. The mixture stirred overnight at room before being poured into vigorously stirred 1 M aqueous sodium bicarbonate. The separated organic phase was dried (Na_2SO_4), filtered and reduced *in vacuo* to give a pink foam. The residue was dried by coevaporation with anhydrous pyridine (2x5ml), dissolved in anhydrous pyridine (4ml) and cooled to 0°C in an ice bath. 4-*tert*-Butylbenzoyl chloride (0.36ml, 1.84mmol) and *N,N*-diisopropylethylamine (0.34ml, 1.9mmol) were added and the reaction stirred overnight at room temperature. The reaction was quenched with MeOH (4ml) and the solvent removed *in vacuo*. The 2'-O-trimethylsilyl group was removed by treatment with 4-toluene sulphonic acid. The residue was taken up in DCM (10ml) and stirred vigorously with

0.5M 4-toluene sulphonic acid monitoring the reaction by TLC. After 10minutes the layers were separated and the organic layer washed with water (10ml), dried (Na_2SO_4), filtered and reduced *in vacuo*. The residue was purified by wet flash chromatography (1-5% MeOH/DCM) to give the *title compound* as a white foam (195mg, 49%). **m.p.** 66-77°C; **tlc** R_f (5% MeOH/DCM) 0.70; **C.H.N.** Found C: 59.56%, H: 7.42%, N: 4.79%, $C_{32}H_{47}N_2O_8Si_2$ Requires C: 59.78%, H: 7.21%, N: 4.36%; **δH** (200MHz, $CDCl_3$) 0.98-1.10 (m, 28H, $CH(CH_3)_2$), 1.31-1.35 (m, 9H, $C(CH_3)_3$), 3.95-4.30 (m, 5H, 2',3',4',5'-H), 5.61 (s, 1H, 1'-H), 5.76 (d, 1H, 5-H, J = 8.2Hz), 7.25-7.87 (m, 4H, aromatic), 8.07 (d, 1H, 6-H, J = 8.2Hz) ppm; **δC** (63MHz, $CDCl_3$) 12.35-13.29 (isopropyl CHs), 16.72-17.44 (isopropyl CH_3s), 30.81 (*tert*-butyl CH_3s), 35.25 (quaternary C of *tert*-butyl) 59.36 (C-5'), 68.00 (C-3'), 76.31 (C-2'), 81.49 (C-4'), 91.19 (C-1'), 101.05 (C-5), 126.17, 128.44, 130.49 (aromatic), 139.25 (C-6), 148.82 (C-2), 162.31 (C-4), 168.36 (benzoyl C=O) ppm; **m/z** (FAB) 642 (M^+); **HRMS** (FAB) Found 643.28461, $C_{32}H_{47}N_2O_8Si_2$ Requires 643.28710.

6-Iodo-hex-4-ynyl benzoate (110).

Iodine (3.03g, 11.9mmol) was added in one portion to a cooled (0°C) solution of compound **(103)** (2.0g, 9.2mmol), triphenylphosphine (0.29g, 1.1mmol), and imidazole (0.09g, 1.3mmol) in anhydrous ether/ acetonitrile (3:1) (100ml) under an atmosphere of nitrogen. Temperature was maintained at 0°C for 1.5 hours with stirring. The reaction mixture was poured into water (100ml) and extracted with ether (3x50ml). The combined extracts were washed with saturated aqueous $Na_2S_2O_3$ (100ml) and water (100ml) and dried (Na_2SO_4), filtered and reduced *in vacuo*. The residue was purified by wet flash chromatography (DCM) to give the *title compound* as a clear oil (2.94g, 98%). **tlc** R_f (DCM) 0.51; **C.H.N.** Found C: 47.86%, H: 3.98% $C_{13}H_{13}O_2I$ Requires C: 47.58%, H: 3.99%; $\nu_{max.}$ 2957 (CH, aromatic), 2229 (carbon-carbon alkyne), 1714 (C=O stretch); **δH** (200MHz, $CDCl_3$) 1.91-2.02 (m, 2H, CH_2), 2.35-2.43 (m, 2H, alkyne-CH_2), 3.66 (t, 2H, I-CH_2, J = 2.4Hz), 4.40 (t, 2H, CH_2-Benzoate, J = 6.2Hz), 7.25-8.06 (m, 5H, aromatic) ppm; **δC** (63MHz, $CDCl_3$) 15.68

(alkyne-CH_2), 27.57 (CH_2), 30.87 (I-CH_2), 63.40 (CH_2-Benzoate), 75.63 (alkyne), 85.86 (alkyne), 128.24, 129.43, 130.02, 132.85 (aromatic), 166.35 (C=O) ppm; **m/z** (FAB) 328 (M^+); **HRMS** (FAB) Found 327.99177, $C_{13}H_{13}O_2I$ Requires 327.99603.

6-Mesyl-hex-4-ynyl benzoate (111).

Compound (**103**) (0.20g, 0.92mmol) was dissolved in anhydrous DCM (20ml) to which pyridine (1.44g, 0.90mmol) was added and cooled to 0°C. Methane sulphonylchloride (0.22ml, 2.8mmol) was added dropwise and the reaction stirred for 2 hours, allowing to warm to room temperature. Water (25ml) and ether (25ml) were added and the organic phase extracted (2x25ml). The extracts were dried ($MgSO_4$)), filtered and reduced *in vacuo*. The residue was purified by wet flash chromatography (DCM) to give the *title compound* as a clear oil (0.26, 94%). **tlc R_f** (DCM) 0.46; **$\nu_{max.}$** 2939 (CH, aromatic), 2307 and 2237 (carbon-carbon alkyne), 1716 (C=O stretch), 1367 and 1174 (-SO_2O-); **δH** (200MHz, $CDCl_3$) 1.97-2.05 (m, 2H, CH_2), 2.41-2.48 (m, 2H, alkyne-CH_2), 3.08 (s, 3H, S-CH_3), 4.40 (t, 2H, CH_2-benzoate, J = 6.2Hz), 4.81 (t, 2H, O-CH_2, J = 2.2Hz), 7.25-8.04 (m, 5H, aromatic) ppm; **δC** (63MHz, $CDCl_3$) 15.62 (alkyne-CH_2), 27.28 (CH_2), 38.77 (CH_3), 58.05 (O-CH_2), 63.21 (CH_2-Benzoate), 72.99 (alkyne), 89.20 (alkyne), 128.29, 129.42, 129.92, 132.95 (aromatic), 166.32 (C=O) ppm; **m/z** (FAB) 297 (MH^+); **HRMS** (FAB) Found 297.08027, $C_{14}H_{17}O_5S$ Requires 297.07967.

6-Trichloroacetimidate-hex-4-ynyl benzoate (112).

Dried potassium carbonate (0.30g, 2.2mmol) and trichloroacetonitrile (0.22ml, 2.2mmol) were added to a solution of compound (**103**) (0.46g, 2.1mmol) in anhydrous DCM (5ml) under an atmosphere of argon. The mixture was stirred for 5 hours before being filtered and concentrated *in vacuo*. The residue was purified by wet flash chromatography (DCM) to give the *title compound* as a clear oil (0.53,

70%). **tlc** R_f (DCM) 0.62; **C.H.N.** Found C: 49.94%, H: 3.87%, N: 3.88% $C_{15}H_{14}NO_3Cl_3$ Requires C: 49.68%, H: 3.89%, N: 3.86%; $\nu_{max.}$ 3338 (N-H), 2955 (CH, aromatic), 2312 and 2238 (carbon-carbon alkyne), 1718 (C=O stretch), 1666 (C=N); **δH** (200MHz, $CDCl_3$) 1.97-2.04 (m, 2H, CH_2), 2.40-2.48 (m, 2H, alkyne-CH_2), 4.40 (t, 2H, CH_2-Benzoate, J = 6.2Hz), 4.88 (t, 2H, CH_2, J = 2.1Hz), 7.25-8.05 (m, 5H, aromatic), 8.42 (s, 1H, NH) ppm; **δC** (63MHz, $CDCl_3$) 15.70 (alkyne-CH_2), 27.45 (CH_2), 57.26 (O-CH_2), 63.40 (CH_2-Benzoate), 74.16 (alkyne), 86.72 (alkyne), 128.26, 129.45, 130.08, 132.85 (aromatic), 161.84 (C=NH), 166.38 (C=O) ppm; **m/z** (FAB) 363 (M^+); **HRMS** (FAB) Found 361.00323, $C_{15}H_{14}NO_3Cl_3$ Requires 361.00393.

Mixture of 2'-O and 3'-O-(Hex-4-ynyl benzoate)uridine (115).

Uridine (1.30g, 5.3mmol), dibutyltin oxide (1.59g, 6.4mmol) and tetrabutyl ammonium bromide (1.88g, 5.8mmol) were suspended in anhydrous DMF (10ml) under an atmosphere of nitrogen. Compound (**110**) (3.97g, 12.1mmol) was added with vigorous stirring and the reaction mixture heated to 60°C. After 22 hours the reaction was filtered, washed with DMF (20ml) and the filtrate reduced *in vacuo*. The residue was purified by wet flash chromatography (1-5% MeOH/DCM) to give the *title compound* as a off white sticky foam (1.92g, 81%). **tlc** R_f (15% MeOH/DCM) 0.45; **m/z** (FAB) 446 (MH^+); **HRMS** (FAB) Found 445.16455, $C_{22}H_{25}O_8N_2$ Requires 445.16109.

3',5'-O-(Tetraisopropyldisiloxane-1,3-diyl)-2'-O-(hex-4-ynyl benzoate) uridine (116).

Compound (**115**) (132mg, 0.30mmol) was dried by coevaporation in anhydrous pyridine (2x2ml) then suspended in anhydrous pyridine (1.5ml) and cooled to 0°C under an atmosphere of argon. 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (0.10ml,

0.30mmol) was added dropwise over 5 minutes. The reaction was allowed to warm to room temperature and stirred overnight. The reaction was quenched with MeOH (5ml) and allowed to stir for 5 minutes before being reduced *in vacuo*. The residue was taken up in DCM and washed with 5% $NaHCO_3$ solution (20ml) and extracted with DCM (2x50ml). The combined extracts were dried (Na_2SO_4), filtered and reduced *in vacuo*. The residue was purified by wet flash chromatography (0-2% MeOH/DCM) to give the *title compound* as an off white foam (93mg, 46%). **tlc R_f** (5% MeOH/DCM) 0.34; **C.H.N.** Found C: 59.52%, H: 7.55%, N: 4.56%, $C_{34}H_{50}N_2O_9Si_2$ Requires C: 59.45%, H: 7.34%, N: 4.08%; **δH** (200MHz, $CDCl_3$) 0.95-1.08 (m, 28H, $CH(CH_3)_2$), 1.93-2.00 (CH_2), 2.35-2.39 (m, 2H, CH_2), 3.97-4.53 (m, 9H, 2xCH_2, 2',3',4',5'-H), 5.66 (d, 1H, 5-H, J = 7.8Hz), 5.74 (d, 1H, 1'-H), 7.38-8.04 (m, 6H, 6-H, aromatic), 9.34 (s, 1H, 3-H) ppm; **δC** (63MHz, $CDCl_3$) 12.06, 12.44, 12.84 (4xCH), 15.34 (CH_2), 16.87-17.46 (8xCH_3), 27.66 (CH_2), 58.40 (CH_2), 60.11 (C-5'), 63.32 (CH_2), 68.90 (C-3'), 73.65 (C-2'), 81.01 (C-4'), 90.64 (C-1'), 101.07 (C-5), 127.78-132.55 (aromatic), 139.93 (C-6), 150.24 (C-2), 163.33 (C-4) ppm; **m/z** (FAB) 687 (MH^+); **HRMS** (FAB) Found, $C_{34}H_{50}N_2O_9Si_2$ Requires 686.30549.

2'-O-(Hex-4-ynyl benzoate) uridine (117).

Compound (**116**) (62mg, 0.09mmol) was dissolved in THF (4ml). Potassium fluoride dihydrate (51mg, 0.54mmol) and a catalytic amount of 18-crown-6-ether were added and the reaction allowed to stir at room temperature for 5 hours. The reaction was filtered, washed and reduced *in vacuo*. The residue was purified by wet flash chromatography (1-5% MeOH in DCM) to give the *title compound* as an off white foam (27mg, 67%). **tlc R_f** (10% MeOH/DCM) 0.32; **C.H.N.** Found C: 59.10%, H: 5.60%, N: 4.07%, $C_{22}H_{24}N_2O_8$ Requires C: 59.45%, H: 5.44%, N: 6.30%; **δH** (200MHz, $CDCl_3$) 1.88-2.01 (m, 2H, CH_2), 2.34-2.41 (m, 2H, CH_2), 3.78-4.41 (m, 9H, 2xCH_2, 2',3',4',5'-H), 5.70 (d, 1H, 5-H, J = 8.0Hz), 5.82 (d, 1H, 1'-H, J = 3.8Hz), 7.25-8.04 (m, 6H, H-6, aromatic) ppm; **δC** (63MHz, $CDCl_3$) 15.46 (CH_2),

27.28 (CH_2), 58.72 (CH_2), 61.46 (C-5'), 63.45 (CH_2), 69.12 (C-3'), 75.92 (alkyne), 80.18 (C-2'), 85.13 (C-4'), 86.82 (alkyne), 89.26 (C-1'), 102.32 (C-5), 128.32-133.03 (aromatic), 141.44 (C-6), 150.27 (C-2), 163.35 (C-4), 166.75 (benzoate C=O) ppm; **m/z** (FAB) 445 (MH^+); **HRMS** (FAB) Found 445.16078, $C_{22}H_{25}N_2O_8$ Requires 445.16109.

2'-O and 3'-O-(Hex-4-ynyl benzoate)-5'-O-(dimethoxytrityl) uridine (119 and 118).

Compound (**115**) (0.60g, 1.35mmol) was coevaporated with anhydrous pyridine (2x5ml) then dissolved in anhydrous pyridine (2ml) and cooled in an ice bath to 0°C under an atmosphere of argon. 4,4'-Dimethoxytrityl chloride (0.50g, 1.48mmol) was added over a 1 hour period portionwise. The reaction was allowed to warm to room temperature and left to stir overnight. MeOH (5ml) was added and the solvent removed *in vacuo*. The residue was taken up in DCM (30ml) and washed with 5% $NaHCO_3$ solution, dried (Na_2SO_4), filtered and reduced *in vacuo* to give a foam. The residue was purified by wet flash chromatography (1-5% MeOH in DCM containing 1% pyridine, v/v/v) to give the *title compounds* as a white foam.

3'-O-(Hex-4-ynyl benzoate)-5'-O-(dimethoxytrityl) uridine (118).

(0.18g, 18%). **tlc** $\mathbf{R_f}$ (10% MeOH/DCM) 0.38; **C.H.N.** Found C: 69.10%, H: 5.60%, N: 4.07%, $C_{43}H_{42}N_2O_{10}$ Requires C: 69.16%, H: 5.67%, N: 3.75%; **δH** (200MHz, $CDCl_3$) 1.84-1.97 (m, 2H, CH_2), 2.24-2.38 (m, 2H, CH_2), 3.41-3.52 (m, 2H, 5'-H) 3.78 (s, 6H, OMe), 4.01-4.48 (m, 7H, 2xCH_2, 2',3',4'-H), 5.27 (d, 1H, 5-H, J = 8.1Hz), 5.93 (d, 1H, 1'-H, J = 1.8Hz), 6.81-8.04 (m, 19H, H-6, aromatic) ppm; δC (63MHz, $CDCl_3$) 15.41 (CH_2), 27.29 (CH_2), 55.05 (OMe), 58.38 (CH_2), 61.41 (C-5'), 63.32 (CH_2), 73.31 (C-2'), 75.79 (C-3'), 82.96 (C-4'), 86.43 (CAr_3), 89.77 (C-1'), 102.33 (C-5), 113.09-136.38 (aromatic), 139.96 (C-6), 150.51 (C-2), 162.30 (C-

4), 166.60 (benzoate C=O) ppm; **m/z** (FAB) 746 (M^+); **HRMS** (FAB) Found 746.28431, $C_{43}H_{42}N_2O_{10}$ Requires 746.28395.

2'-O-(Hex-4-ynyl benzoate)-5'-O-(dimethoxytrityl) uridine (119).

(0.53g, 53%).**tlc** R_f (10% MeOH/DCM) 0.58; **C.H.N.** Found C: 69.10%, H: 5.60%, N: 4.07%, $C_{43}H_{42}N_2O_{10}$ Requires C: 69.16%, H: 5.67%, N: 3.75%; **δH** (200MHz, $CDCl_3$) 1.93-2.02 (m, 2H, CH_2), 2.36-2.40 (m, 2H, CH_2), 3.53 (d, 2H, 5'-H), 3.78 (s, 6H, OMe), 3.80-4.40 (m, 7H, 2xCH_2, 2',3',4'), 5.70 (d, 1H, 5-H, J = 8.1Hz), 5.80 (d, 1H, 1'-H, J = 4.0Hz), 6.78-8.06 (m, 19H, H-6, aromatic) ppm; **δC** (63MHz, $CDCl_3$) 15.45 (CH_2), 27.22 (CH_2), 55.13 (OMe), 58.72 (CH_2), 61.64 (C-5'), 63.42 (CH_2), 69.28 (C-3'), 80.07 (C-2'), 85.24 (C-4'), 86.86 (CAr_3), 89.36 (C-1'), 102.39 (C-5), 113.44-136.25 (aromatic), 140.18 (C-6), 150.08 (C-2), 162.75 (C-4), 166.77 (benzoate C=O) ppm; **m/z** (FAB) 746 (M^+); **HRMS** (FAB) Found 746.28431, $C_{43}H_{42}N_2O_{10}$ Requires 746.28395.

5'-O-(Dimethoxytrityl)-2'-O-(hex-4-ynyl benzoate)-uridine-3'-O-(2-cyanoethyl N,N-diisopropylphosphoramidite) (120).

Compound (**119**) (0.50g, 0.67mmol) was coevaporated in anhydrous CH_3CN (3x5ml) and dissolved in anhydrous DCM (2.5ml) under an atmosphere of argon. N,N-Diisopropylethylamine (0.28ml, 1.27mmol) was added dropwise followed by 2-cyanoethoxy N,N-diisopropylaminochlorophosphine (0.35ml, 1.65mmol) with vigorous stirring and left overnight. MeOH (1ml) then EtOAc (10ml) were added and then washed with saturated aqueous $NaHCO_3$ and saturated NaCl solutions. The organic layer was extracted, dried (Na_2SO_4), filtered and reduced *in vacuo*. The residue was purified by wet flash chromatography (0-2% MeOH/DCM containing 1% pyridine) and then taken up in 1ml of dry toluene and precipitated in petroleum ether under vigorous stirring (150ml, cooled to -65°C) to give the *title compound* as a white

foam (0.46g, 72%). **tlc R_f** (5% MeOH/DCM) 0.61; **δH** (200MHz, $CDCl_3$) 0.98-1.28 (m, 12H, CH_3), 1.93-1.96 (m, 2H, CH_2), 2.31-2.42 (m, 2H, CH_2), 2.60-2.67 (m, 2H, CH_2CN), 3.38-3.68 (m, 6H, OCH_2, CH, 5'-H), 3.77 (s, 6H, OMe), 3.80-4.42 (m, 7H, 2xCH_2, 2',3',4'), 5.25 (d, 1H, 5-H, J = 8.7Hz), 5.98 (d, 1H, 1'-H, J = 2.9Hz), 6.79-8.03 (m, 19H, H-6, aromatic) ppm; **δC** (63MHz, $CDCl_3$) 15.57 (CH_2), 20.12 (CH_2CN), 24.26-24.49 (4xMe), 27.22 (CH_2), 42.66-43.24 (2xCH), 55.13 (OMe), 58.72 (CH_2), 61.64 (C-5'), 63.42 (CH_2), 69.28 (C-3'), 80.07 (C-2'), 85.24 (C-4'), 86.82 (CAr_3), 87.32 (C-1'), 101.79 (C-5), 113.04-135.08 (aromatic), 117.50 (CN), 139.93 (C-6), 149.82 (C-2), 162.65 (C-4), 166.31 (benzoate C=O) ppm; **δP** (63MHz, $CDCl_3$) 151.15, 151.27; **m/z** (FAB) 948 (MH^+); **HRMS** (FAB) Found 947.39716, $C_{52}H_{59}N_4O_{11}P$ Requires 946.39180.

5'-O-Dimethoxytrityl-uridine (121).[23]

Uridine (1.00g, 4.1mmol) was suspended in anhydrous pyridine under an atmosphere of argon. $AgNO_3$ (0.76g, 4.5mmol) and 4,4'-Dimethoxytrityl chloride (1.45g, 4.5mmol) were added. After 1.5 hours the reaction mixture was filtered and washed with DCM (20ml). The filtrate was washed with 5% $NaHCO_3$ sol. and extracted with DCM (2x50ml). The extracts were dried (Na_2SO_4), filtered and reduced *in vacuo*. The residue was purified by wet flash chromatography (1-5% MeOH in DCM containing 1% pyridine, v/v/v) to give the *title compound* as a white foam (1.15g, 51%). **tlc R_f** (10% MeOH/DCM) 0.25; **C.H.N.** Found C: 65.68%, H: 6.04%, N: 5.55%, $C_{30}H_{30}N_2O_8$ Requires C: 65.92%, H: 5.53%, N: 5.13%; **δH** (200MHz, $CDCl_3$) 3.48-3.49 (s, 2H, 5'-H), 3.75 (s, 6H, OMe), 4.16-4.46 (m, 3H, 2',3',4'-H), 5.32 (d, 1H, 5-H, J = 8.1Hz), 5.93 (d, 1H, 1'-H, J = 2.7Hz), 6.78-7.71 (m, 13H, aromatic), 7.99 (d, 1H, 6-H, J = 8.1Hz) ppm; **δC** (63MHz, $CDCl_3$) 55.06 (OMe), 61.75 (C-5'), 69.46 (C-3'), 75.19 (C-2'), 83.48 (C-4'), 86.79 (CAr_3), 90.12 (C-1'), 102.07 (C-5), 113.09-136.05 (aromatic), 144.20 (C-6), 150.95 (C-2), 163.73 (C-4) ppm; **m/z** (FAB) 546 (M^+); **HRMS** (FAB) Found 546.19995, $C_{30}H_{30}N_2O_8$ Requires 546.20022.

5'-O-Dimethoxytrityl-[3',2',N^3-tri(benzoyl)]-uridine (122).[105]

Compound **(121)** (77mg, 0.14mmol) was dissolved in anhydrous pyridine (0.5ml) and cooled to 0°C under an atmosphere of nitrogen. Benzoyl chloride (0.066ml, 0.56mmol) was added to and the reaction stirred overnight at room temperature. MeOH (2ml) and water (20ml) were added and then extracted with DCM (2x20ml). The combined extracts were dried (Na_2SO_4), filtered and reduced *in vacuo*. The residue was purified by wet flash chromatography (DCM containing 1% pyridine, v/v/v) to give the *title compound* as a white foam (62mg, 51%). **tlc R_f** (DCM) 0.22; **C.H.N.** Found C: 70.95%, H: 5.07%, N: 2.77%, $C_{51}H_{42}N_2O_{11}$ Requires C: 71.32%, H: 4.93%, N: 3.26%; **δH** (200MHz, $CDCl_3$) 3.53-3.68 (m, 4H, 3', 4', 5'-H), 3.78 (s, 6H, OMe), 4.47 (d, 1H, 2'-H, J = 2.4Hz), 5.37 (d, 1H, 5-H, J = 8.2Hz), 5.92 (d, 1H, 1'-H, J = 2.8Hz), 6.83-7.98 (m, 29H, aromatic, 6-H) ppm; **δC** (63MHz, $CDCl_3$) 55.14 (OMe), 62.33 (C-5'), 72.03 (C-3'), 73.57 (C-2'), 82.47 (C-4'), 85.75 (CAr_3), 87.56 (C-1'), 102.97 (C-5), 113.31-139.79 (aromatic), 143.80 (C-6), 150.09 (C-2), 162.33 (C-4), 165.07, 165.31, 168.10 (C=O) ppm; **m/z** (FAB) (M^+); **HRMS** (FAB) Found 858.27873, $C_{51}H_{42}N_2O_{11}$ Requires 858.27886.

3',2',N^3-tri(benzoyl)-uridine (123).[105]

Compound (**122**) (40mg, 0.47mmol) was dissolved in DCM (2ml). Trifluoroacetic acid (23μL) was added and the reaction stirred for 30 minutes at room temperature. This was diluted with DCM (20ml) and washed with saturated $NaHCO_3$ (20ml) and water (20ml). The organic layer was separated, dried (Na_2SO_4), filtered and reduced *in vacuo*. The residue was purified by wet flash chromatography (1% MeOH/DCM) to give the *title compound* as a white foam (25mg, 96%). **m.p.** 102-5°C; **tlc R_f** (10% MeOH/DCM) 0.59; **δH** (200MHz, DMSO) 3.77 (s, 2H, 5'-H), 4.46 (d, 1H, 2'-H, J = 2.6Hz), 5.53 (s, br, 1H), 5.69-5.80 (m, 3H), 5.37 (d, 1H, 5-H, J = 8.2Hz), 5.92 (d,

1H, 1'-H, J = 2.8Hz), 7.37-8.01 (m, 16H, aromatic, 6-H) ppm; **δC** (63MHz, $CDCl_3$) 61.03 (C-5'), 72.19 (C-3'), 73.64 (C-2'), 83.33 (C-4'), 86.23 (C-1'), 102.75 (C-5), 128.46-134.02 (aromatic), 150.70 (C-2), 163.16 (C-4), 158.68, 164.63, 164.95, 167.25 (C=O) ppm; **m/z** (FAB) 557 (MH^+); **HRMS** (FAB) Found 557.15555, $C_{30}H_{25}N_2O_9$ Requires 557.15600.

Fully Protected Diribonucleotide Derivative (124).

A mixture of compound (**123**) (25mg, 0.045mmol) and 1*H*-tetrazole (10mg, 0.213mmol) was rendered anhydrous by coevaporations three times each with anhydrous toluene and anhydrous pyridine and with anhydrous acetonitrile. The mixture was then taken up in acetonitrile and mixed with (**120**) (63mg, 0.067mmol) under an atmosphere of argon. After stirring at room temperature for 2.5 hours, the mixture was treated with a solution of iodine (55mg, 0.21mmol) in pyridine-water (1ml, 98:2, v/v). The resulting mixture was stirred at room temperature for 40 minutes, and the asturated Na_2SO_3 was added to reduce the excess iodine. The mixture was diluted with DCM and washed two times with saturated $NaHCO_3$ and water. The organic phase was separated, dried (Na_2SO_4), filtered and reduced *in vacuo*. The residue was purified by wet flash chromatography (DCM containing 1% pyridine, v/v/v) to give the *title compound* as a foam (42mg, 66%). **tlc** $\mathbf{R_f}$ (DCM) 0.25; **δH** (200MHz, $CDCl_3$) 1.87-1.96 (m, 2H, CH_2), 2.32-2.36 (m, 2H, CH_2), 2.75-2.85 (m, 2H, CH_2CN), 3.78 (s, 6H, OMe), 3.90 (m, 2H, CH_2), 4.30-4.58 (m, 12H, 2', 3', 4', 5'-H, CH_2), 5.16 (m, 1H), 5.64-6.02 (m, 3H), 6.83-7.98 (m, 35H, aromatic, 6-H) ppm; **δC** (63MHz, $CDCl_3$) 15.43 (CH_2), 27.20, (CH_2), 55.11 (OMe), 58.65 (CH_2), 62.69, 62.84 (C-5'), 63.58 (CH_2), 70.72, 72.42 (C-3'), 73.38 (C-2'), 81.27 (C-2'), 84.07 (C-4'), 87.08, 87.62 (C-1'), 102.56, 102.66 (C-5), 113.01-139.79 (aromatic), 149.33, 150.33 (C-2), 158.71, 158.46, 162.93 (C-4), 165.27, 165.41, 166.84 168.26 (C=O) ppm; **m/z** (FAB) 1418 (M^+); **HRMS** (FAB) Found 1418.42279, $C_{76}H_{69}N_5O_{21}P$ Requires 1418.42242.

3'-O-(Hex-4-ynyl benzoate)-2'-O-(succinate)-5'-O-(dimethoxytrityl) uridine (125).

Compound (**118**) (0.30g, 0.40mmol) was coevaporated in anhydrous pyridine (2x2ml) and then dissolved up in anhydrous pyridine (2ml) under an atmosphere of argon. Succinic anhydride (0.12g, 1.21mmol) and dimethylaminopyridine (25mg, 0.2mmol) were added. After 3 days the pyridine was removed *in vacuo* and the residue coevaporated with toluene (3x5ml). The residue was taken up in DCM and washed with saturated brine, dried (Na_2SO_4), filtered and reduced *in vacuo* to give the *title compound* as a light brown foam (0.31g, 91%).; **tlc** $\mathbf{R_f}$ (10% MeOH/DCM) 0.35; **δH** (200MHz, $CDCl_3$) 1.84-1.90 (m, 2H, CH_2), 2.26-2.35 (m, 2H, CH_2), 2.71 (br, 4H, CH_2CH_2), 3.51 (m, 2H, 5'-H) 3.77 (s, 6H, OMe), 4.18-4.56 (m, 7H, 2xCH_2, 2',3',4'-H), 5.32 (d, 1H, 5-H, J = 8.8Hz), 6.06 (1H, 1'-H), 6.81-7.98 (m, 19H, H-6, aromatic) ppm; **δC** (63MHz, $CDCl_3$) 15.62 (CH_2), 27.50 (CH_2), 28.91 (CH_2), 55.08 (OMe), 58.65 (CH_2), 61.21 (C-5'), 63.44 (CH_2), 73.91, 74.01 (C-2', C-3'), 81.68 (C-4'), 86.58 (CAr_3), 87.37 (C-1'), 102.39 (C-5), 113.01-136.75 (aromatic), 139.95 (C-6), 150.36 (C-2), 163.68 (C-4), 166.36 (benzoate C=O), 170.95, 175.22 (C=O) ppm; **m/z** (FAB) 846 (M^+); **HRMS** (FAB) Found 846.29678, $C_{47}H_{46}N_2O_{13}$ Requires 846.29999.

Derivatization of Long Chain Aminoalkyl Silica.

A mixture of compound (**125**) (0.10g, 0.12mmol), 50mg of long chain aminoalkyl silica (500A° pore size, 110μ particle size), DHC (0.10g, 0.49mmol), DMAP (7mg, 0.06mmol) were mixed thoroughly in anhydrous pyridine (4ml) and triethylamine (0.1ml). After stirring overnight under an atmosphere of argon, a small sample of the support was washed with MeOH followed by DCM and then dried. The dried support (1.9mg) was treated with perchloric acid solution (4ml) (70% $HClO_4$, 51.4ml + MeOH 46ml) and the absorbance measured at 498nm. The loading was calculated to

be 26μmol g^{-1}. The bulk support was washed with pyridine and the residual amino groups capped by treatment with pyridine (5ml), acetic anhydride (0.5ml) and DMAP (50mg) for 30 minutes. The support was then washed with MeOH, DCM and ether before being dried and the final loading determined. Final loading, 16μmol g^{-1}.

2'-O-(Hex-4-ynyl benzoate) uridine (127).

Compound (**119**) (67mg, 0.90mmol) was dissolved in DCM (5ml) to which trifluoroacetic acid (45μl, 0.045ml) was added. After stirring at room temperature for 20 minutes the reaction was diluted with DCM (25ml) and washed with saturated aqueous $NaHCO_3$ then water. The extracts were dried (Na_2SO_4), filtered and reduced *in vacuo* to give a foam. The residue was purified by wet flash chromatography (1-5% MeOH in DCM) to give the *title compound* as a white foam (39mg, 98%). Analysis by HPLC (Vydac reverse phase C_{18}, 250 x 2.6mm, 5μ, A = CH_3CN, B = H_2O, 0.1% TFA; 1ml/min. 0-2min. 0%A, 2-32min. 0-90%; R_t = 20.1 minutes; **tlc** $\mathbf{R_f}$ (10% MeOH/DCM) 0.32; **C.H.N.** Found C: 59.10%, H: 5.60%, N: 4.07%, $C_{22}H_{24}N_2O_8$ Requires C: 59.45%, H: 5.44%, N: 4.30%; **δH** (200MHz, $CDCl_3$) 1.88-2.01 (m, 2H, CH_2), 2.34-2.41 (m, 2H, CH_2), 3.78-4.41 (m, 9H, 2xCH_2, 2',3',4',5'-H), 5.70 (d, 1H, 5-H, J = 8.0Hz), 5.82 (d, 1H, 1'-H, J = 3.8Hz), 7.25-8.04 (m, 6H, H-6, aromatic) ppm; **δC** (63MHz, $CDCl_3$) 15.46 (CH_2), 27.28 (CH_2), 58.72 (CH_2), 61.46 (C-5'), 63.45 (CH_2), 69.12 (C-3'), 75.92 (alkyne), 80.18 (C-2'), 85.13 (C-4'), 86.82 (alkyne), 89.26 (C-1'), 102.32 (C-5), 128.32-133.03 (aromatic), 141.44 (C-6), 150.27 (C-2), 163.35 (C-4), 166.75 (benzoate C=O) ppm; **m/z** (FAB) 444 (M^+); **HRMS** (FAB) Found 445.16012, $C_{22}H_{25}N_2O_8$ Requires 445.15876.

2'-O-(Hex-4-yn-1-ol)uridine (128).

Compound **(127)** (115mg, 0.26mmol) was suspended in ammonia/ethanol (3 : 1) solution (24ml) and sealed in a vial. After 95 hours no starting material remained. The

reaction mixture was cooled to -20°C and the solvent removed *in vacuo*. The residue was washed with DCM to give the title compound as a solid. Analysis by HPLC (Vydac reverse phase C_{18}, 250 x 2.6mm, 5μ, A = CH_3CN, B = H_2O, 0.1% TFA; 1ml/min. 0-2min. 0%A, 2-32min. 0-90%; R_t = 12.7 minutes; **tlc** $\mathbf{R_f}$ (15% MeOH/DCM) 0.26; **δH** (200MHz, DMSO) 1.49-1.70 (m, 2H, CH_2), 2.18-2.24 (m, 2H, CH_2), 3.39-3.44 (m, 2H, CH_2, 5'-H), 3.53-3.66 (m, 2H, CH_2), 3.86-4.31 (m, 5H, 2',3',4'-H, CH_2), 5.67 (d, 1H, 5-H, J = 8.1Hz), 5.88 (d, 1H, 1'-H, J = 5.3Hz), 8.04 (d, 1H, H-6, J = 8.1Hz) ppm; **δC** (63MHz, DMSO) 14.79 (CH_2), 31.47 (CH_2), 57.43 (CH_2), 59.45 (CH_2), 60.76 (C-5'), 68.49 (C-3'), 76.15 (alkyne), 79.64 (C-2'), 85.61 (C-4'), 85.87 (C-1'), 87.09 (alkyne), 102.01 (C-5), 140.61 (C-6), 150.71 (C-2), 163.23 (C-4) ppm; **m/z** (FAB) 341 (MH^+); **HRMS** (FAB) Found 341.13528, $C_{15}H_{21}N_2O_7$ Requires 341.13488.

6-(Tetrahydropyranyloxy)-hex-4-ynyl thiobenzoate (131).

Compound (**101**) (11.40g, 52.6mmol) and dried sodium thiobenzoate (11.13g, 70mmol) in DMF were heated at reflux for 16 hours under an atmosphere of argon. The reaction was cooled, filtered and the solvent removed *in vacuo*. The residue was dissolved in DCM, filtered and then reduced *in vacuo* before being passed through a silica plug (DCM:Hexane, 2:1) to give the *title compound* as an oil (11.90g, 71%). **tlc** $\mathbf{R_f}$ (5% MeOH/DCM) 0.76; $\mathbf{\nu_{max.}}$ 2941 (CH, aromatic), 2221 (carbon-carbon alkyne), 1664 (C=O stretch); **m/z** (FAB) 319 (MH^+); **HRMS** (FAB) Found 319.13668, $C_{18}H_{23}O_3S$ Requires 319.13679.

6-Hydroxy-hex-4-ynyl thiobenzoate (132).

Compound (**131**) (11.90g, 37.4mmol) was dissolved in MeOH (500ml) to which 10% oxalic acid (210ml) was added. This was allowed to stir at room temperature for 24 hours before the MeOH was removed *in vacuo*. Water (200ml) and EtOAc (100ml)

were added and the organic layer separated with EtOAc (2x100ml). The combined extracts were dried ($MgSO_4$), filtered and reduced *in vacuo*. The residue was purified by wet flash chromatography (DCM) to give the *title compound* as a clear oil (4.35g, 50%). **tlc R_f** (5% MeOH/DCM) 0.54; **$\nu_{max.}$** 3408 (OH), 2929 (CH, aromatic), 2224 (carbon-carbon alkyne), 1661 (C=O stretch); **δH** (200MHz, $CDCl_3$) 1.81-1.92 (m, 2H, CH_2), 2.05 (br, 1H, OH), 2.32-2.39 (m, 2H, alkyne-CH_2), 3.16 (t, 2H, CH_2-S, J = 7.2Hz), 4.24 (t, 2H, CH_2-OH, J = 2.2Hz), 7.25-7.97 (m, 5H, aromatic) ppm; **δC** (63MHz, $CDCl_3$) 17.79 (alkyne-CH_2), 27.70 (CH_2), 28.27 (CH_2), 51.06 (HO-CH_2), 79.36 (alkyne), 84.64 (alkyne), 127.03, 128.44, 133.24, 135.64 (aromatic), 191.81 (C=O) ppm; **m/z** (FAB) 235 (MH^+); **HRMS** (FAB) Found 234.07097, $C_{13}H_{14}O_2S$ Requires 234.07302.

6-Iodo-hex-4-ynyl thiobenzoate (133).

Iodine (0.65g, 2.56mmol) was added in one portion to a cooled (0°C) solution of compound (**132**) (0.46g, 1.97mmol), triphenylphosphine (0.62g, 2.37mmol), and imidazole (0.19g, 2.76mmol) in anhydrous ether/acetonitrile (3:1) (80ml). Temperature was maintained at 0°C for 2 hours with stirring. The reaction mixture was poured into water (60ml) and extracted with ether (3x50ml). The combined extracts were washed with saturated aqueous $Na_2S_2O_3$ (100ml) and water (100ml) and dried (Na_2SO_4), filtered and reduced *in vacuo*. The residue was purified by wet flash chromatography (DCM) to give the *title compound* as an oil (0.51g, 76%). **tlc R_f** (DCM) 0.59; **$\nu_{max.}$** 2939 (CH, aromatic), 2225 (carbon-carbon alkyne), 1661 (C=O stretch); **δH** (200MHz, $CDCl_3$) 1.85-1.93 (m, 2H, CH_2), 2.31-2.39 (m, 2H, alkyne-CH_2), 3.15 (t, 2H, CH_2-thiobenzoate, J = 7.1Hz), 3.66 (t, 2H, I-CH_2, J = 2.4Hz), 7.25-7.98 (m, 5H, aromatic) ppm; **δC** (63MHz, $CDCl_3$) -17.38 (CH_2), 18.23 (alkyne-CH_2), 27.80 (CH_2), 28.14 (CH_2), 77.93 (alkyne), 84.92 (alkyne), 127.08, 128.47, 133.24, 136.91 (aromatic), 191.56 (C=O) ppm; **m/z** (FAB) 345 (MH^+); **HRMS** (FAB) Found 344.98232, $C_{13}H_{14}OSI$ Requires 344.98101.

Mixture of 2'-O and 3'-O-(Hex-4-ynyl thiobenzoate) uridine (134).

Compound **(133)** (2.95g, 8.57mmol) was added with vigorous stirring to a suspension of uridine (1.50g, 6.12mmol), dibutyltin oxide (1.83g, 7.35mmol) and tetrabutyl ammonium bromide (2.16g, 6.70mmol) in anhydrous DMF (10ml) under an atmosphere of nitrogen at 60°C. After 20 hours the reaction was filtered, washed with DMF (30ml) and the filtrate reduced *in vacuo*. The residue was purified by wet flash chromatography (1-5% MeOH/DCM) to give the *title compound* as a sticky foam (2.07g, 74%). **tlc R_f** (10% MeOH/DCM) 0.28; **m/z** (FAB) 461 (MH^+); **HRMS** (FAB) Found 461.13672, $C_{22}H_{24}O_7N_2S$ Requires 461.13825.

2'-O and 3'-O-(Hex-4-ynyl thiobenzoate)-5'-O-(dimethoxytrityl) uridine (136 and 135).

Compound (**134**) (1.91g, 4.16mmol) was coevaporated with anhydrous pyridine (2x5ml) then dissolved in anhydrous pyridine (3ml) and cooled to 0°C under an atmosphere of argon. 4,4'-Dimethoxytrityl chloride (1.69g, 4.99mol) was added over a 1 hour period portionwise. The reaction was allowed to warm to room temperature and left to stir overnight. MeOH (2ml) was added and the solvent removed *in vacuo*. The residue was taken up in DCM (40ml) and washed with 5% $NaHCO_3$ solution, dried (Na_2SO_4), filtered and reduced *in vacuo* to give a foam. The residue was purified by wet flash chromatography (1-5% MeOH in DCM containing 1% pyridine, v/v/v) to give the *title compounds* as foams.

3'-O-(Hex-4-ynyl thiobenzoate)-5'-O-(dimethoxytrityl) uridine (135).

(0.62g, 20%). **tlc R_f** (10% MeOH/DCM) 0.44; **C.H.N.** Found C: 67.05%, H: 5.67%, N: 3.89%, $C_{43}H_{42}N_2O_9S$ Requires C: 67.70%, H: 5.55%, N: 3.67%; **δH** (200MHz,

$CDCl_3$) 1.73-1.84 (m, 2H, CH_2), 2.23-2.29 (m, 2H, CH_2), 3.07 (t, 2H, CH_2), 3.43-3.54 (m, 2H, 5'-H) 3.76 (s, 6H, OMe), 3.99-4.46 (m, 5H, CH_2, 2',3',4'-H), 5.34-5.38 (d, 1H, 5-H, J = 8.1Hz), 5.93 (d, 1H, 1'-H, J = 2.1Hz), 6.81-7.92 (m, 19H, H-6, aromatic), 9.80 (br, 1H, N-3) ppm; **δC** (63MHz, $CDCl_3$) 17.80 (CH_2), 27.66 (CH_2), 28.14 (CH_2), 55.05 (OMe), 58.20 (CH_2), 61.62 (C-5'), 73.64 (C-2'), 75.30 (C-3'), 81.26 (C-4'), 86.79 (CAr_3), 89.94 (C-1'), 102.22 (C-5), 113.12-136.74 (aromatic), 139.97 (C-6), 150.57 (C-2), 163.30 (C-4), 191.40 (thiobenzoate C=O) ppm; **m/z** (FAB) 762 (M^+); **HRMS** (FAB) Found 746.25758, $C_{43}H_{42}N_2O_9S$ Requires 762.26011.

2'-O-(Hex-4-ynyl thiobenzoate)-5'-O-(dimethoxytrityl) uridine (136).

(0.96g, 30%). **tlc R_f** (10% MeOH/DCM) 0.59; **C.H.N.** Found C: 67.54%, H: 5.64%, N: 4.19%, $C_{43}H_{42}N_2O_9S$ Requires C: 67.70%, H: 5.55%, N: 3.67%; **δH** (200MHz, $CDCl_3$) 1.83-1.91 (m, 2H, CH_2), 2.31-2.37 (m, 2H, CH_2), 3.15 (t, 2H, CH_2), 3.52-3.53 (m, 2H, 5'-H), 3.77 (s, 6H, OMe), 4.03-4.07 and 4.19-4.22 (m, 2H, 3',4'-H), 4.39-4.55 (m, 3H, CH_2, 2'-H), 5.33 (d, 1H, 5-H, J = 8.1Hz), 5.96 (d, 1H, 1'-H, J = 2.1Hz), 6.79-8.00 (m, 19H, H-6, aromatic), 9.31 (br, 1H, N-3) ppm; **δC** (63MHz, $CDCl_3$) 17.87 (CH_2), 27.66 (CH_2), 28.19 (CH_2), 55.10 (OMe), 58.69 (CH_2), 61.32 (C-5'), 68.47 (C-3'), 76.01 (alkyne), 81.08 (C-2'), 83.21 (C-4'), 86.64 and 86.86 (CAr_3, alkyne), 87.27 (C-1'), 101.94 (C-5), 113.15-136.82 (aromatic), 139.90 (C-6), 149.99 (C-2), 163.16 (C-4), 191.66 (thiobenzoate C=O) ppm; **m/z** (FAB) 762 (M^+); **HRMS** (FAB) Found 762.26427, $C_{43}H_{42}N_2O_9S$ Requires 762.264011.

2'-O-(Hex-4-ynyl thiobenzoate) uridine (137).

Compound (**136**) (150mg, 0.20mmol) was dissolved in DCM (10ml) to which triflouroacetic acid (140μl, 0.14ml) was added. After stirring at room temperature for 30 minutes the reaction was diluted with DCM (25ml) and washed with saturated

aqueous $NaHCO_3$ then water. The extracts were dried (Na_2SO_4), filtered and reduced *in vacuo*. The residue was purified by wet flash chromatography (1-5% MeOH in DCM) to give the *title compound* as a foam (69mg, 76%). Analysis by HPLC (Vydac reverse phase C_{18}, 250 x 2.6mm, 5μ, A = CH_3CN, B = H_2O, 0.1% TFA; 1ml/min. 0-2min. 0%A, 2-32min. 0-90%; R_t = 22.4 minutes; **tlc R_f** (10% MeOH/DCM) 0.24; **δH** (200MHz, $CDCl_3$) 1.79-1.90 (m, 2H, CH_2), 2.29-2.36 (m, 2H, CH_2), 3.10-3.16 (m, 2H, CH_2), 3.78-4.09 (m, 3H, 2',3',4'-H), 4.27-4.46 (m, 4H, CH_2, 5'-H), 5.71 (d, 1H, 5-H, J = 8.1Hz), 5.81 (d, 1H, 1'-H, J = 3.6Hz), 7.25-7.95 (m, 6H, H-6, aromatic), 9.51 (br, 1H, N-3) ppm; **δC** (63MHz, $CDCl_3$) 17.88 (CH_2), 27.75 (CH_2), 28.07 (CH_2), 58.80 (CH_2), 61.39 (C-5'), 68.99 (C-3'), 76.03 (alkyne), 80.21 (C-2'), 85.08 (C-4'), 86.91 (alkyne), 89.38 (C-1'), 102.30 (C-5), 127.08-136.76 (aromatic), 141.50 (C-6), 150.29 (C-2), 163.42 (C-4), 192.17 (thiobenzoate C=O) ppm; **m/z** (FAB) 461 (MH^+); **HRMS** (FAB) Found 461.13972, $C_{22}H_{25}N_2O_7S$ Requires 461.13825.

2'-O-(Hex-4-yn-1-thiol) uridine (138).

Compound **(137)** (24mg, 0.052mmol) was suspended in ammonia/ethanol (3 : 1) solution (18ml) and sealed in a vial. After 72 hours no starting material remained. The reaction mixture was cooled to -20°C and the solvent removed *in vacuo*. The residue was washed with DCM to give the title compound as a solid. Analysis by HPLC (Vydac reverse phase C_{18}, 250 x 2.6mm, 5μ, A = CH_3CN, B = H_2O, 0.1% TFA; 1ml/min. 0-2min. 0%A, 2-32min. 0-90%; R_t = 18.0 minutes; **tlc R_f** (15% MeOH/DCM) 0.37; **δH** (200MHz, $CDCl_3$) 1.95-2.05 (m, 2H, CH_2), 2.56-2.60 (m, 2H, CH_2), 3.02-3.07 (m, 2H, CH_2), 3.78-3.84 (m, 2H, CH_2), 3.94- 4.34 (m, 6H, 2',3',4', 5'-H), 5.53 (d, 1H, 5-H, J = 8.3Hz), 5.71 (d, 1H, 1'-H, J = 4.2Hz), 7.59-7.63 (m, 1H, H-6, J = 8.2Hz), 8.53 (br, 1H, N-3)ppm; **δC** (63MHz, $CDCl_3$) 29.10 (CH_2), 34.21 (CH_2), 37.52 (CH_2), 61.69 (C-5'), 69.16 (C-3'), 69.58 (CH_2), 77.10 (alkyne), 79.68 (C-2'), 85.12 (C-4'), 90.83 (C-1'), 102.34 (C-5), 141.87 (C-6), 150.12 (C-2), 162.68 (C-4); **m/z** (FAB) 357 (MH^+); **HRMS** (FAB) Found 357.11280, $C_{15}H_{21}N_2O_6S$ Requires 357.11065.

6-(Tetrahydropyranyloxy)-hex-4-yne cyanide (147).

NaCN (0.47g, 9.6mmol) was added to a suspension of **101** (1.04g, 4.8mmol) taken in DMSO (15ml) and the reaction stirred for 3.5 hours at 55°C under an atmosphere of nitrogen. Water (40ml) was added and the extracted with ether (2x30ml). The combined extracts were dried (Na_2SO_4), filtered and reduced *in vacuo*. The residue was purified by wet flash chromatography (DCM) to give the *title compound* as a clear oil (0.95g, 96%). **tlc** $\mathbf{R_f}$ (5% MeOH/DCM) 0.73; $\mathbf{\nu_{max.}}$ 2942 (CH), 2245 (CN); **δH** (200MHz, $CDCl_3$) 1.51-1.89 (m, 8H, CH_2), 2.37-2.52 (m, 4H, CH_2), 3.45-3.57 and 3.76-3.89 (m, 2H, CH_2-O), 4.12-4.34 (m, 2H, O-CH_2-alkyne), 4.78-4.80 (m, 1H, CH) ppm; **δC** (63MHz, $CDCl_3$) 15.47 (alkyne-CH_2), 18.38 (CH_2), 23.77 (CH_2), 24.63 (CH_2), 29.57 (CH_2), 31.01 (CH_2), 53.78 (O-CH_2-alkyne), 61.39 (CH_2-O), 77.34 (alkyne), 82.82 (alkyne), 96.45 (CH), 118.54 (CN) ppm; **m/z** (FAB) 208 (MH^+); **HRMS** (FAB) Found 208.13393, $C_{12}H_{18}O_2N$ Requires 208.13375.

7-(Tetrahydropyranyloxy)-hept-5-ynoic acid (148).

Compound (**147**) (33.72g, 0.163mol) in 25% NaOH solution (200ml) and MeOH (350ml) were heated at reflux for 6 hours. The reaction mixture was cooled to 0°C and acidified to pH 4-5 with 2M aq. HCl before being extracted with ethyl acetate. The combined organic extracts were dried (Na_2SO_4), filtered and reduced *in vacuo*. The residue was purified by wet flash chromatography (0-3% MeOH/DCM) to give the *title compound* as a clear oil (25.56g, 69%). **tlc** $\mathbf{R_f}$ (5% MeOH/DCM) 0.38; $\mathbf{\nu_{max.}}$ 3400-3100 (OH), 2942 (CH), 1708 (C=O); **δH** (200MHz, $CDCl_3$) 1.50-1.82 (m, 8H, CH_2), 2.23-2.47 (m, 4H, CH_2), 3.39-3.58 and 3.76-3.90 (m, 2H, CH_2-O), 4.12-4.34 (m, 2H, O-CH_2-alkyne), 4.75-4.77 (m, 1H, CH) ppm; **δC** (63MHz, $CDCl_3$) 16.75 (alkyne-CH_2), 17.59 (CH_2), 22.06 (CH_2), 23.90 (CH_2), 28.81 (CH_2), 31.30 (CH_2), 53.14 (O-CH_2-alkyne), 60.56 (CH_2-O), 75.51 (alkyne), 83.72 (alkyne), 95.34 (CH),

177.73 (C=O) ppm; **m/z** (FAB) 227 (MH^+); **HRMS** (FAB) Found 227.12819, $C_{12}H_{19}O_4$ Requires 227.12833.

Methyl-7-(tetrahydropyranyloxy)-hept-5-ynoate (149).

Compound (**148**) (0.68g, 3.0mmol) was dissolved in anhydrous THF (25ml) and cooled to 0°C before being treated with an ethereal solution of diazomethane. The solvent was removed *in vacuo* to give the *title compound* in quantitative yield (0.72g, 100%). **tlc R_f** (5% MeOH/DCM) 0.69; **$\nu_{max.}$** 2945 (CH), 1738 (C=O); **δH** (200MHz, $CDCl_3$) 1.50-1.86 (m, 8H, CH_2), 2.26-2.33 (m, 2H, alkyne-CH_2), 2.43 (t, 2H, CH_2, J = 7.4Hz), 3.46-3.58 and 3.74-3.88 (m, 2H, CH_2-O), 3.67 (s, 3H, CH_3), 4.12-4.34 (m, 2H, O-CH_2-alkyne), 4.77-4.80 (m, 1H, CH) ppm; **δC** (63MHz, $CDCl_3$) 17.58 (alkyne-CH_2), 18.41 (CH_2), 23.03 (CH_2), 24.67 (CH_2), 29.57 (CH_2), 32.12 (CH_2), 50.88 (CH_3), 53.87 (O-CH_2-alkyne), 61.33 (CH_2-O), 84.57 (alkyne), 96.07 (CH), 173.02 (C=O) ppm; **m/z** (FAB) 241 (MH^+); **HRMS** (FAB) Found 241.14364, $C_{13}H_{21}O_4$ Requires 241.14398.

Methyl-7-hydroxy-hept-5-ynoate (150).

Compound (**149**) (14.43g, 0.060mol) was dissolved in MeOH (350ml). 15% Oxalic acid (300ml) was added and the reaction allowed to stir for 48 hours. The MeOH was removed *in vacuo*, water (200ml) was added and then extracted with EtOAc (3x150ml). The combined organic extracts were dried (Na_2SO_4), filtered and reduced *in vacuo*. The residue was purified by wet flash chromatography (0-5% MeOH/DCM) to give the *title compound* as a clear oil (8.27g, 88%). **tlc R_f** (5% MeOH/DCM) 0.51; **$\nu_{max.}$** 3435 (OH), 2950 (CH), 1735 (C=O); **δH** (200MHz, $CDCl_3$) 1.74-1.84 (m, 2H, CH_2), 2.20-2.42 (m, 5H, alkyne-CH_2, CH_2, OH), 3.62 (s, 3H, CH_3), 4.16-4.19 (m, 2H, O-CH_2-alkyne) ppm; **δC** (63MHz, $CDCl_3$) 16.73 (alkyne-CH_2), 22.24 (CH_2), 31.39 (CH_2), 49.78 (CH_2), 50.21 (CH_3), 77.98 (alkyne), 83.57 (alkyne), 172.43

(C=O) ppm; **m/z** (FAB) 157 (MH^+); **HRMS** (FAB) Found 157.08675, $C_8H_{13}O_3$ Requires 157.08498.

Methyl-7-iodo-hept-5-ynoate (151).

Iodine (12.34g, 48.2mmol) was added in one portion to a cooled (0°C) solution of compound (**150**) (5.92g, 37.9mmol), triphenylphosphine (11.93g, 45.5mmol), and imidazole (3.35g, 49.3mmol) in anhydrous ether/acetonitrile (3:1) (250ml). Temperature was maintained at 0°C for 1.5 hours with stirring. The reaction mixture was poured into water (200ml) and extracted with ether (3x100ml). The combined organic extracts were washed with saturated aqueous $Na_2S_2O_3$ (200ml) and water (100ml) and then dried (Na_2SO_4), filtered and reduced *in vacuo*. The residue was purified by wet flash chromatography (DCM) to give the *title compound* as a clear oil (4.65g, 46%). **tlc** $\mathbf{R_f}$ (DCM) 0.51; $\mathbf{\nu_{max.}}$ 2953 (CH), 1743 (C=O); **δH** (200MHz, $CDCl_3$) 1.72-1.90 (m, 2H, CH_2), 2.21-2.30 (m, 2H, alkyne-CH_2), 2.42 (t, 2H, J = 7.4Hz), 3.67 (s, 3H, CH_3), 3.89-3.91 (m, 2H, I-CH_2) ppm; **δC** (63MHz, $CDCl_3$) 16.75 (alkyne-CH_2), 21.94 (CH_2), 31.30 (CH_2), 50.21 (CH_3), 77.66 (alkyne), 82.46 (alkyne), 172.23 (C=O) ppm; **m/z** (FAB) 267 (MH^+); **HRMS** (FAB) Found 266.98906, $C_8H_{12}O_2I$ Requires 266.98821.

Ethyl-7-(tetrahydropyranyloxy)-hept-5-ynoate (152).

Ethyl chloroformate (4.18g, 38.5mmol) was added to a suspension of (**148**) (8.71g, 38.5mmol), DMAP (1.88g, 15.4mmol), and triethylamine (5.90ml, 42.4mmol) in anhydrous DCM (50ml) cooled to 0°C. The reaction was stirred at 0°C for 40 minutes. The reaction mixture was diluted with DCM (50ml) and washed with saturated aqueous NH_4CL before being extracted with DCM (2x50ml), dried (Na_2SO_4), filtered and reduced *in vacuo* to give an oil. The residue was purified by wet flash chromatography (20-0%Hexane/DCM) to give the *title compound* as a clear

oil (8.52g, 85%). **tlc R_f** (5% MeOH/DCM) 0.64; $\nu_{max.}$ 2942 (CH), 1734 (C=O); **δH** (200MHz, $CDCl_3$) 1.20 (t, 3H, CH_3, J = 7.2Hz), 1.45-1.83 (m, 8H, CH_2), 2.20-2.28 (m, 2H, alkyne-CH_2), 2.36 (t, 2H, CH_2, J = 7.4Hz), 3.43-3.51 and 3.73-3.83 (m, 2H, CH_2-O), 4.19 (q, 2H, O-CH_2, J = 7.1Hz), 4.14-4.27 (m, 2H, O-CH_2-alkyne), 4.72-4.75 (m, 1H, CH) ppm; **δC** (63MHz, $CDCl_3$) 12.65 (CH_3), 18.06 (alkyne-CH_2), 18.90 (CH_2), 23.57 (CH_2), 25.16 (CH_2), 30.07 (CH_2), 32.87 (CH_2), 50.88 (CH_3), 54.30 (O-CH_2-alkyne), 60.08 (O- CH_2), 61.75 (CH_2), 76.50 (alkyne), 85.03 (alkyne), 96.47 (CH), 172.85 (C=O) ppm; **m/z** (FAB) 255 (MH^+); **HRMS** (FAB) Found 255.15942 $C_{14}H_{23}O_4$ Requires 255.15963.

Ethyl-7-hydroxy-hept-5-ynoate (153).

Compound (**152**) (7.47, 29.3mmol) was dissolved in MeOH (210ml). 10% Oxalic acid (150ml) was added and the reaction allowed to stir for 48 hours. The MeOH was removed *in vacuo* then water (150ml) was added and extracted with EtOAc (3x100ml). The combined extracts were dried (Na_2SO_4), filtered and reduced *in vacuo*. The residue was purified by wet flash chromatography (0-5% MeOH/DCM) to give the *title compound* as a clear oil (4.18g, 84%). **tlc R_f** (5% MeOH/DCM) 0.42; $\nu_{max.}$ 3437 (OH), 2951 (CH), 1736 (C=O); **δH** (200MHz, $CDCl_3$) 1.21 (t, 3H, CH_3, J = 7.2Hz), 1.69-1.84 (m, 2H, CH_2), 2.19-2.27 (m, 2H, alkyne- CH_2), 2.38 (t, 2H, CH_2, J = 7.3Hz), 2.43 (br, OH), 4.09 (q, 2H, CH_2, J = 7.2Hz), 4.18 (t, 2H, O-CH_2-alkyne, J = 2.2Hz) ppm; **δC** (63MHz, $CDCl_3$) 13.99 (CH_3), 17.99 (alkyne-CH_2), 23.55 (CH_2), 32.91 (CH_2), 50.92 (HO-CH_2), 51.43 (CH_2), 79.18 (alkyne), 84.67 (alkyne), 173.13 (C=O) ppm; **m/z** (FAB) 171 (MH^+); **HRMS** (FAB) Found 171.10268, $C_9H_{15}O_3$ Requires 171.10212.

Ethyl-7-iodo-hept-5-ynoate (154).

Iodine (7.89, 31.1mmol) was added in one portion to a cooled (0°C) solution of compound (**153**) (4.07g, 23.9mmol), triphenylphosphine (7.52g, 28.7mmol), and imidazole (2.28g, 33.5mmol) in anhydrous ether/acetonitrile (3:1) (166ml). Temperature was maintained at 0°C for 1.5 hours with stirring. The reaction mixture was poured into water (200ml) and extracted with ether (3x150ml). The combined extracts were washed with saturated aqueous $Na_2S_2O_3$ (200ml) and water (100ml) and dried (Na_2SO_4), filtered and reduced *in vacuo*. The residue was purified by wet flash chromatography (DCM) to give the *title compound* as a clear oil (5.81g, 87%). **tlc** $\mathbf{R_f}$ (DCM) 0.51; $\mathbf{\nu_{max.}}$ 2978 (CH), 2227 (alkyne), 1731 (C=O); **δH** (200MHz, $CDCl_3$) 1.24 (t, 3H, CH_3, J = 7.2Hz), 1.75-1.86 (m, 2H, CH_2), 2.21-2.30 (m, 2H, alkyne-CH_2), 2.38 (t, 2H, CH_2, J = 7.4Hz), 3.66-3.68 (m, 2H, I-CH_2), 4.10 (q, 2H, CH_2, J = 6.9Hz) ppm; **δC** (63MHz, $CDCl_3$) 12.80 (CH_3), 17.07 (alkyne-CH_2), 22.12 (CH_2), 31.56 (CH_2), 58.96 (CH_2), 76.52 (alkyne), 83.86 (alkyne), 171.68 (C=O) ppm; **m/z** (APCI) 267 ($M\text{-}H^+$); **HRMS** (FAB) Found 281.00498, $C_9H_{14}O_2I$ Requires 281.003857.

Mixture of 2'-O and 3'-O-(Methyl-hept-5-ynoate) uridine (155).

Uridine (1.09g, 4.4mmol), dibutyltin oxide (1.33g, 5.3mmol) and tetrabutyl ammonium bromide (1.58g, 4..9mmol) were suspended in anhydrous DMF (10ml) under an atmosphere of nitrogen. Compound (**151**) (1.46g, 5.5mmol) was added with vigorous stirring and the reaction mixture heated to 60°C. After 24 hours the reaction was filtered, washed with DMF (20ml) and the filtrate reduced *in vacuo*. The residue was purified by wet flash chromatography (1-5% MeOH/DCM) to give the *title compound* as a off white sticky foam (0.82g, 81%). **tlc** $\mathbf{R_f}$ (15% MeOH/DCM) 0.41; **m/z** (FAB) 383 (MH^+).

2'-O and 3'-O-(Methyl-hept-5-ynoate)-5'-O-(dimethoxytrityl) uridine (157 and 156).

Compound (**155**) (0.81g, 2.11mmol) was coevaporated with anhydrous pyridine (2x5ml) then dissolved in anhydrous pyridine (2ml) and cooled in an ice bath to 0°C under an atmosphere of argon. 4,4'-dimethoxytrityl chloride (0.93g, 2.74mmol) was added over a 1 hour period portionwise. The reaction was allowed to warm to room temperature and left to stir overnight. MeOH (1ml) was added and the solvent removed *in vacuo*. The residue was taken up in DCM (40ml) and washed with 5% $NaHCO_3$ solution, dried (Na_2SO_4), filtered and reduced *in vacuo* to give a foam. The residue was purified by wet flash chromatography (1-5% MeOH in DCM containing 1% pyridine, v/v/v) to give the *title compounds* as foams.

3'-O-(Methyl-hept-5-ynoate)-5'-O-(dimethoxytrityl) uridine (156).

(0.43g, 30%). **tlc** $\mathbf{R_f}$ (10%MeOH/DCM) 0.41; **C.H.N.** Found C: 66.98%, H: 6.00%, N: 4.66%, $C_{38}H_{40}N_2O_{10}$ Requires C: 66.66%, H: 5.89%, N: 4.09%; **δH** (200MHz, $CDCl_3$) 1.75-1.83 (m, 2H, CH_2), 2.21-2.25 (m, 2H, CH_2), 2.36-2.44 (m, 2H, CH_2), 3.51 (s, br, 2H, 5'-H), 3.64 (s, 3H, OMe), 3.77 (s, 6H, 2xOMe), 4.05-4.46 (m, 5H, CH_2, 2',3',4',), 5.29 (d, 1H, 5-H, J = 8.1Hz), 5.97 (d, 1H, 1'-H, J = 2.2Hz), 6.80-7.39 (m, 13H, aromatic), 7.98 (d, 1H, 6-H, J = 8.4Hz), 9.63 (s, br, N-3) ppm; δC (63MHz, $CDCl_3$) 16.79 (CH_2), 22.18 (CH_2), 31.33 (CH_2), 50.24 (OMe), 53.85 (2xOMe), 57.44 (CH_2), 60.11 (C-5'), 67.25 (C-2'), 74.65 (C-3'), 82.05 (C-4'), 85.70 (CAr_3), 85.98 (C-1'), 100.79 (C-5), 111.95-134.01 (aromatic), 138.71 (C-6), 148.90 (C-2), 162.16 (C-4), 172.23 (C=O) ppm; **m/z** (FAB) 684 (M^+); **HRMS** (FAB) Found 684.26683, $C_{38}H_{40}O_{10}N_2$ Requires 684.26830.

2'-O-(Methyl-hept-5-ynoate)-5'-O-(dimethoxytrityl) uridine (157).

(0.33g, 23%). **tlc R_f** (10%MeOH/DCM) 0.62; **C.H.N.** Found C: 66.30%, H: 6.18%, N: 4.58%, $C_{38}H_{40}N_2O_{10}$ Requires C: 66.66%, H: 5.89%, N: 4.09%; **δH** (200MHz, $CDCl_3$) 1.66-1.77 (m, 2H, CH_2), 2.09-2.20 (m, 2H, CH_2), 2.28-2.36 (m, 2H, CH_2), 3.46-3.58 (m, 2H, 5'-H), 3.65 (s, 3H, OMe), 3.78 (s, 6H, 2xOMe), 3.82-4.42 (m, 5H, CH_2, 2',3',4',), 5.21 (d, 1H, 5-H, J = 8.1Hz), 6.16 (d, 1H, 1'-H, J = 7.6Hz), 6.69-7.51 (m, 13H, aromatic), 7.71-7.79 (m, 1H, 6-H)ppm; **δC** (63MHz, $CDCl_3$) 15.45 (CH_2), 27.22 (CH_2), 55.13 (OMe), 58.72 (CH_2), 61.64 (C-5'), 63.42 (CH_2), 69.28 (C-3'), 80.07 (C-2'), 85.24 (C-4'), 86.86 (CAr_3), 89.36 (C-1'), 102.39 (C-5), 113.44-136.25 (aromatic), 140.18 (C-6), 150.08 (C-2), 162.75 (C-4), 166.77 (C=O); **m/z** (FAB) 683 ($M-H^+$); **HRMS** (FAB) Found 683.25912, $C_{38}H_{39}O_{10}N_2$ Requires 683.26047.

2'-O-(Methyl-hept-5-ynoate) uridine (158).

Trifluoroacetic acid (14μl) was added to (**157**) (22mg, 0.03mmol) in DCM (2ml). After stirring at room temperature for 25 minutes the reaction was diluted with DCM (15ml) and washed with saturated aqueous $NaHCO_3$ then water. The extracts were dried (Na_2SO_4), filtered and reduced *in vacuo* to give a foam. The residue was purified by wet flash chromatography (1-5% MeOH in DCM) to give the *title compound* as a foam (7mg, 64%). Analysis by HPLC (Vydac reverse phase C_{18}, 250 x 2.6mm, 5μ, A = CH_3CN, B = H_2O, 0.1% TFA; 1ml/min. 0-2min. 0%A, 2-32min. 0-90%; R_t = 18.1 minutes; **tlc R_f** (10% MeOH/DCM) 0.14; **δH** (200MHz, $CDCl_3$) 1.67-1.80 (m, 2H, CH_2), 2.13-2.21 (m, 2H, CH_2), 2.28-2.40 (m, 2H, CH_2), 3.34-4.41 (m, 7H, CH_2, 2',3',4',5'-H), 3.66 (s, 3H, OMe), 5.61-5.78 (m, 2H, 5-H, 1'-H), 7.72 (d, 1H, 6-H, J = 8.2Hz) ppm; **δC** (63MHz, $CDCl_3$) 15.77 (CH_2), 28.02 (CH_2), 55.21 (OMe), 58.75 (CH_2), 59.92 (C-5'), 63.70 (CH_2), 68.02 (C-3'), 81.54 (C-2'), 85.10 (C-4'), 89.37 (C-1'), 102.29 (C-5), 140.23 (C-6), 149.88 (C-2), 162.51 (C-4), 166.29

(C=O) ppm; **m/z** (FAB) 383 (MH^+); **HRMS** (FAB) Found 382.13976, $C_{17}H_{22}N_2O_8$ Requires 382.13762.

Mixture of 2'-O and 3'-O-(Ethyl-hept-5-ynoate) uridine (159).

Uridine (2.74g, 11.2mmol), dibutyltin oxide (3.36, 13.5mmol) and tetrabutyl ammonium bromide (3.99g, 12.4mmol) were suspended in anhydrous DMF (24ml) under an atmosphere of nitrogen. Compound (**154**) (4.72g, 17.0mmol) was added with vigorous stirring and the reaction mixture heated to 60°C. After 24 hours the reaction was filtered, washed with DMF (20ml) and the filtrate reduced *in vacuo*. The residue was purified by wet flash chromatography (1-5% MeOH/DCM) to give the *title compound* as a sticky foam (3.59g, 80%). **tlc R_f** (15% MeOH/DCM) 0.40; **m/z** (FAB) (M^+); **HRMS** (FAB) Found 396.15510, $C_{18}H_{24}O_8N_2$ Requires 396.15327.

2'-O and 3'-O-(Ethyl-hept-5-ynoate)-5'-O-(dimethoxytrityl) uridine (161 and 160).

Compound (**159**) (1.87g, 4.7mmol) was coevaporated with anhydrous pyridine (2x5ml) then dissolved in anhydrous pyridine (2ml) and cooled in an ice bath to 0°C under an atmosphere of argon. 4,4'-dimethoxytrityl chloride (1.92, 5.7mmol) was added over a 1 hour period portionwise. The reaction was allowed to warm to room temperature and left to stir overnight. MeOH (1ml) was added and the solvent removed *in vacuo*. The residue was taken up in DCM (40ml) and washed with 5% $NaHCO_3$ solution, dried (Na_2SO_4), filtered and reduced *in vacuo* to give a foam. The residue was purified by wet flash chromatography (1-5% MeOH in DCM containing 1% pyridine, v/v/v) to give the *title compounds* as foams.

3'-O-(Ethyl-Hept-5-ynoate)-5'-O-(dimethoxytrityl) uridine (160).

(0.27g, 8%). **tlc R_f** (10%MeOH/DCM) 0.40; **C.H.N.** Found C: 67.16%, H: 6.19%, N: 4.29%, $C_{39}H_{40}N_2O_{10}$ Requires C: 67.04%, H: 6.06%, N: 4.01%; **δH** (200MHz, $CDCl_3$) 1.22 (t, 3H, CH_3, J = 7.2Hz), 1.66-1.77 (m, 2H, CH_2), 2.09-2.14 (m, 2H, CH_2), 2.27-2.37 (m, 2H, CH_2), 3.51-3.52 (m, 2H, 5'-H), 3.76 (s, 6H, OMe), 4.05-4.46 (m, 7H, O-CH_2, CH_2, 2',3',4',), 5.28 (d, 1H, 5-H, J = 8.0Hz), 5.95 (d, 1H, 1'-H, J = 1.9Hz), 6.80-7.36 (m, 13H, aromatic), 7.97 (d, 1H, 6-H, J = 8.0Hz), 9.78 (br, 1H, N-3) ppm; **δC** (63MHz, $CDCl_3$) 12.80 (CH_3), 16.81 (CH_2), 22.34 (CH_2), 31.65 (CH_2), 53.85 (OMe), 57.46 (CH_2), 59.07 (CH_2), 60.08 (C-5'), 67.22 (C-2'), 74.62 (C-3'), 82.00 (C-4'), 85.70 (CAr_3), 85.99 (C-1'), 100.78 (C-5), 111.95-134.76 (aromatic), 138.74 (C-6), 148.95 (C-2), 162.30 (C-4), 171.82 (C=O) ppm; **m/z** (FAB) 697 ($M\text{-}H^+$); **HRMS** (FAB) Found 697.27582, $C_{39}H_{41}O_{10}N_2$ Requires 697.27612.

2'-O-(Ethyl-Hept-5-ynoate)-5'-O-(dimethoxytrityl) uridine (161).

(1.20g, 36%). **tlc R_f** (10%MeOH/DCM) 0.64; **C.H.N.** Found C: 67.00%, H: 6.03%, N: 4.17%, $C_{39}H_{42}N_2O_{10}$ Requires C: 67.04%, H: 6.06%, N: 4.01%; **δH** (200MHz, $CDCl_3$) 1.22 (t, 3H, CH_3, J = 7.2Hz), 1.66-1.77 (m, 2H, CH_2), 2.09-2.14 (m, 2H, CH_2), 2.27-2.37 (m, 2H, CH_2), 3.53-3.63 (m, 2H, 5'-H), 3.76 (s, 6H, OMe), 3.82-4.42 (m, 7H, O-CH_2, CH_2, 2',3',4',), 5.16 (d, 1H, 5-H, J = 8.0Hz), 6.15 (d, 1H, 1'-H, J = 4.3Hz), 6.70-7.76 (m, 14H, 6-H, aromatic) ppm; **δC** (63MHz, $CDCl_3$) 14.02 (CH_3), 17.95 (CH_2), 23.48 (CH_2), 32.85 (CH_2), 55.04 (OMe), 57.97 (CH_2), 60.16 (CH_2), 63.76 (C-5'), 71.32 (C-3'), 75.58 (C-2'), 82.52 (C-4'), 86.65 (CAr_3), 86.74 (C-1'), 102.39 (C-5), 112.98-135.80 (aromatic), 140.06 (C-6), 150.48 (C-2), 163.20 (C-4), 172.82 (C=O) ppm; **m/z** (FAB) 697 ($M\text{-}H^+$); **HRMS** (FAB) Found 697.27497 $C_{39}H_{41}N_2O_{10}$ Requires 697.27612.

2'-O-(Ethyl-hept-5-ynoate) uridine (162).

Trifluoroacetic acid (0.086ml) was added to (**161**) (132mg, 0.19mmol) in DCM (4ml). After stirring at room temperature for 25 minutes the reaction mixture was diluted with DCM (25ml) and washed with saturated aqueous $NaHCO_3$ then water. The extracts were dried (Na_2SO_4), filtered and reduced *in vacuo* to give a foam. The residue was purified by wet flash chromatography (1-5% MeOH in DCM) to give the *title compound* as a white foam (62mg, 83%). Analysis by HPLC (Vydac reverse phase C_{18}, 250 x 2.6mm, 5μ, A = CH_3CN, B = H_2O, 0.1% TFA; 1ml/min. 0-2min. 0%A, 2-32min. 0-90%; R_t = 18.2 minutes; **tlc R_f** (10% MeOH/DCM) 0.13; **δH** (200MHz, $CDCl_3$) 1.25 (t, 3H, CH_3, J = 7.2Hz), 1.76-1.85 (m, 2H, CH_2), 2.21-2.32 (m, 2H, CH_2), 2.35-2.44 (m, 2H, CH_2), 3.21-4.48 (m, 9H, O-CH_2, CH_2, 2',3',4',5'-H), 5.68-5.77 (m, 2H, 5-H, 1'-H), 7.66 (d, 1H, 6-H, J = 8.1Hz)ppm; **δC** (63MHz, $CDCl_3$) 12.77 (CH_3), 16.76 (CH_2), 22.21 (CH_2), 31.73 (CH_2), 57.41 (CH_2), 59.25 (CH_2), 60.31 (C-5'), 67.91 (C-3'), 78.61 (C-2'), 81.68 (C-4'), 85.84 (C-1'), 101.19 (C-5), 140.61 (C-6), 149.27 (C-2), 162.04 (C-4), 172.02 (C=O); **m/z** (APCI) 397 (MH^+); **HRMS** (FAB) Found 396.15439, $C_{18}H_{24}N_2O_8$ Requires 396.15327.

(Hex-4-ynyl benzoate)-1-phosphodichloridite (164).

Compound **103** (916mg, 4.20mmol) was added drop-wise to a cooled (0°C) 2M solution of phosphorous trichloride in DCM (2.10ml, 4.20mmol) and allowed to stir for 2 hours. Analysis by ^{31}P NMR indicated no presence of PCl_3. The solution was removed in *vacuo* to give the title compound without any purification. **δP** (200MHz, $CDCl_3$) 178.7.

N,N-Diisopropyl-(hex-4-ynyl benzoate)-1-phosphonamidyl chloride (165).

N,N-Diisopropylamine (440mg, 4.3mmol) was added drop-wise to a stirred solution of **164** in anhydrous ether cooled to 0°C. After 2 hours the reaction was filtered, washed and reduced in *vacuo* and used without purification in the synthesis of the phosphoramidite **167**.

N^4-Benzoyl Cytidine.[105]

Cytidine (0.20g, 0.8mmol) was dissolved in anhydrous pyridine (10ml) under an atmosphere of argon. Trimethylsilyl chloride (0.37ml, 2.9mmol) was added at room temperature and allowed to stir for 1 hour. Benzoyl chloride (0.15ml, 1.2mmol) was added dropwise at 0°C and the reaction allowed to stir for 4 hours. MeOH (5ml) and water (5ml) were added. The solvents and hydrolysed silyl derivatives were removed *in vacuo*. The residue was then treated with ice cold water. The solid residue obtained was washed with hexane-ethyl acetate (1:1) mixture and then dried *in vacuo* giving the title compound (232mg, 80%). **m.p.** 229-234°C; **tlc** $\mathbf{R_f}$ (10% MeOH/DCM) 0.07; **C.H.N.** Found C: 55.17%, H: 5.00%, N: 12.04%, $C_{16}H_{17}N_3O_8$ Requires C: 55.33%, H: 4.93%, N: 12.10%; **δH** (200MHz, DMSO) 3.59-4.04 (m, 5H, 2',3',4',5'-H), 5.06 (d, 1H, 5'-OH, J = 5.6Hz), 5.19 (t, 1H, 3'-OH, J = 5.0Hz), 5.51 (t, 1H, 2'-OH), 5.81 (d, 1H, 1'-H, J = 2.8Hz), 7.34 (d, 1H, 5-H, J = 5.0Hz), 7.48-8.01 (m, 5H, aromatic), 8.49 (d, 1H, 6-H, J = 7.2Hz), 11.22 (s, 1H, 4-N) ppm; **δC** (63MHz, DMSO) 60.04 (C-5'), 68.78 (C-3'), 74.68 (C-2'), 84.36 (C-4'), 90.36 (C-1'), 96.11 (C-5), 128.57, 132.86, 133.24 (aromatic), 145.48 (C-6), 154.78 (C-2), 163.18 (C-4), 167.46 (benzoyl C=O) ppm; **m/z** (FAB) 348 (MH^+); **HRMS** (FAB) Found 348.11943, $C_{16}H_{18}N_3O_8$ Requires 348.11956.

5'-O-Dimethoxytrityl- (N^4-Benzoyl) cytidine (166).[105]

N^4-Benzoyl cytidine (0.80g, 2.3mmol) was suspended in anhydrous pyridine (5ml) under an atmosphere of nitrogen. 4,4'-Dimethoxytrityl chloride (0.86g, 2.5mmol) was added portionwise over a period of 1 hour and then left to stir for 3.75 hours. EtOH (5ml) was added to quench the reaction and the solvent reduced *in vacuo*. The residue was taken up in DCM (30ml) and washed with cold saturated $NaHCO_3$ then saturated NaCl sol. before being dried (Na_2SO_4), filtered and reduced *in vacuo*. The residue was purified by wet flash chromatography (1-5% MeOH in DCM containing 1% pyridine, v/v/v) to give the *title compound* as a white foam (624mg, 42%). **m.p.** 77-78°C; **tlc R_f** (10% MeOH/DCM) 0.34; **C.H.N.** Found C: 68.03%, H: 5.67%, N: 7.10%, $C_{37}H_{35}N_3O_8$ Requires C: 68.40%, H: 5.43%, N: 6.47%; **δH** (200MHz, DMSO) 3.37 (s, 2H, 5'-H), 3.75 (s, 6H, OMe), 4.07-4.24 (m, 3H, 2',3',4'-H), 5.21 (d, 1H, 3'-OH, J = 7.0Hz), 5.76 (d, 1H, 5-H, J = 4.7Hz), 5.84 (d, 1H, 1'-H, J = 1.4Hz), 6.90-7.79 (m, 18H, aromatic), 8.41 (d, 1H, 6-H, J = 7.5Hz), 11.31 (s, 1H, 4-N) ppm; **δC** (63MHz, DMSO) 55.13 (OMe), 62.05 (C-5'), 68.66 (C-3'), 74.59 (C-2'), 86.15 (C-4'), 86.15 (CAr_3), 91.20 (C-1'), 96.21 (C-5), 113.44-136.25 (aromatic), 144.55 (C-6), 154.49 (C-2), 163.18 (C-4), 167.46 (benzoyl C=O) ppm; **m/z** (FAB) 650 (MH^+); **HRMS** (FAB) Found 650.24954, $C_{37}H_{36}N_3O_8$ Requires 650.25062.

5'-O-4,4'-Dimethoxytrityl-(N^4-Benzoyl)-cytidine-3'-O-[N,N-diisopropyl-(hex-4-ynyl benzoate)-1-phosphoramidite] (167).

A solution of **166** (321mg, 0.05mmol) in anhydrous DCM was added to a solution of **165** in the presence of *N,N*-diisopropylethylamine (0.35ml, 1.65mmol) and allowed to stir overnight under an atmosphere of argon. MeOH (1ml) then EtOAc (10ml) were added and then washed with saturated aqueous $NaHCO_3$ and saturated NaCl solutions before being dried (Na_2SO_4), filtered and reduced *in vacuo*. The residue was purified by wet flash chromatography (0-2% MeOH/DCM containing 1% pyridine) and then taken up in 1ml of dry toluene and precipitated in petroleum ether under vigorous

stirring (150ml, cooled to -65°C) to give the *title compound* as a white foam (0.28g, 72%). **tlc R_f** (5% MeOH/DCM) 0.72; **δH** (200MHz, $CDCl_3$) 0.98-1.28 (m, 12H, CH_3), 1.93-1.96 (m, 2H, CH_2), 2.31-2.42 (m, 2H, CH_2), 2.60-2.67 (CH_2CN), 3.38-3.68 (m, 6H, OCH_2, CH, 5'-H), 3.77 (s, 6H, OMe), 3.80-4.42 (m, 7H, 2xCH_2, 2',3',4'), 5.25 (d, 1H, 5-H, J = 8.7Hz), 5.98 (d, 1H, 1'-H, J = 2.9Hz), 6.79-8.03 (m, 19H, H-6, aromatic) ppm; **δC** (63MHz, $CDCl_3$) 15.57 (CH_2), 27.22 (CH_2), 55.13 (OMe), 58.72 (CH_2), 61.64 (C-5'), 63.42 (CH_2), 69.28 (C-3'), 80.07 (C-2'), 85.24 (C-4'), 86.82 (CAr_3), 87.32 (C-1'), 101.79 (C-5), 113.04-135.08 (aromatic), 117.50 (CN), 139.93 (C-6), 149.82 (C-2), 162.65 (C-4), 166.31 (benzoate C=O) ppm; **δP** (200MHz, $CDCl_3$) 140.51 ppm; **m/z** (FAB) 982 (MH^+); **HRMS** (FAB) Found 982.41590, $C_{56}H_{61}N_4O_{10}P$ Requires 982.41374.

Bis-(4-methylphenyl)-4-(10-(17-tetrabenzo[a,c,g,I]fluorenyl)-decyloxy) phenyl methanol (173).

A solution of 4-bromotoluene (0.55g, 3.24mmol) in anhydrous THF (10ml) was slowly added to magnesium turnings (83mg, 3.4mmol) and a crystal of iodine. After stirring under an atmosphere of nitrogen for 30 minutes the reaction mixture was heated to reflux for 1 hour. The reaction mixture was cooled to room temperature and a solution of ethyl-4-(10-(17-tetrabenzo[a,c,g,I]fluorenyl)decyclooxy)benzoate (0.20g, 0.23mmol) in THF was added dropwise. After 3 hours heating at reflux the mixture was cooled and poured into saturated aqueous ammonium chloride (20ml). The product was extracted with DCM, dried ($MgSO_4$) and reduced *in vacuo* to give a light brown oil. The residue was purified by wet flash chromatography (DCM) and crystallised in DCM/Hexane, to give the *title compound* as a beige crystalline solid (125mg, 52%). **tlc R_f** (DCM) 0.40; **C.H.N.** Found C: 89.36%, H: 7.40% $C_{60}H_{56}O_2$ Requires C: 89.07%, H: 6.98%; **$\nu_{max.}$** 3542 (OH), 2927, 2854 (CH stretch); **δH** (200MHz, $CDCl_3$) 0.33-1.64 (m, 16H, CH_2), 2.33 (s, 6H, CH_3), 2.58-2.66 (m, 2H, -$OCH_2\underline{CH_2}$-), 3.83 (t, 2H, -$O\underline{CH_2}CH_2$-, J = 6.6Hz), 5.07 (t, 1H, CH, J = 4.3Hz), 6.75-6.80 (m, 2H, aromatic), 7.07-7.17 (m, 10H, aromatic, Tbf, toluene), 7.59-7.74 (m,

8H, aromatic, Tbf), 8.25-8.29 (m, 2H, aromatic), 8.67-8.83 (m, 6H, aromatic, Tbf) ppm; δC (63MHz, $CDCl_3$) 20.90 (2xCH_3), 22.01, 25.72, 28.62, 28.80, 28.94, 28.99, 29.04, 29.25 (CH_2), 47.16 (allylic C), 67.75 (OCH_2), 81.33 (quat. -OH), 113.50 (aromatic), 123.41, 124.37, 124.92, 125.53, 125.78, 126.70, 127.37 (aromatic, Tbf), 127.61, 127.95, 128.41, 128.69, 129.34, 131.16, 136.56, 136.78, 139.09, 144.27, 144.29, 158.01 (aromatic) ppm; λ_{max}/nm (ε/ $dm^3mol^{-1}cm^{-1}$) 238 (48889), 254 (54444), 288 (26667), 301 (29444), 365 (12778), 381 (12222); m/z (FAB) 809 (M^+).

3.3. Deprotection and Stability Studies

3.3.1. Stage 1: Ammonia Cleavage Conditions

The conditions used for the first stage of the deprotection of the proposed 2'-hydroxyl protecting groups, where those required for the removal of the phosphate protecting groups and cleavage of the completed chain from the solid support. The 2'-O-protected uridine monomers were treated with freshly prepared 33% aqueous ammonia solution in ethanol (3 : 1) at room temperature in a screw -capped vial.

Base Hydrolysis of 2'-O-(Hex-4-ynyl benzoate) uridine (127) and 2'-O-(Hex-4-ynyl thiobenzoate) uridine (137).

HPLC data obtained (Vydac reverse phase C_{18}, 250 x 2.6mm, 5μ, A = CH_3CN, B = H_2O, 0.1% TFA; 1ml/min., 0-2min. 0%A, 2-32min. 0-90%A);

COMPOUND	RETENTION TIME (MINUTES)
2'-O-(Hex-4-ynyl benzoate) uridine (127).	**20.1**
2'-O-(Hex-4-yn-1-ol) uridine (128).	**12.7**
2'-O-(Hex-4-ynyl thiobenzoate) uridine (137).	**22.4**
2'-O-(Hex-4-yn-1-thiol) uridine (138).	**18.0**

Base Hydrolysis of 2'-O-(Methyl-hept-5-ynoate) uridine (158).

Compound **(158)** (2mg) was suspended in ammonia/ethanol (3 : 1) solution (2ml) and sealed in a vial. After 72 hours no starting material remained. The reaction mixture was cooled to -20°C and the solvent removed *in vacuo*. The residue was dissolved in 2ml of water and analysed by HPLC. Several peaks were observed on the HPLC trace and showed approximately 25% deprotection to the uridine.

COMPOUND	RETENTION TIME (MINUTES)
3'-O-(Methyl-hept-5-ynoate) uridine (158).	**18.1**
2'-O-(Hept-5-ynoic acid) uridine	**12.8,13.2,14.6,14.8**
Uridine	**4.4**

Base Hydrolysis of 2'-O-(Ethyl-hept-5-ynoate)uridine (162).

Compound **(162)** (24mg, 0.052mmol) was suspended in ammonia/ethanol (3 : 1) solution (18ml) and sealed in a vial. After 72 hours no starting material remained. The reaction mixture was cooled to -20°C and the solvent removed *in vacuo*. The residue was analysed be HPLC. Several peaks were observed on the HPLC trace and showed approximately 25% deprotection to the uridine. Mass spec. indicated the presence of the carboxylic acid. **m/z** (FAB) 368 (M^+); **HRMS** (FAB) Found 368.12193, $C_{16}H_{20}N_2O_8$ Requires 368.12197.

COMPOUND	RETENTION TIME (MINUTES)
2'-O-(Ethyl-hept-5-ynoate) uridine (162).	**18.2**
2'-O-(Hept-5-ynoic acid) uridine.	**12.8,13.2,14.6,14.8**
Uridine	**4.4**

3.3.2. Stage 2: Acid cleavage

The deprotection of 2'-O-(Hex-4-yn-1-ol) uridine (**128**) and 2'-O-(hex-4-yn-1-thiol) uridine (**138**) were studied over the pH range 1 to 3 using $0.1dm^{-1}$ (pH 1.1), $0.01dm^{-1}$ (pH 2.0) hydrochloric acid, and a buffer solution of 50mM ammonium formate (pH 3.1). The uridine monomer (1mg) was dissolved in the appropriate aqueous acid or buffer solution, and monitored by HPLC. The deprotection studies of 2'-O-(2-hexynthiol) uridine (**138**) were carried out under an atmosphere of argon. See Sections 2.5. (Figure 52) and 2.6.2. of the discussion.

4.0. References

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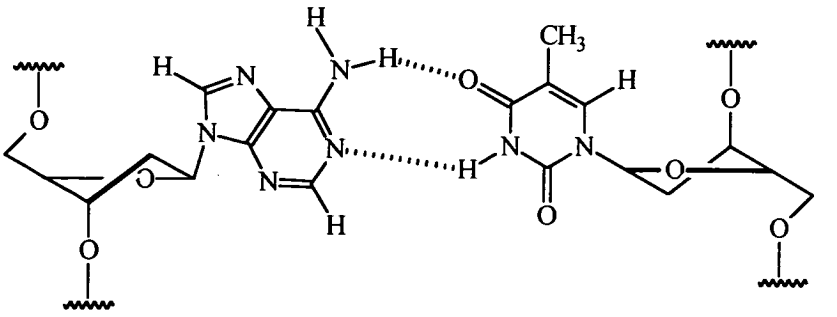
5.0. Branched Oligodeoxynucleotides (ODNs) : Automated Synthesis and Triple Helical Hybridization Studies

5.1.Introduction

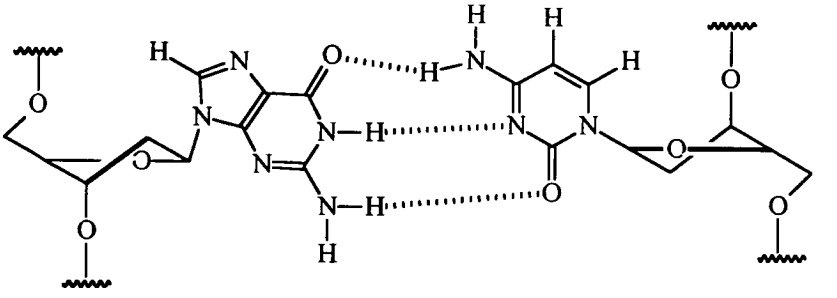
5.1.1. Antisense Oligonucleotides

In the search for new drugs it is still necessary to synthesise and test, on average, 10,000 compounds in order to discover a new active substance that is considered to be worth developing. Generally the active substance is directed against proteins such as enzymes, receptors or ion channels of which the structure and mode of action are usually very complex and often incompletely understood. If the active substance could be directed at the level of the nucleic acid, a number of advantages would be gained.

At present there are a number of drugs on the market whose activity is based on direct interaction with deoxyribonucleic acid (DNA).[3] These are mainly used for chemotherapy in which the compound interchelates or binds specifically to DNA. However, drugs like Cisplatin are unable to use the specificity that is available in the sequence information in the nucleic acids of particular genes. Sequence specific recognition of nucleic acids can be obtained using synthetic oligonucleotides. These small, synthetic oligonucleotides, resembling single stranded DNA are called **antisense** oligonucleotides. These bind through hybridization to coding sequences (sense strand) in a specific messenger RNA (mRNA) target by Watson-Crick base pairing (Figure 1). Adenosine (A) and thymidine (T) or guanosine (G) and cytidine (C) interact through hydrogen bonding. These simple base pairing rules govern the interaction between the antisense ODNs and the cellular RNA allowing the design of ODNs to target any gene of a known sequence giving great specificity of action.



adenine (A-T) thymine



guanine (G-C) cytosine

Figure 1. Base Pairs of DNA

Due to this specificity, antisense ODNs have been classified as a major class of new pharmaceuticals, stimulating huge interest and increased activity in this area of research.

Statistically, a small ODN of 15 nucleotides in length has the specificity capable to inhibit the expression of a single target gene through complementary hybridisation with a cellular messenger ribonucleic acid (mRNA).[1] It was Zamecnik and Stephenson who first reported in 1978 the use of synthetic antisense oligonucleotides for therapeutic purposes.[2] They showed the inhibition of Rous sarcoma viral replication with the addition of a 13-mer nucleotide that was complementary to its RNA.

The synthesised antisense single strand ODN can attack the complementary sense strand in 3 different ways. It can form a triple helix with a DNA target molecule, a

duplex with RNA target molecules or a complex of protein and oligodeoxynucleotides. This is represented schematically in figure 2.

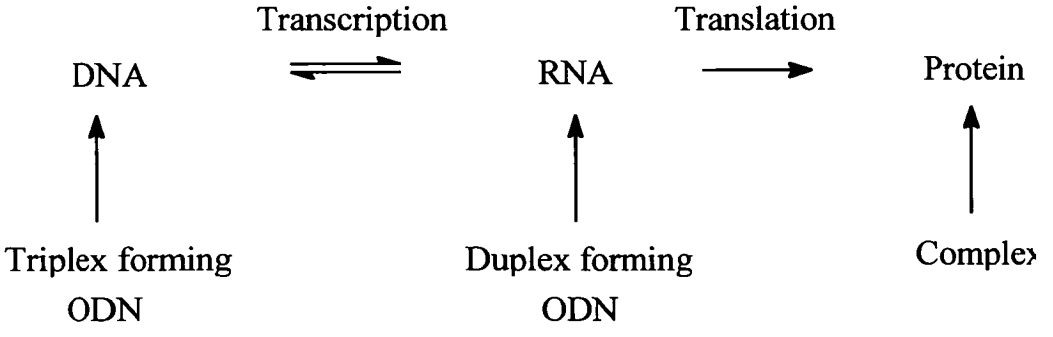


Figure 2. Mechanism of attack

For the antisense oligonucleotide to be able to inhibit transcription or translation it must reach the interior of the cell unaltered. First, the ODN must be able to cross the cellular membrane to reach the cytoplasm or nucleus. When the ODN is in the cell it must be resistant to degradation and be able to bind specifically, and with high affinity to the target sequence in order to inhibit expression of the disease causing gene. Figure 3 shows a simple representation of this process.

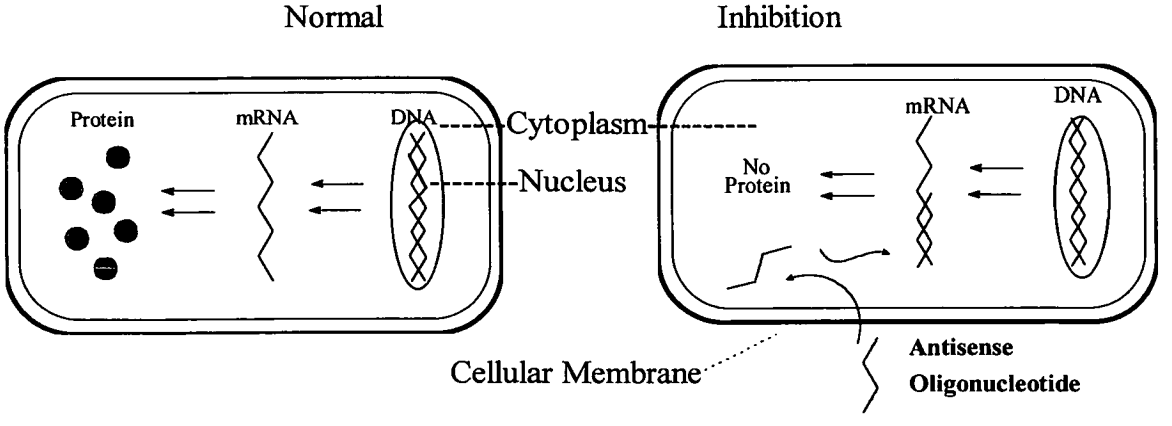


Figure3. Action of antisense oligonucleotides

In order to satisfy these requirements to reach the target sequence unaltered, ODNs have to be chemically modified. Modifications in the sugar moiety, phosphodiester bond and at the phosphorus atom have been extensively studied and reviewed.[3,4,5,6]

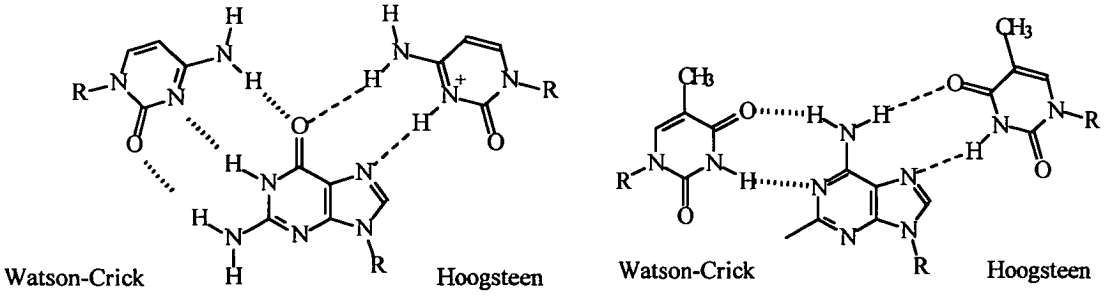
Enhanced resistance towards nucleases has often been achieved, however, a decrease in the stability of the resulting duplexes has frequently been observed.

5.1.2. Triplex Formation

Oligonucleotides can interact with double-stranded DNA to form triple-stranded complexes. RNA and DNA triple-stranded complexes were first recognised by Felsenfeld *et al* in 1957.[7] The formation of a stable 2:1 complex was described between two strands of polyuridylic acid and one strand of polyadenylic acid in the presence of $MgCl_2$. Subsequently, triple-stranded complexes containing other combinations of polynucleotide strands were described and reviewed.[8,9,10,11]

Site specific binding of an oligonucleotide to the major groove of a DNA duplex *via* intermolecular triple helix formation was demonstrated by Moser and Dervan in 1987.[12] This study, along with others,[13] demonstrated a new strategy for recognition of specific double-stranded DNA sequences with far reaching implications in the field of genetics, biochemistry, and medicine.

At least two classes of DNA triplexes have been characterised based on specific, Hoogsteen[14] or reversed Hoogsteen type hydrogen bonding interactions, between the bases in the homopurine strand of a Watson-Crick duplex and an additional oligonucleotide strand (Figure 4).



C-G-C$^+$ **T-A-T**

Figure 4. Triple Helix Formation : Watson-Crick and Hoogsteen Base Pairing

In the pyrimidine-purine-pyrimidine triplexes, a pyrimidine rich third strand is aligned in parallel with the purine strand of the Watson-Crick duplex to form T-AT and C^+-GC base triple combinations (Figure 4). In the purine-purine-pyrimidine triplexes, a purine rich third strand is aligned anti-parallel to the purine strand, yielding G-GC, A-AT and T-AT base triple combinations.

5.1.3. Branched Oligodeoxynucleotides

The targeting of single stranded nucleic acids by bi-molecular triplex formation has previously been addressed by use of back folding linear DNA[15] and circular oligonucleotides.[16,17,18] Entropic reasons, increased affinities and selectivities for such bimolecular complexes led to an extension of this approach. Branched ODNs were synthesised and studied as possible probes for high affinity targeting of complementary ODNs with the rationale being that full duplex formation (Watson-Crick hydrogen bonds) between the branched DNA segment and the target is followed by alignment of the branch in the major groove of the duplex, thus forming a triple helix (Hoogsteen hydrogen bonds).[19] Figure 5 shows a schematic representation of the structure formed between the branched oligodeoxynucleotide and the target complementary strand. The majority of research in the field of synthetic branched nucleic acids has been focused on synthesising, investigating and mimicking the branching moiety of lariat-RNA occurring in eukaryotic mRNA splicing.[20,21,22,23,24,25] Synthesis of branched oligonucleotides has been performed largely by use of rather laborious solution phase methods.[20-24] However, there have been accounts of solid phase synthesis as well.[25] The hybridisation results obtained by Azhayeva *et al.*[26] employing 3'-deoxypsicothymidine (structure **A**, Figure 6) as the branching monomer were rather disappointing. It was thought that the unnatural structure of the branching point nucleoside and destabilisation caused by the presence

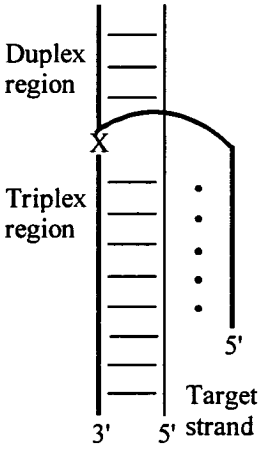


Figure 5.

of the branch resulted in substantial decreases in melting points compared with linear controls.

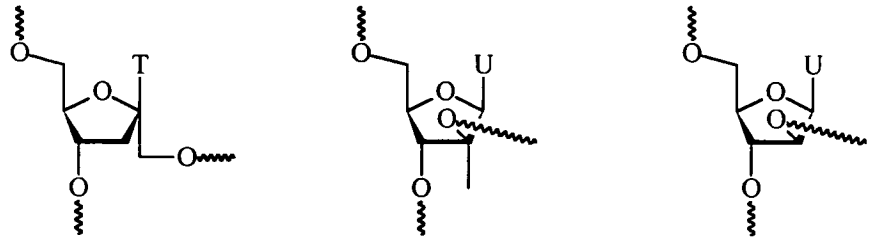


Figure 6. Branching monomers

Previously Wengel *et al*[27,28] used 1-(2-methyl-β-D-aribinofuranosyl)-uracil (structure **B**, Figure 6) as a branching monomer. A fully automated synthetic strategy was described for the preparation of novel mono-branched ("Y-shaped") and double branched ("H-shaped") ODN analogues with sequences of arbitrary length and base composition. Increased thermal stabilities compared to linear references containing one modified monomer (structure **B**, 2'-O-unbranched, Figure 6), due to the formation of short triple helical complexes, was reported.[27,28] However, compared to unmodified linear ODNs, the branched analogues, containing the modified monomer, exhibited lower melting temperatures (11-14°C). This was thought to be caused by disruption of the normal Watson-Crick duplex structure upon incorporation of the modified monomer.

A more favourable branching point geometry seemed necessary to achieve thermal stabilities comparable or even higher than those of unmodified controls. Previously it had been shown that the destabilising effect of incorporating one 2'-O-methylarabinonucleoside into an 11-mer or a 17-mer was a decrease in melting points of 1-6°C per modification[29] which was lower than the decrease observed for the 1-(2-methyl-β-D-aribinofuranosyl)-uracil monomer (structure **B**, Figure 6). It was, thus, decided to synthesise and evaluate branched ODNs using *arabino*-uridine [1-(β-D-aribinofuranosyl)uracil] as the branching monomer (structure **C**, Figure 6). This

utilises the 2'-hydroxyl group which orientates itself into the major groove as an attachment site for the branch.

5.2. Results and Discussion

5.2.1. Synthesis of 1-(5',2'-O-(4,4'-Dimethoxytrityl)-β-D-arabinofuranosyl) uracil

Initially, 1-(5',2'-O-(4,4'-Dimethoxytrityl)-β-D-arabinofuranosyl) uracil (**4**) was chosen as the branching monomer for the synthesis of the branched ODNs. Incorporation of the 4,4'-dimethoxytrityl protecting group at 2'-hydroxyl position, as well as at the 5'-hydroxyl, would allow for the same deprotection, activation/coupling and capping methodologies employed in ODN synthesis using the established phosphoramidite chemistry. It would, therefore, be unnecessary to deprotect from off the solid support before branching. From the branching monomer identical branches would, therefore, be synthesised allowing a model study for the possible formation of antiparallel triplexes.

The phosphoramidite of 1-(5',2'-O-(4,4'-dimethoxytrityl)-β-D-arabinofuranosyl) uracil was synthesised in 5 steps (Figure 7). *Arabino*-uridine was initially protected at the 5' and 3' hydroxyl positions using Markiewicz reagent[30] (1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane) in anhydrous pyridine giving 1-[5',3'-O-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-β-D-arabinofuranosyl] uracil (**1**) in a 93% yield. 4,4'-Dimethoxytritylation of compound **1** using dimethoxytrityl chloride and DMAP in anhydrous triethylamine gave compound **2** in 52%. Desilylation using potassium fluoride dihydrate with 18-crown-6-ether in tetrahyrofuran (THF) gave compound (**3**) in 89% yield.[31] This was followed by selective protection of the 5'-hydroxyl functionality by reaction with 4,4'-dimethoxytrityl chloride (DMT-Cl) and silver

nitrate in anhydrous pyridine affording 1-(5',2'-O-(4,4'-dimethoxytrityl)-β-D-arabinofuranosyl) uracil (**4**) in 65% yield.

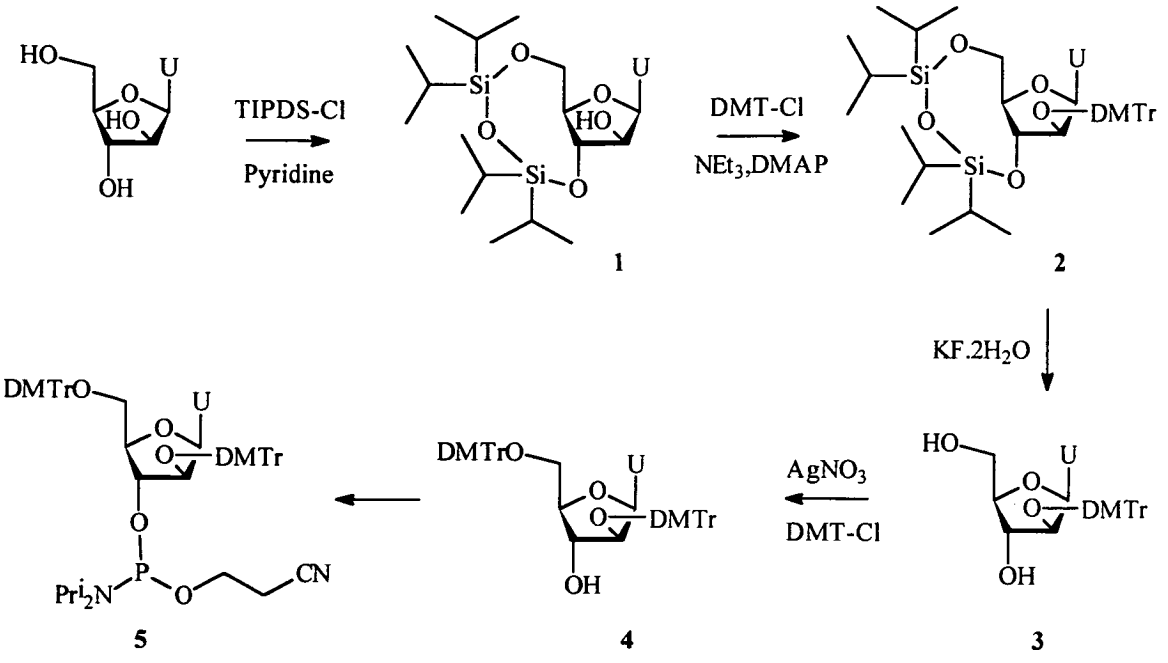


Figure 7. 1-(5',2'-O-(4,4'-Dimethoxytrityl)-β-D-arabinofuranosyl) uracil

Phosphitylation of (**4**) was carried out in anhydrous dichloromethane (DCM) with N,N-diisopropylethylamine and 2-cyanoethyl-N,N-diisopropylethylaminochloro-phosphoramidite. The phosphoramidite (**5**) was purified by wet flash chromatography and precipitation from anhydrous toluene in petroleum ether cooled to -65°C giving a yield of 40%. The branching monomer was then ready to be incorporated into ODNs.

5.2.2. Synthesis of Branched ODNs

The synthesis *via* phosphoramidites according to Caruthers[32] (Section 1.6.4.), originally introduced by Letsinger[33] as the phosphite triester method, is currently the most efficient method for preparing ODNs. It entails the 5'-hydroxyl group of the growing DNA chain being reacted with a nucleoside 3'-β-cyanoethyl N,N-diisopropyl phosphoramidite with catalysis by 1H-tetrazole. The resulting phosphite triester is then immediately oxidised with iodine to the phosphotriester (see Chapter 1).

The ODNs were synthesised using standard phosporamidite chemistry[34] employing the usual cycles. The base sequences and lengths of the ODNs corresponded to previous research that had been carried out by Wengel *et al.*[28]

Base Sequences of ODNs	
A	5'-T T T T T-$_{5'}$X$_{3'}$-T T T T T T-3'
	5'-T T T T T $_{2'}$
B	5'-T T T-$_{5'}$X$_{3'}$-T T T T T T T T-3'
	5'-T T T $_{2'}$
C	5'-T T T T T T T-$_{5'}$X$_{3'}$-T T T T T T-3'
	5'-T T T T T T T $_{2'}$
D	5'-C C T C C-$_{5'}$X$_{3'}$-T T T T T T-3'
	5'-C C T C C $_{2'}$
E	5'-C T C C T C C-$_{5'}$X$_{3'}$-T T T T T T-3'
	5'-C T C C T C C $_{2'}$
F	5'-A A A A A A A G G A G G A G-3'
G	5'-A A A A A A A G G A G G-3'
H	5'-A A A A A A A A A A A A A A-3'
I	5'-A A A A A A A A A A A A A-3'
A = 2'deoxyadenosine, C = 2'deoxycytidine, T = thymidine, X = branching monomer (*arabino*-uridine).	

Figure 8. Sequences Synthesised.

A fully automated strategy using phophoramidite chemistry for the synthesis of the novel branched oligonucleotides **A-E** and complementary strands **F-I** was devised.

Using a Pharmacia Gene Assembler Special synthesiser the synthesis was controlled by use of different concentrations and coupling times of amidites commencing from the incorporated branching monomer. For the initial sequence to the branching monomer commercial amidites were used in 0.1M concentration with 2 minute coupling times. The modified branching monomer (X) was then incorporated using a 0.3M concentration with a 24 minute coupling time. To circumvent the sterical hindrance at the unprotected 2'-hydroxyl position and the need to synthesise two strands at the branching step, the concentrations of the amidites were trebled to 0.3M and the coupling times increased to 12 minutes. This procedure gave coupling efficiencies of amidite (**5**) (Figure 7) from 60% to 80%, as VIS-spectrometrically determined by the release of the DMT-cation during detritylation. The branching step gave an increased coupling yield to that of (**5**) since the release of DMT-cation is expected to double due to the two symmetrical branches. The DMT group of the last nucleotide incorporated was retained as a purification aid for all the ODNs.

The DMT protected ODNs were cleaved from the solid support by incubation in concentrated aqueous ammonia at room temperature for 3 days. Subsequent purification using disposable reverse-phase cartridges afforded the pure oligomers. Since all modified ODNs were left with the final DMT group on, they were separated from all the truncated sequences which were capped during the syntheses and, therefore, lacked the DMT handle.

The composition of the branched ODNs was confirmed by matrix assisted laser desorption mass spectrometry (Figure 9) using internal PNA calibrants.[35] The masses of ODNs **A** and **D** were experimentally found to be within 0.06% of the calculated values.

Oligodeoxynucleotide	Calculated Mass	Mass Found
A	**5110.33**	**5113.44**
D	**4990.24**	**4992.84**

Figure 9. MALDI-mass spectrometry

5.2.3. Hybridization Studies

The thermal stabilities of the novel branched ODNs were compared to their corresponding linear analogues in hybridization experiments. The melting point samples were run using 2.5μM of each participating oligonucleotide in a 10 mM Tris-HCl pH 7 (at 25°C), 10mM $MgCl_2$, 100mM NaCl buffer solution. The mixtures were heated to 80°C for 10 minutes, allowed to cool to room temperature for 30 minutes and then kept between 0-5°C for at least 1 hour prior to measurement. The melting curves were acquired on a Perkin-Elmer Lambda 2 UV/VIS spectrometer equipped with a temperature controlling system (consisting of a Peltier Temperature Programmer PTP-6 and a Heto constant temperature cooling cycle). Absorption at 260nm and 284nm were measured while the temperature was raised from 10 to 80°C with a linear rise of 1°C min^{-1}. The melting points were determined, using the PECSS software provided by Perkin-Elmer, as the maximum of the first derivative of the melting curve.

The ODNs were designed to orient the additional branches antiparallel to the complementary dA_{12} strand which should allow formation of antiparallel triple helices. The length of the branches were varied to evaluate the effect of the length. The strand dissociation's were detected through hyperchromicities at 260nm and 284nm, the latter being characteristic for triple-helix dissociation. Figure 10 shows the derivative of a UV melting curve at 260nm, of branched oligomer **A** with the complementary strand **I** (dA_{12}). The measured melting point (T_m) was 32°C, which is a drop of 4°C compared with unmodified controls (dT_{12} with dA_{12}). At 284nm no transition was detected, therefore, no triple-helix formation had been achieved. Similar T_m results were obtained in the other branched ODNs with their complementary strands.

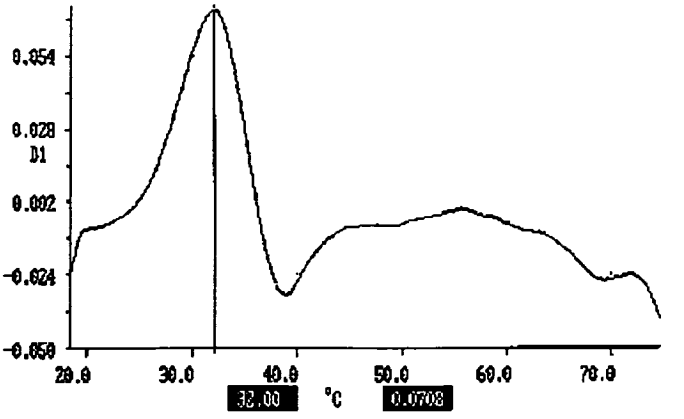
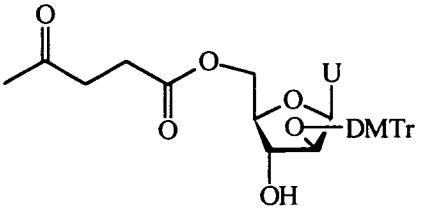


Figure 10. Derivative of a UV Melting Curve at 260nm

The length of the additional branches were thought to be too short to allow the correct orientation to generate triplex formation. The decrease in T_m compared to the corresponding linear reference was attributed to disruption of the Watson-Crick duplex structure caused by the incorporation of the branching monomer as well as unfavourable steric interactions in the branching point. For example between the nucleobase and the 2'-phosphate group. It was, therefore, decided to synthesise and further evaluate, branched ODNs using *arabino*-uridine but with non-identical and extended branched strands. Incorporation of non-identical strands would allow the possibility of further triplexes being formed. This could be achieved by replacement of either of the dimethoxytrityl groups, of (5',2'-O-(4,4'-Dimethoxytrityl)-β-D-arabinofuranosyl) uracil (**4**), with levulinyl (Figure 11).



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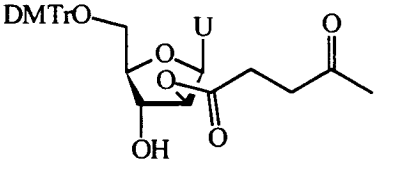

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Figure 11. Branching Monomers

The levulinyl protecting group is removed by treating the support with 0.5M hydrazine hydrate in pyridine-acetic acid (4:1, v/v) for 90 minutes, thereby, freeing a 5' or 2'-hydroxyl for the preparation of branching.[36]

5.2.4. Future Work

5.2.4.1. Synthesis of Modified Branching Monomers

Synthesis of 1[2'-O-(4,4'-dimethoxytrityl)-5'-O- levulinyl-β-D-arabinofuranosyl] uracil (**6**) was achieved by selective levulination of the primary hydroxyl group of nucleoside **3** using the enzyme Novozym 435® in anhydrous dioxan in a yield of 64%.

Similarly, nucleoside (**1**) was levulinated using levulinic anhydride and DMAP and anhydrous pyridine to give 1[2-O-levulinyl-3,5-O-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-β-D-arabinofuranosyl] uracil (**8**) in 65% yield.

Due to time constraints, the synthesis of the branching monomers were not continued by myself. However, work by Meldgaard *et al*[36] discussed the synthesis of the 5' and 2'-levulinyl monomers. Desilylation using tetrabutylammonium fluoride, followed by dimethoxytritylation of the 5'-hydroxyl using standard procedures gave 1[5-O-(4,4'-dimethoxytrityl)-2-O- levulinyl-β-D-arabinofuranosyl] uracil (**7**) (Figure 12).

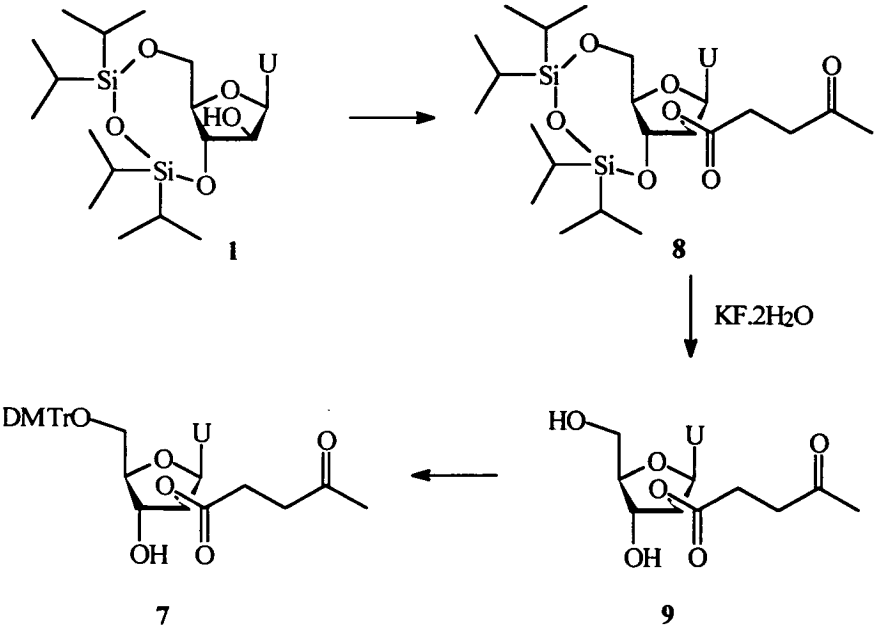

Figure 12. Synthesis of 1[5-O-(4,4'-dimethoxytrityl)-2-O- levulinyl-β-D-arabinofuranosyl]uracil (7)

5.2.4.2. Synthesis of Branched Oligoribonucleotides

Meldgaard *et al*[36] incorporated the branching monomers **6** and **7** in the synthesis of looped and branched ODNs using similar protocols that have been described in this chapter. The synthetic strategy allowed the synthesis of branched ODNs with sequences of arbitrary length and base composition. The melting results for the looped and branched ODNs showed that eight nucleotides in the additional branch were sufficient to achieve a stable triplex structure. However, compared with the corresponding unmodified linear reference, no significant increase in T_m could achieved. It was suggested that the use of arabinonucleosides containing short, non-phosphorus 2'-O-linkers would relieve the disruption to the Watson-Crick duplex structure by steric interactions at the branching point.

5.3. Experimental

5.3.1. Notes

NMR spectra were obtained on a Brucker AC250 spectrometer at 250 MHz for ^{1}H NMR, 63 MHz for ^{13}C NMR and 101 MHz for ^{31}P NMR spectroscopy. δ-Values are reported in ppm relative to internal $SiMe_4$ for ^{1}H and ^{13}C and relative to external 85% H_3PO_4 for ^{31}P spectra. Fast-atom bombardment mass spectrometry (FAB-MS) was performed on a Kratos MS 50 RF spectrometer. The enzyme Novozym 435® is commercially available from Novo Nordisk A/S, Bagsvaerd, Denmark. Unmodified amidites were purchased from Cruachem. Matrix-assisted laser desorption mass spectrometry was performed using Micromass TofSpec E mass spectrometer.

5.3.2. Synthetic Procedures

1-(5',3'-1,1,3,3-Tetraisopropyldisiloxane-β-D-arabinofuranosyl) uracil (1).

1-(β-D-Arabinofuranosyl)uracil (2.0g, 8.19mmol) was dissolved in anhydrous pyridine (15ml) under an atmosphere of nitrogen. This was cooled on an ice bath while 1,1,3,3-tetraisopropyldisiloxane (3.00ml, 8.8mmol) was added over a 5min. period. After 3 hours no starting material remained and the reaction was quenched with methanol (10ml) and the solvent then removed *in vacuo*. The residue was taken up in DCM (50ml) and washed with 5% $NaHCO_3$ solution (50ml). The organic phase was dried (Na_2SO_4), filtered and evaporated *in vacuo*. The residue was then purified by wet flash chromatography (1-5% MeOH in CH_2Cl_2, v/v) to give the *title compound* as a white foam (3.72g, 93%). **m.p.** 187-8°C; **tlc** R_f (10% MeOH/DCM) 0.47; **C.H.N.** Found C: 51.92%, H: 8.10%, N: 5.41%, $C_{21}H_{38}N_2O_7Si_2$ Requires C: 51.57%, H: 7.78%, N: 5.73%; **δH** (250MHz, DMSO) 0.96 - 1.07 (m, 28H, $CH(CH_3)_2$), 3.69 - 3.72 (m, 2H, 5'-H), 3.90 - 4.13 (m, 2H, 3'-H, 4'-H), 4.27 - 4.35 (m, 1H, 2'-H), 5.85 (d, 1H, 1'-H, $J_{1'-2'}$ = 5.79Hz), 6.02 (d, 1H, 5-H, J_{5-6} = 6.37Hz), 7.46 (d, 1H, 6-H, J_{6-5} = 8.1Hz), 11.28 (s, 1H, 3-H) ppm; δC (63MHz, DMSO) 11.87 - 17.16 (12C, TIPDS), 60.77 (C-5'), 74.39 (C-2'), 75.07 (C-3'), 79.23 (C-1'), 82.46 (C-4'), 100.32 (C-5), 141.19 (C-6), 150.32 (C-2), 162.91 (C-4) ppm; **m/z** (EI) 443 (M^+ - ^iPr).

1-[2'-O-(4,4'-Dimethoxytrityl)-5',3'-O-1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-β-D-arabinofuranosyl] uracil (2).

Method A:

Compound **(1)** (1.8g,3.68mmol) was dissolved in triethylamine (15ml) under an atmosphere of argon. Dimethylaminopyridine (0.022g, 0.184mmol) and 5equivalents of 4,4'-dimethoxytrityl chloride (6.234g, 18.4mmol,) were then added. This was allowed to stir under an atmosphere of argon for 24 hours at room temperature. The reaction was quenched with methanol (5ml) and reduced *in vacuo*. The residue was

taken up in DCM and washed with 5% $NaHCO_3$ solution (50ml). The extracts were dried (Na_2SO_4), filtered and evaporated *in vacuo*. The residue was purified by wet flash chromatography (1-5% MeOH in CH_2Cl_2 containing 1% pyridine, v/v/v) to give the *title compound* as a white foam (1.52g, 52%).

Method B:

Compound **(1)** (0.72g, 1.47mmol) was dissolved in anhydrous pyridine (5ml) under an atmosphere of argon. Silver nitrate (1.25g, 7.35mmol, 5 equivalents) then 4,4'-dimethoxytrityl chloride (2.49g, 7.35mmol, 5equivalents) were then added. After 36 hours the reaction was quenched with methanol (5ml) and filtered. This was then washed with 5% $NaHCO_3$ solution (50ml) and then extracted with DCM. The extracts were dried (Na_2SO_4), filtered and evaporated *in vacuo*. The residue was the purified by wet flash chromatography (1-5% MeOH in CH_2Cl_2 containing 1% pyridine, v/v/v) to give the *title compound* as a white foam (1.52g, 1.9mmol, 52%).

m.p. 94-7°C; **tlc** R_f (10% MeOH/DCM) 0.71; **C.H.N.** Found C: 63.55%, H: 7.35%, N: 3.52%, $C_{42}H_{56}N_2O_9Si_2$ Requires C: 63.72%, H: 7.08%, N: 3.54%; **δH** (250MHz, DMSO) 0.96 - 1.07 (m, 28H, $CH(CH_3)$), 3.65 (m, 5'-H), 3.70-3.75 (s, 6H, OCH_3), 3.83-4.14 (m, 2H, 3'-H, 4'-H), 4.63-4.71 (m, 1H, 2'-H), 5.31 (d, 1H, 1'-H), 5.50 (d, 1H, 5-H, $J_{5\text{-}6}$ = 8.1Hz), 6.76-7.40 (m, aromatic, 6-H), 11.04 (s, 1H, 3-H) ppm; **δC** (63MHz, DMSO) 12.14-17.09 (12C, TIPDS), 54.87 (OCH_3), 60.77 (C-5'), 75.73 (C-2'), 76.98 (C-3'), 78.74 (C-1'), 87.30 (C-4'), 100.42 (C-5), 113.04-135.95 (aromatic), 144.42 (C-6), 149.74 (C-2), 162.60 (C-4) ppm; **m/z** (FAB) 811 (M^+ + Na)

1-[2'-O-(4,4'-Dimethoxytrityl)-β-D-arabinofuranosyl] uracil (3).

Compound **(2)** (1.52g, 1.90mmol) was dissolved in THF (30ml). Potassium fluoride dihydrate (1.07g, 11.4mmol)) and 18-crown-6-ether (0.207g, 0.57mmol) were added to a vigorously stirred solution at room temperature. After 48 hours the reaction was filtered and the solids washed with THF. The filtrate was reduced *in vacuo* and the

residue purified wet flash chromatography (1-5% MeOH in CH_2Cl_2 containing 1% pyridine, v/v/v) to give the *title compound* as a white foam (0.927g, 1.70mmol, 89%). **m.p.** 69-70°C; **tlc** R_f (10% MeOH/DCM) 0.35; **C.H.N.** Found C: 66.16%, H: 5.74%, N: 4.98%, $C_{30}H_{30}N_2O_8$ Requires C: 65.87%, H: 5.49%, N: 5.12%; **δH** (250MHz, DMSO) 3.22 (s, 1H, 4'-H);3.41-3.46 (m. 1H, 3'-H); 3.58-3.60 (m, 2H, 5'-H); 3.73 (s, 6H, OCH_3); 3.84-3.91 (m, 1H, 2'-H), 5.69 (d, 1H, 5-H, J_{5-6} = 8.1Hz), 6.03 (d, 1H, 1'-H), 6.85-7.42 (m, 13H, aromatic), 11.52 (s, 1H, 3-H) ppm; **δC** (63MHz, DMSO) 54.90 (OCH_3), 66.44 (C-5'), 74.71 (C-3'), 76.03 (C-2'), 83.59 (CAr_3), 84.57 (C-1'), 87.04 (C-4'), 100.34 (C-5), 113.02-135.95 (aromatic), 144.63 (C-6), 149.48 (C-2), 162.60 (C-4) ppm; **m/z** (FAB) 547 (MH^+).

1-[5',2'-O-Di(4,4'-dimethoxytrityl)-β-D-arabinofuranosyl] uracil (4).

Compound **(3)** (0.29g, 0.53mmol) was dissolved in anhydrous pyridine (0.5ml). $AgNO_3$ (0.09g, 0.53mmol) and 4,4'-dimethoxytrityl chloride (0.18g,0.53mmol) were added under an atmosphere of argon. After 24 hours the reaction mixture was filtered and washed with 5% $NaHCO_3$ solution (25ml) then extracted with DCM. The extracts were dried (Na_2SO_4), filtered and evaporated *in vacuo*. The residue was the purified by wet flash chromatography (1-5% MeOH in CH_2Cl_2 containing 1% pyridine, v/v/v) to give the *title compound* as a white foam (0.291g, 0.34mmol, 65%). **m.p.** 104-5°C; **tlc** R_f (10% MeOH/DCM) 0.69; **C.H.N.** Found C: 72.63%, H: 6.20%, N: 3.85%, $C_{51}H_{48}N_2O_{10}$ Requires C: 72.09%, H: 5.65%, N: 3.30%; **δH** (250MHz, $CDCl_3$) 3.19-3.23 (m, 2H, 5'-H), 3.36-3.43 (m. 1H, 4'-H), 3.55(m, 1H, 3'-H), 3.74 (m, 12H, OCH_3), 4.22-4.24 (m, 1H, 2'-H), 5.65 (d, 1H, 5-H, J_{5-6} = 8.1Hz), 6.19 (d, 1H, 1'-H, J = 2.5Hz), 6.73-7.32 (m, 27H, 6-H, aromatic) ppm; **δC** (63MHz, $CDCl_3$) 55.22 (OCH_3), 63.17 (C-5'), 78.55 (C-2'), 81.96 (C-3'), 84.27 (CAr_3), 86.34 (C-1'), 88.28 (C-4'), 101.28 (C-5), 113.16-135.98 (aromatic), 144.63 (C-6), 149.72 (C-2), 162.98 (C-4) ppm; **m/z** (FAB) 849 (M^+).

1-{3'-O-[2-Cyanoethoxy(diisopropylamino)phosphino]-5',2'-O-di(4,4'-dimethoxytrityl)-β-D-arabinofuranosyl} uracil (5).

Compound **(4)** (0.200g, 0.236mmol) was coevaporated with anhydrous CH_3CN and dissolved in anhydrous CH_2Cl_2 (2ml) under an atmosphere of argon. N,N-diisopropylethylamine (0.9ml) was added dropwise followed by 2-cyanoethyl-N,N-diisopropylaminochlorophosphoramidite (0.136ml, 0.779mmol). After 60 minutes 0.5 equivalents of reagents were then added and left for a further 30 minutes. Methanol (1ml) was added to quench the reaction. EtOAc (6ml) was added and the reaction mixture was washed with saturated $NaHCO_3$ (3x25ml) and NaCl (3 x 25ml). The organic phase was dried (Na_2SO_4), filtered and reduced *in vacuo*. The residue was purified by wet flash chromatography (30-50% ethyl acetate-cyclohexane, 1% pyridine) which was taken up in 1ml of dry toluene and precipitated in petroleum ether with vigorous stirring (150ml, cooled to -65°C) to give the *title compound* as a white foam (100mg, 0.095mmol, 40%). **tlc R_f** (5% MeOH/DCM) 0.49; **δP** (250 MHz, $CDCl_3$) 151.2 and 151.3 ppm.

1-[5'-O-Levulinyl-2'-O-(4,4'-dimethoxytrityl)-β-D-arabinofuranosyl] uracil (6).

Compound **(3)** (100mg, 0.183mmol) was dissolved in anhydrous dioxane (2ml) under an atmosphere of argon. The Novazym 435® (1g) and freshly prepared levulinic anhydride (1.76ml,2mmol) were added and the reaction left to stir overnight. After 24hours the enzyme was filtered off and the reaction washed with 5% $NaHCO_3$ and extracted with EtOAc. The extracts were dried ($NaSO_4$), filtered and reduced *in vacuo*. The residue was then purified by wet flash chomatography (1-5% MeOH in CH_2Cl_2 containing 1% pyridine, v/v/v) to give the *title compound* as a foam (0.075g, 64%). **tlc R_f** (10% MeOH/DCM) 0.68; **δH** (250MHz, DMSO) 2.12 (s, 3H, CH_3), 2.46-2.74 (m, 4H, CH_2CH_2), 3.12 (s, 1H, 3'-OH), 3.75 (s, 6H, OCH_3), 4.00-4.11 (m, 2H, 3'-H and 4'-H), 4.29-4.36 (m, 1H, 2'H), 5.29 (d, 1H,), 5.69 (d, 1H, 5-H, J = 7.6), 5.90 (s, 1H, 1'-H), 6.83-6.91 (m, 4H, ArH), 7.12-7.40 (m,9H, ArH), 7.86 (s,

1H, 6-H), 8.58 (s, 1H, 3-H) ppm; δC (63MHz, DMSO) 27.45 and 29.42 (CH_2CH_2), 37.27 (CH_3), 54.97 (OCH_3), 63.13 (C-5'), 74.27 and 76.78 (C-2' and C-3'), 80.84, 83.83 and 87.46 (C-1' and C-4', Car_3), 100.58 (C-5), 113.21-136.00 (aryl), 142.78 (C-6), 150.08 (C-2), 158.31 (aryl), 162.89 (C-4), 172.08 (C=O) and 206.62 (C=O) ppm; **m/z** (FAB) 644 (M^+).

1-(2'-O-levulinyl-5',3'-1,1,3,3-tetraisopropyldisiloxane-β-D-arabinofuranosyl) uracil (8).

Compound **(1)** (0.7g, 1.43mmol) was dissolved in anhydrous pyridine (5ml). DMAP(5%) and 3 equivalents of freshly prepared levulinic anhydride in dioxane were added and was left to stir under an atmosphere of argon overnight. Methanol (5ml) was added and the reaction reduced *in vacuo*. The residue was taken up in EtOAc and washed with 5% $NaHCO_3$ The extracts were dried ($NaSO_4$), filtered and reduced *in vacuo*. The residue was then purified by wet flash chomatography (1-5% MeOH in CH_2Cl_2) to give the *title compound* as a foam (0.504g, 65%). **tlc** R_f (10% MeOH/DCM) 0.72; **δH** (250 MHz, $CDCl_3$) 0.99-1.19 (m, 28H, $CH(CH_3)_2$), 2.17 (s, 3H CH_3), 2.36-2.78 (m, 4H, CH_2CH_2), 3.82 (m, 1H, 4'-H), 3.98-4.19 (m, 2H, 5'-H), 4.44 (t, 1H, 3'-H, J = 8.2), 5.55 (dd, 1H, 2'-H, J = 6.3 and 7.9), 5.7 (d, 1H, 5-H, J = 8.1), 6.2 (d, 1H, 1'-H, J = 6.2), 7.6 (d, 1H, 6-H, J = 8.2), 9.1 (s, 1H, 3-H) ppm; **δC** (63MHz, $CDCl_3$) 12.23-17.30 (TIPDS), 27.30 (CH_3), 29.54 (CH_2), 37.44 (CH_2), 60.22 (C-5'), 71.18 (C-3'), 75.83 (C-2'), 80.56 and 81.64 (C-1', C-4'), 101.75 (C-5), 140.15 (C-6), 149.87 (C-2), 163.07 (C-4), 171.18 (C=O), 205.60 (C=O) ppm; **m/z** (FAB) 585 (MH^+).

1-[5'-O-(4,4'-Dimethoxytrityl)-β-D-arabinofuranosyl] uracil.

1-(β-D-Arabinofuranosyl)uracil) (0.5g, 2.05mmol) was dissolved in anhydrous pyridine (2ml) under an atmosphere of argon. $AgNO_3$ (0.382g, 2.26mmol) then 4,4'-

dimethoxytrityl chloride (0.76g, 2.26mmol) were added and the reaction left to stir overnight. The reaction was quenched with methanol (5ml) and then filtered. The filtrate was washed with 5% $NaHCO_3$ and extracted with DCM (2 x 30ml). The extracts were dried (Na_2SO_4), filtered and reduced *in vacuo*. The residue was then purified by wet flash chomatography (1-5% MeOH in CH_2Cl_2 containing 1% pyridine, v/v/v) to give the *title compound* as a white foam (0.567g, 1.04mmol, 51%). **m.p.** °C; **tlc** $\mathbf{R_f}$ (10% MeOH/DCM) 0.35; **C.H.N.** Found C: 66.22%, H: 5.81%, N: 5.07%, $C_{30}H_{30}N_2O_8$ Requires C: 65.87%, H: 5.49%, N: 5.12%; **δH** (250 MHz, DMSO) 3.21-3.29 (m, 2H, 5'-H), 3.74 (s, 6H, OCH_3), 3.87-3.91 (m. 2H, 3'-H, 4'-H), 4.01 (m, 1H, 2'-H), 5.39 (d, 1H, 5-H, $J_{5\text{-}6}$ = 8.1Hz), 6.04 (d, 1H, 1'-H, J = 4.5Hz), 6.88-7.47 (m, 13H, aromatic), 8.56 (d, 2H, 6-H, J = 7.6), 11.27 (s, 1H, 3-H); **δC** (250 MHz,DMSO) 54.94 (OCH_3), 63.06 (C-5'), 74.98 (C-2'), 76.03 (C-3'), 82.58 (CAr_3), 84.72 (C-1'), 85.46 (C-4'), 99.86 (C-5), 113.13-135.48 (aromatic), 144.63 (C-6), 149.49 (C-2), 163.09 (C-4); **m/z** (FAB) 547 (MH^+).

1-{3'-O-[2-Cyanoethoxy(diisopropylamino)phosphino]-5'-O-(4,4'-Dimethoxytrityl)-β-D-arabinofuranosyl} uracil.

Compound **(4)** (0.160g, 0.293mmol) was coevaporated with anhydrous CH_3CN and dissolved in anhydrous CH_2Cl_2 (2ml) under an atmosphere of argon. N,N-Diisopropylethylamine (0.3ml) was added dropwise followed by 2-cyanoethyl-N,N-diisopropylaminochlorophophoramidite (0.056ml, 0.317mmol, 1.1equivalents). After 60 minutes methanol (1ml) was added to quench the reaction. EtOAc (6ml) was added and the reaction mixture washed with saturated $NaHCO_3$ (3 x 25ml) and NaCl (3 x 25ml). The organic phase was dried (Na_2SO_4), filtered and reduced *in vacuo*. The residue was purified by wet flash chromatography (30-50% ethyl acetate-cyclohexane, 1% pyridine) which was taken up in 1ml of dry toluene and precipitated in petroleum ether under vigorous stirring (150ml, cooled to -65°C) to give the *title compound* as a white foam (160mg, 0.214mmol, 73%). **tlc** $\mathbf{R_f}$ (10% MeOH/DCM) 0.27; **δH** (250 MHz, $CDCl_3$) 0.99-1.29 (m, 12H, $(CH_3)_2$), 2.76 (t, 2H, CH_2CN, J = 6.3Hz), 3.30-

3.67 (m, 5H, 5'-H, OCH_2, CH(N)), 3.79 (s, 6H, OCH_3), 4.10-4.41 (m, 3H, 2'-H, 3'-H,4'-H), 5.49 (d, 1H, 5-H, $J_{5\text{-}6}$ = 1.88Hz), 6.79-7.45(m, 14H, aromatic, 6-H); **δC** (250 MHz, $CDCl_3$) 22.75 (CH_2CN), 24.43 ($CH(CH_3)_2$), 43.06 (CHN), 55.11 (OCH_3), 58.08 (OCH_2), 113.02-130.02(aromatic); **δP** (250 MHz, $CDCl_3$) 149.6 and 149.8.

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Courses and Conferences Attended

Departmental Colloquia, University of Edinburgh, 1994-1997, various speakers.

Organic Research Seminars, University of Edinburgh, 1994-1997, various speakers.

"Medicinal Chemistry", Professor R. Baker and colleagues, Merck, Sharp and Dohme, University of Edinburgh, 1994-1997.

Royal Society of Chemistry, Perkin Division Scottish Meeting, Dundee 1994, Glasgow 1995, Edinburgh 1996, various speakers.

Solid Phase Synthesis and Combinatorial Libraries, Forth International Symposium, Edinburgh, 1995, various speakers.

NMR Spectroscopy, Drs. I. Sadler and P. Barlow, University of Edinburgh, 1996.

SCI Graduate Symposium in Novel Organic Chemistry, St. Andrews University, 1996, various speakers.

Twenty-fourth International Symposium of the European Peptide Society, Edinburgh, 1996, various speakers.

"Current Awareness in Organic Chemistry", Drs. I. Gosney, A.N. Hulne, H. M^{c}Nab, R.M. Paton, N. Turner, (sponsored by Zeneca Grangemouth), University of Edinburgh, 1997.

Edinburgh Centre for Protein Technology (ECPT) lectures, University of Edinburgh, 1997, various speakers.

Automated synthesis of branched oligodeoxynucleotide analogues using *arabino*-uridine as branching nucleotide

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Fully automated synthesis of branched oligodeoxynucleotides (branched ODNs) has been accomplished using 2′-*O*-levulinyl- or 5′-*O*-levulinyl-protected *arabino*-uridine derivatives as branching monomers. Selective removal of the levulinyl groups is accomplished using 0.5 M hydrazine hydrate in a pyridine–acetic acid–water (4:3:0.35, v/v/v) buffer. The affinity of the branched ODNs towards complementary DNA has been evaluated at 260 nm and 284 nm during thermal denaturing experiments. Enhanced affinity of a branched ODN compared with the corresponding linear reference is attributed to bimolecular triple helix formation.

Introduction

Recently, development of Y-shaped branched oligodeoxynucleotides (branched ODNs) as possible probes for high-affinity targeting of complementary ODNs has been attempted using the strategies outlined in Fig. 1A,[1-4] the rationale being that full-length duplex formation (Watson–Crick hydrogen bonds) between the branched DNA segment and the target is followed by alignment of the branch in the major groove of the duplex thus forming a triple helix (Hoogsteen hydrogen bonds). For entropic reasons, increased affinities and selectivities for such bimolecular complexes are envisaged in analogy with results reported for circular oligonucleotides.[5,6]

The hybridisation results obtained by Azhayeva *et al.*[2] employing 3′-deoxypsicothymidine (structure **A**, Fig. 2) as branching monomer were rather disappointing. Thus, two-phasic transitions and appreciable decreases in melting points (mps) compared with linear controls were obtained. These results were explained by the unnatural structure of the branching point nucleoside and destabilisation caused by the presence of the branch. We have used 1-(2-methyl-β-D-arabinofuranosyl)-uracil (structure **B**, Fig. 2) as branching monomer[3,4] to give branched ODN analogues capable of hybridising to target ODNs as depicted in Fig. 1A. Increased thermal stabilities compared with the corresponding linear reference containing one modified monomer (structure **B**, 2′-*O*-unbranched, Fig. 2) were obtained in monophasic transitions.

However, to achieve thermal stabilities comparable to or even higher than those of unmodified controls, a more favourable branching-point geometry seems necessary. Earlier, we have shown an only moderately destabilising effect of incorporating one 2′-*O*-methylarabinonucleoside into 11-mer or 17-mer ODNs.[7] Decreases in mps of 1–6 °C per modification, compared with observed decreases of 11–14 °C for one unbranched 1-(2-methyl-β-D-arabinofuranosyl)uracil monomer **B**,[3,4] were achieved. Therefore, we decided to synthesize and evaluate the novel branched ODNs **B**–**M** (Tables 1–3) using *arabino*-uridine [1-(β-D-arabinofuranosyl)uracil] as branching monomer utilising the 2′-hydroxy group oriented into the major groove as attachment site for the branch. Besides the potential entropic advantages, the C-type branched ODNs (Fig. 1) might be capable of forming π-interactions at the ends of the two triplex-forming strands thereby inducing cooperativity between the two strands which has been reported to increase the stability of a related complex.[8]

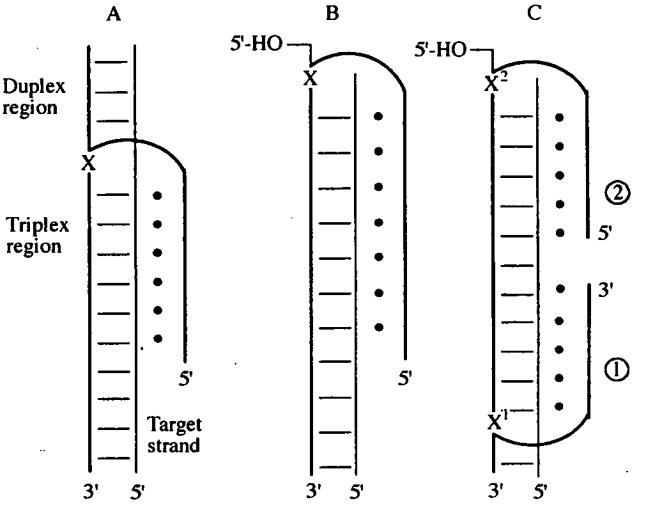

Fig. 1 Schematic representation of the structures of complexes formed between branched ODNs and complementary oligonucleotides

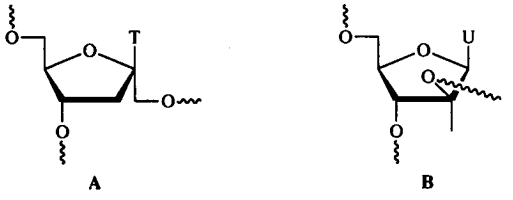

Fig. 2 Structures around branching nucleotides in previously described branched ODNs used in oligonucleotide targeting as depicted in Fig. 1A. T = thymin-1-yl, U = uracil-1-yl.

Results and discussion

Nucleoside **1**[9] was levulinated using levulinic anhydride and 4-(dimethylamino)pyridine (DMAP) in anhydrous diethyl ether

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Table 1 Sequences of synthesized branched ODNs (plus non-branched reference strand A) and measured mps (T_m) from hybridisation with complementary dA_{12}

	Sequence[a]	T_m/°C[b]	Triple helix[c]
A	3′-TTTTTTTT-$_3$-X_5-TTT-5′	30	
B	3′-TTTTTTTT-$_3$-X_5-TTT-5′ 5′-TTTTTTTCC-$_2$′	27	+
C	3′-TTTTTTTT-$_3$-X_5-TTT-5′ 5′-TTTTTTTCCC-$_2$′	27	+
D	3′-TTTTTTTT-$_3$-X_5-TTT-5′ 5′-TTTTTTTCCCC-$_2$′	27	+
E	3′-TTTTTTTT-$_3$-X_5-TTT-5′ 5′-TTTGTTTCC-$_2$′	24	
F	3′-TTTTTTTT-$_3$-X_5-TTT-5′ 5′-TGTGTGTCC-$_2$′	23	

[a] C = 2′-Deoxycytidine; G = 2′-deoxyquanosine; T = thymidine; X = modified ara-U nucleotide. [b] Mps were determined as the maximum of the first derivative of the absorption *vs.* temperature curve at 260 nm. [c] + Denotes confirmation of transition at 284 nm.

Table 2 Sequences of synthesized branched ODNs of the B-type and measured mps (T_m) from hybridisation with complementary dA_{12}

	Sequence[a]	T_m/°C[b]	Triple helix[c]
G	3′-CATTTTTTTTTTT-$_3$-X 5′-TTTTT-$_2$′	37.6	
H	3′-CATTTTTTTTTTT-$_3$-X 5′-TTTTTTTTT-$_2$′	41.2	+
I	3′-CATTTTTTTTTTT-$_3$-X 5′-TTTTTTCCCCC-$_2$′	35.8	+
J	3′-CATTTTTTTTTT-$_3$-X 5′-TTTTTTTTTCCCCC-$_2$′	41.0	+
K	3′-CATTTTTTTTTT-$_3$-X 5′-TTTTTGTTTT-$_2$′	29.8	

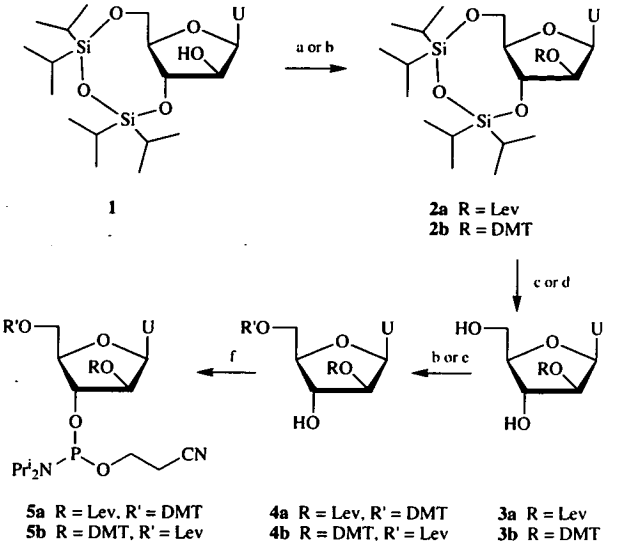
[a] C = 2′-Deoxycytidine; A = 2′-deoxyadenosine; G = 2′-deoxyguanosine; T = thymidine; X = modified ara-U nucleotide. [b] Mps were determined as the maximum of the first derivative of the absorption *vs.* temperature curve at 260 nm. [c] + Denotes confirmation of transition at 284 nm.

Table 3 Sequences of synthesized branched ODNs of the C-type and measured mps (T_m) from hybridisation with complementary dA_{12}

	Type[a]	① [a,b]	② [a,b]	T_m/°C[c]	Triple helix[d]
L	C	T_4-3′	T_4-5′	29.6	
M	C	T_8-3′	T_8-5′	34.0	+

[a] Refers to Fig. 1. [b] T = Thymidine. [c] Mps were determined as the maximum of the first derivative of the absorption *vs.* temperature curve at 260 nm. [d] + Denotes confirmation of transition at 284 nm.

and anhydrous pyridine to give 1-[2-*O*-levulinyl-3,5-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-β-D-arabinofuranosyl]uracil **2a** in 87% yield. Desilylation using tetrabutylammonium fluoride (TBAF) in tetrahydrofuran (THF) to give compound **3a** in 76% yield followed by selective protection of the 5′-hydroxy functionality by reaction with 4,4′-dimethoxytrityl chloride (DMTCl) and silver nitrate in anhydrous pyridine afforded 1-[5-*O*-(4,4′-dimethoxytrityl)-2-*O*-levulinyl-β-D-arabinofuranosyl]uracil **4a** in 96% yield. Subsequent phosphitylation of the 3′-hydroxy group using 2-cyanoethyl *N,N*-diisopropylphosphoramidochloridite and *N,N*-diisopropylamine in dichloromethane gave the desired amidite **5a** in 67% yield after precipitation from light petroleum (Scheme 1). 4,4′-Dimethoxytritylation of compound **1** using silver nitrate in anhydrous THF afforded nucleoside **2b** in quantitative yield. Subsequent desilylation to give compound **3b** was achieved in 97% yield using KF·2H_2O in THF. Selective levulination of the primary hydroxy group using the enzyme Novozym 435® under conditions described earlier[10] afforded derivative **4b** in 93% yield. The corresponding acylation with pyridine as base afforded the undesired diacylated by-product in addition to compound **4b**.



Scheme 1 *Reagents:* (a) Levulinic anhydride, DMAP, pyridine; (b) DMTCl, $AgNO_3$, THF, pyridine; (c) TBAF, THF; (d) KF·2H_2O, 18-crown-6, THF; (e) levulinic anhydride, Novozym 435®, 1,4-dioxane; (f) 2-cyanoethyl *N,N*-diisopropylphosphoramidochloridite, *N,N*-diisopropylethylamine, CH_2Cl_2. DMT = 4,4′-dimethoxytrityl: Lev = levulinyl; U = uracil-1-yl.

Nucleoside **4b** was phosphitylated as described above for synthesis of compound **5a** to give phosphoramidite **5b** in 64% yield.

All ODNs were synthesized using standard phosphoramidite chemistry[11] with cycles including detritylation, coupling, capping and oxidation. Synthesis of the type A branched ODNs (Fig. 1) was initiated by constructing the linear strand using standard amidite concentration (0.1 M) and coupling time (2 min). However, when incorporating the modified nucleotide **X**, a 0.15 M solution of amidite **5a** and a coupling time of 12 min were applied. A separate end-capping cycle (consisting of detritylation and acetylation) was used to block further linear elongation at the 5′-end, thus preparing for branching. The support was treated with 0.5 M hydrazine hydrate in pyridine–acetic acid (4:1, v/v) for 90 min to remove the 2′-*O*-levulinyl protecting group. The first nucleotide in the branch was subsequently attached at the 2′-hydroxy group of nucleotide **X** by 12 min couplings at standard amidite concentration whereupon the remaining part of the branch was synthesized using standard conditions. The ODNs of the B- and C-type (Fig. 1) were synthesized by using compound **5b** at standard concentration and a coupling time of 2 × 12 min (coupling, washing, coupling with a fresh solution of amidite, capping, oxidation and detritylation). This procedure increased the coupling efficiency of amidite **5b** from ~65% (single coupling) to ~95%. As the next step, the first nucleotide in the branch originating from the 2′-hydroxy group was attached using a coupling time of 2 × 12 min. After finishing this branch, the looped B-type ODNs were subjected to standard ammonolytic deprotection which, besides nucleobase and phosphate deprotection and cleavage from the support, effected delevulination, thus liberating the 5′-hydroxy group of monomer **X**. Synthesis of the C-type branched and looped ODNs was accomplished using commercially available 'reversed' (5′-*O*-phosphitylated and 3′-*O*-4,4′-dimethoxytritylated) phosphoramidites in branch ① (Fig. 1C). After completion of branch ①, end-capping at the 3′-end was performed as described above. Subsequently, the support was treated with a freshly prepared 0.5 M solution of hydrazine hydrate in a buffer consisting of pyridine–acetic acid–water (4:3:0.35, v/v/v) for 5 min in order selectively to 5′-*O*-delevulinate at the branching point X^1. The remaining part of the type-C ODNs were synthesized using amidite **5b** (to incorporate monomer X^2) and standard phosphoramidites. The

DMT group of the last nucleotide incorporated was in all syntheses left on for the purpose of purification. The coupling yields determined by a VIS spectrophotometer as the amount of the DMT carbenium ion released during each detritylation step was >99% for commercial amidites and ~60% for the modified amidite **5a**. The branched ODNs were cleaved from the solid support and deprotected by treatment with 32% aq. ammonia for 72 h at room temperature. The crude ODNs were desalted, purified and detritylated on disposable reversed-phase cartridges, taking advantage of the hydrophobicity of the DMT group. The composition of ODNs **C**, **F**, **H**, **J** and **M**, chosen as representative probes, was confirmed by matrix-assisted laser desorption mass spectrometry (**C**: Found, 6589 Da. Calc., 6587 Da; **F**: Found, 6374 Da. Calc., 6373 Da; **H**: Found, 7237 Da. Calc., 7235 Da; **J**: Found, 8678 Da. Calc., 8681 Da; **M**: Found, 9060 Da. Calc., 9062 Da. The purity was confirmed for all ODNs using capillary gel electrophoresis.

The ODNs were designed to orient the additional branches parallel to the complementary dA_{12} strand which should allow the formation of stable triple helices.[12] The sequence of branching in the A-type oligodeoxynucleotides was varied to evaluate the effect of the length of the cytidine linker. Furthermore, thymidines in the branch were replaced with guanosines to test the effect of mismatches on triple-helix formation and thermal stability. Mp (T_m) determinations were carried out in medium salt buffer as described earlier.[4] The strand dissociations (Tables 1–3) were detected through hyperchromicities at 260 nm and 284 nm, the latter being characteristic for triple-helix dissociations.[13,14] The insertion of the modified nucleotide **X** without attaching an additional branch (ODN **A**) causes a drop in T_m of 6 °C compared with unmodified controls (T_{12} and 3′-T_8dUT_3-5′). As expected, no transition was detected at 284 nm with the linear ODN **A**. For the branched ODNs **B**–**D**, triple-helix dissociations were confirmed, and, as expected, ODNs **E** and **F** having mismatches in the branch showed no sign of dissociation when measured at 284 nm. It may therefore be concluded that the T_8-branches in ODNs **B**–**D** are involved in complexes with dA_{12} as depicted in Fig. 1A. The mp of mismatched ODNs **E** and **F** reveal that a non-binding branch has a significant destabilising effect on the duplex (decrease in T_m compared with that of **A**: ~7 °C). This could, at least in part, be due to unfavourable steric interactions at the branching point. This effect is partly compensated for by the stabilising effect of triple-helix formation as seen for ODNs **B**–**D**, but the mps are still lower than for the linear reference **A**. The results for the different linkers indicate that the length of the cytidine linker (C_2–C_4) is of no importance.

The looped ODNs **G**–**K** (Fig. 1B, Table 2) show, in contrast to earlier results for unmodified triplex-forming back-looped ODNs,[15] that there seems to be no preference for the sequence with a five-nucleotide loop *versus* the corresponding sequence without a connecting loop (ODN **J** *versus* ODN **H**). This supports our original idea that direct entropically favoured backfolding should be possible having an attachment site oriented into the major groove. However, incorporation of the modification causes a decrease in the mp of 4 °C (3′-CAT_{12}-5′: 28.2 °C, 3′-CAT_{11}X-5′: 24.0 °C), a decrease that can be overcome by bimolecular triplex formation (Table 2, ONDs **H** and **J**). The looped ODNs form triplexes of comparable stability to complexes formed between a target strand and unmodified ODNs with a loop consisting of five nucleotides (3′-$CAT_{12}C_5T_6$-5′: 36.0 °C, 3′-$CAT_{12}C_5T_{10}$-5′: 40.4 °C). Incorporation of one or two mismatches in the triplex-forming strand causes a decrease in the mp and no detectable transition at 284 nm, indicating the expected selectivity in the binding of the third strand, and also showing that binding involving the third strand causes stabilisation of the complex.

The looped and branched ODNs **L** and **M** (Fig. 1C, Table 3) show that eight nucleotides in the third strand are sufficient for triplex formation, whereas four are not. Triplex formation for ODN **M** causes an increase in the T_m to 34 °C compared with 28.5 °C for the corresponding linear ODN (3′-$CAXT_{10}$X-5′). The results indicate that the two branches do not interact cooperatively and do not stabilise the triplexes by π-interactions. In contrast to the A-type ODNs, there seems to be no destabilising effect in the B- and C-type ODNs due to branches not involved in Hoogsteen hydrogen bonding. This may originate from the difference in incorporating **X** in the middle (in A-type ODNs) or in the ends, the latter generally known to be less detrimental to duplex formation. In all cases where triplex structures were found, concomitant dissociation of the triplex and duplex complexes was observed (monophasic bimolecular transitions), indicating a cooperativity between the Watson–Crick bonds and the Hoogsteen bonds.

To summarise, synthesis of branched ODNs using *arabino*-uridine as branching point **X** has been accomplished. The synthetic strategy allows synthesis of branched ODNs with sequences of arbitrary length and base composition. The thermal stability of branched ODNs is increased compared with the branched mismatched references. The melting results for the looped and branched ODNs showed that eight nucleotides in the additional branch are sufficient to achieve a stable triplex structure. However, compared with the corresponding unmodified linear reference, no significant increase in T_m could be obtained. This is probably due to disruption of the Watson–Crick duplex structure caused by the modification **X** as well as unfavourable steric interactions in the branching point, *e.g.* between the nucleobase and the 2′-phosphate group. This suggests that arabinonucleosides containing short, non-phosphorus 2′-*O*-linkers should be examined as the next class of *arabino*-configurated branched ODNs.

Experimental

NMR spectra were obtained on a Bruker AC250 spectrometer at 250 MHz for ^{1}H NMR, 63 MHz for ^{13}C NMR and 101 MHz for ^{31}P NMR spectroscopy. δ-Values are reported in ppm relative to internal $SiMe_4$ for ^{1}H and ^{13}C and relative to external 85% H_3PO_4 for ^{13}P spectra. All coupling constants are in Hz. Fast-atom bombardment mass spectrometry (FAB-MS) was performed on a Kratos MS 50 RF spectrometer. The enzyme Novozym 435® is commercially available from Novo Nordisk A/S, Bagsvaerd, Denmark. Unmodified and reversed (5′-*O*-phosphitylated and 3′-*O*-4,4′-dimethoxytritylated) amidites were purchased from Cruachem. Matrix-assisted laser desorption mass spectrometry was performed using a Micromass TofSpec E mass spectrometer using a matrix of diammonium hydrogen citrate and 2,6-dihydroxyacetophenone. Capillary gel electrophoresis was performed using a Beckman P/ACE System 5000 (ss DNA 100 Gel Column). Light petroleum refers to the fraction with distillation range 60–80 °C.

1-[2′-*O*-Levulinyl-3′,5′-O-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-β-D-arabinofuranosyl]uracil 2a

Compound **1**[9] (497 mg, 1.02 mmol) was dissolved in anhydrous pyridine (10 cm^3) and DMAP (6 mg, 0.05 mmol) was added. The mixture was kept under N_2 and cooled to 0 °C. An ethereal solution of levulinic anhydride (3.1 cm^3, 3.1 mmol) was added dropwise. After being stirred for 1.5 h, the reaction was quenched with saturated aq. $NaHCO_3$ (45 cm^3). The mixture was extracted with ethyl acetate (2 × 100 cm^3) and the organic phase was dried (Na_2SO_4), filtered, and evaporated *in vacuo*. The residue was purified by column chromatography (5% MeOH in CH_2Cl_2, v/v) to give compound **2a** as a foam (522 mg, 87%), $\delta_H(CDCl_3)$ 0.9–1.2 (m, 28 H, 4 × Pr^i), 2.2 (s, 3 H, CH_3), 2.4–2.8 (m, 4 H, CH_2CH_2), 3.8 (m, 1 H, 4′-H), 4.0–4.2 (m, 2 H, 5′-H_2), 4.4 (t, *J* 8.3, 1 H, 3′-H), 5.6 (dd, *J* 6.3 and 7.9, 1 H, 2′-H), 5.7 (d, *J* 8.2, 1 H, 5-H), 6.2 (d, *J* 6.2, 1 H, 1′-H), 7.6 (d, *J* 8.2, 1 H, 6-H) and 8.8 (br, 1 H, NH); $\delta_C(CDCl_3)$ 12.3–17.4 (TIPDS), 27.4 (CH_3), 29.7 (CH_2), 37.5 (CH_2), 60.3 (C-5′), 71.1

(C-3'), 75.9 (C-2'), 80.6 and 81.7 (C-1', C-4'), 101.9 (C-5), 140.3 (C-6), 149.9 (C-2), 163.0 (C-4), 171.3 (C=O) and 205.7 (C=O).

1-(2'-*O*-Levulinyl-β-D-arabinofuranosyl)uracil 3a

Compound **2a** (495 mg, 0.85 mmol) was dissolved in anhydrous THF (25 cm^3) under N_2 and a solution of TBAF in THF (2.5 cm^3, 2.5 mmol) was added. After being stirred for 15 min, the reaction was complete, and the mixture was loaded directly onto a silica gel column where it was eluted with 10% MeOH in CH_2Cl_2 (v/v) to give compound **3a** as a pale yellow foam (221 mg, 76%). $\delta_H(CD_3OD)$ 2.3 (s, 3 H, CH_3), 2.5–2.9 (m, 4 H, CH_2CH_2), 3.8–4.3 (m, 6 H, 3'- and 4'-H, 5'-H_2, 3'- and 5'-OH), 5.4 (dd, *J* 3.9 and 5.0, 1 H, 2'-H), 5.8 (d, *J* 8.1, 1 H, 5-H), 6.4 (d, *J* 5.0, 1 H, 1'-H) and 7.9 (d, *J* 8.1, 1 H, 6-H); $\delta_C(CD_3OD)$ 28.6 (CH_3), 29.5 (CH_2), 38.4 (CH_2), 61.8 (C-5'), 74.5 (C-3'), 79.1 (C-2'), 84.9 and 85.3 (C-1' and -4'), 102.0 (C-5), 143.2 (C-6), 152.0 (C-2), 166.26 (C-4), 172.9 (C=O) and 208.7 (C=O).

1-[5'-*O*-(4,4'-Dimethoxytrityl)-2'-*O*-levulinyl-β-D-arabinofuranosyl]uracil 4a

Compound **3a** (162.5 mg, 0.47 mmol) was dissolved in anhydrous THF (15 cm^3) under argon and anhydrous pyridine (1 cm^3) was added. Silver nitrate (89 mg, 0.52 mmol) and DMTCl (177 mg, 0.52 mmol) were added and the mixture was stirred at room temperature for 24 h. The mixture was filtered, poured into 5% aq. $NaHCO_3$ (10 cm^3) and was extracted with ethyl acetate (2 × 50 cm^3). The organic phase was dried (Na_2SO_4), filtered, and evaporated *in vacuo*. The residue was purified by column chromatography (5% MeOH in CH_2Cl_2, v/v) to give compound **4a** as a pale yellow foam (287 mg, 96%), $\delta_H(CDCl_3)$ 2.1 (s, 3 H, CH_3), 2.4–2.8 (m, 4 H, CH_2CH_2), 3.5 (m, 2 H, 5'-H_2), 3.6 (br, 1 H, 3'-OH), 3.8 (s, 6 H, OCH_3), 4.0 (m, 1 H, 4'-H), 4.4–4.5 (m, 1 H, 3'-H), 5.3 (dd, *J* 4.7 and 5.6, 1 H, 2'-H), 5.5 (d, *J* 8.1, 1 H, 5-H), 6.3 (d, *J* 5.7, 1 H, 1'-H), 7.1–7.5 (m, 13 H, ArH), 7.7 (d, *J* 8.2, 1 H, 6-H) and 9.0 (br, 1 H, NH); $\delta_H(CDCl_3)$ 27.7 (CH_3), 29.6 (CH_2), 37.7 (CH_2), 55.1 (OCH_3), 55.2 (OCH_3), 61.7 (C-5'), 74.2 (C-3'), 79.0 (C-2'), 81.9, 83.2 and 86.7 (C-1' and -4', *C*Ar_3), 101.6 (C-5), 113.2, 113.3, 127.0, 127.9, 128.1, 130.1, 130.1, 135.4 and 135.5 (aryl), 141.0 (C-6), 144.4 (aryl), 150.0 (C-2), 158.6 (aryl), 163.1 (C-4), 172.4 (C=O) and 206.1 (C=O).

1-{3'-*O*-[2-Cyanoethoxy(diisopropylamino)phosphino]-5'-*O*-(4,4'-dimethoxytrityl)-2'-*O*-levulinyl-β-D-arabinofuranosyl}uracil 5a

Nucleoside **4a** (192 mg, 0.298 mmol) was coevaporated with anhydrous acetonitrile (3 × 2.5 cm^3) and dried overnight *in vacuo*. After dissolution of compound **4a** in anhydrous CH_2Cl_2 (1.5 cm^3) under argon, *N,N*-diisopropylethylamine (0.20 cm^3, 1.2 mmol) and 2-cyanoethyl *N,N*-diisopropylphosphoramidochloridite (0.13 cm^3, 0.48 mmol) was added under stirring at room temperature. After 1 h, the reaction was quenched with MeOH (1 cm^3), dissolved in ethyl acetate (6 cm^3) and washed successively with saturated aq. $NaHCO_3$ (3 × 5 cm^3) and saturated aq. NaCl (3 × 5 cm^3). The organic phase was dried (Na_2SO_4), filtered, and evaporated *in vacuo*. The residue was dissolved in toluene (1 cm^3) and the product was precipitated from light petroleum (70 cm^3; cooled to −65 °C) under vigorous stirring. The precipitate was collected by filtration, re-dissolved and coevaporated with anhydrous acetonitrile (3 × 2.5 cm^3) to give compound **5a** as a foam (168 mg, 67%), $\delta_P(CDCl_3)$ 151.8 and 151.9.

1-[2'-*O*-(4,4'-Dimethoxytrityl)-3',5'-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-β-D-arabinofuranosyl]uracil 2b

Compound **1**[9] (1.62 g, 3.33 mmol) was coevaporated with anhydrous pyridine (2 × 5 cm^3) and dissolved in a mixture of anhydrous THF (30 cm^3) and anhydrous pyridine (3 cm^3) under argon. Silver nitrate (2.64 g, 15.5 mmol) was added followed by DMTCl (5.33 g, 15.7 mmol) and the mixture was stirred overnight. The reaction mixture was subsequently filtered through a layer of silica gel, and evaporated *in vacuo*. The residue was taken up in ethyl acetate and washed successively with saturated aq. $NaHCO_3$ (3 × 20 cm^3) and saturated aq. NaCl (2 × 20 cm^3), and the organic phase was dried (Na_2SO_4), filtered, and evaporated *in vacuo*. The residue was purified by column chromatography (20–40% ethyl acetate–light petroleum, 1% pyridine) to give compound **2b** as a yellow foam (2.62 g, 100%), $\delta_H(CDCl_3)$ 1.0–1.1 (m, 28 H, Pr^i), 3.7 (m, 1 H, 4'-H), 3.8 (s, 6 H, OCH_3), 3.9–4.6 (m, 4 H, 2'- and 3'-H, 5'-H_2), 5.5 (d, *J* 5.0, 1 H, 1'-H), 5.6 (d, *J* 8.2, 1 H, 5-H), 6.8 (m, 4 H, ArH), 7.1–7.4 (m, 9 H, ArH), 7.5 (d, *J* 8.2, 1 H, 6-H) and 8.4 (s, 1 H, NH); $\delta_C(CDCl_3)$ 12.8–17.3 (Pr^i), 55.1 (OCH_3), 61.8 (C-5'), 75.1 (C-2'), 77.4 (C-3'), 80.7, 81.8 and 88.4 (C-1' and -4', *C*Ar_3), 101.0 (C-5), 113.2, 127.2–135.1, 142.8 and 144.2 (aryl), 149.6 (C-2), 158.9 (aryl) and 162.7 (C-4); FAB-MS: *m/z* 811 (M + 23).

1-[2'-*O*-(4,4'-Dimethoxytrityl)-β-D-arabinofuranosyl]uracil 3b

Compound **2b** (1.97 g, 2.50 mmol) was dissolved in THF (36 cm^3). KF·$2H_2O$ (1.42 g, 15 mmol) and 18-crown-6 (233 mg, 0.88 mmol) was added to the vigorously stirred solution at room temperature. After 48 h, the reaction mixture was filtered, evaporated *in vacuo* and the residue was purified by column chromatography (2–5% MeOH in CH_2Cl_2 containing 1% pyridine, v/v/v) to give compound **3b** as a foam (1.33 g, 97%); $\delta_H(CD_3OD)$ 3.4–3.5 (m, 1 H, 4'-H), 3.7–3.9 (m, 3 H, 3'-H and 5'-H_2), 3.9 (s, 6 H, OCH_3), 4.4 (dd, *J* 3.1 and 4.6, 1 H, 2'-H), 5.8 (d, *J* 8.1, 1 H, 5-H), 6.1 (d, *J* 4.4, 1 H, 1'-H), 6.9–7.5 (m, 13 H, ArH) and 8.1 (d, *J* 7.0, 1 H, 6-H); $\delta_C(CD_3OD)$ 56.0 (OCH_3), 63.1 (C-5'), 76.8 (C-2'), 79.3 (C-3'), 85.9, 86.0 and 89.9 (C-1' and -4', *C*Ar_3), 101.8 (C-5), 114.6, 128.5–132.0 and 136.9 (aryl), 145.7 (C-6), 146.5 (aryl), 152.1 (C-2), 160.8 (aryl) and 166.5 (C-4); FAB-MS: *m/z* 569 (M + 23).

1-[2'-*O*-(4,4'-Dimethoxytrityl)-5'-*O*-levulinyl-ß-D-arabinofuranosyl]uracil 4b

Compound **3b** (306 mg, 0.56 mmol) was dissolved in anhydrous dioxane (3 cm^3) under argon. Novozym 435® (200 mg) was added followed by a freshly prepared solution of levulinic anhydride in 1,4-dioxane (10 cm^3, 3.68 mmol), and the mixture was stirred overnight at room temperature. The mixture was filtered, washed with saturated aq. $NaHCO_3$ (3 × 5 cm^3), and the organic phase was dried (Na_2SO_4), filtered, and evaporated *in vacuo*. The residue was purified by column chromatography (1–5% MeOH in CH_2Cl_2 containing 1% pyridine, v/v/v) to give compound **4b** as a pale yellow foam (332 mg, 93%), $\delta_H(CDCl_3)$ 2.2 (s, 3 H, CH_3), 2.6–2.8 (m, 4 H, CH_2CH_2), 3.5 (br, 1 H, 3'-OH), 3.8 (s, 6 H, OCH_3), 3.8–4.4 (m, 5 H, 2'-, 3'- and 4'-H and 5'-H_2), 5.8 (d, *J* 8.1, 1 H, 5-H), 6.1 (br, 1 H, 1'-H), 6.8–6.9 (m, 4 H, ArH), 7.2–7.3 (m, 9 H, ArH), 7.8 (br, 1 H, 6-H) and 9.1 (s, 1 H, NH); $\delta_C(CDCl_3)$ 27.8 and 29.7 (CH_2CH_2), 37.8 (CH_3), 55.1 (OCH_3), 63.1 (C-5'), 76.0 and 78.0 (C-2' and -3'), 80.2, 84.0 and 88.3 (C-1' and -4', *C*Ar_3), 101.4 (C-5), 113.3 and 127.3–135.3 (aryl), 143.0 (C-6), 144.2 (aryl), 150.0 (C-2), 158.8 (aryl), 163.0 (C-4), 172.6 (C=O) and 206.7 (C=O); FAB-MS: *m/z* 667 (M + 23).

1-{3'-*O*-[2-Cyanoethoxy(diisopropylamino)phosphino]-2'-*O*-(4,4'-dimethoxytrityl)-5'-*O*-levulinyl-β-D-arabinofuranosyl}uracil 5b

Nucleoside **4b** (291 mg, 0.45 mmol) was coevaporated with anhydrous acetonitrile (3 × 2 cm^3) and re-dissolved in anhydrous CH_2Cl_2 (3.6 cm^3) under argon. *N,N*-Diisopropylethylamine (1.7 cm^3, 9.9 mmol) and 2-cyanoethyl *N,N*-diisopropylphosphoramidochloridite (0.18 cm^3, 0.67 mmol) were added to the stirred mixture at room temperature. After 1 h, the reaction was quenched with anhydrous methanol (1 cm^3), diluted with ethyl acetate and washed successively with saturated aq. $NaHCO_3$ (3 × 5 cm^3) and NaCl (3 × 5 cm^3).

The organic phase was dried (Na_2SO_4), filtered, and evaporated *in vacuo*. The residue was purified by column chromatography [a 1:1 (v/v) mixture of light petroleum and a mixture of CH_2Cl_2, ethyl acetate and Et_3N (9:9:2, v/v/v)], evaporated, re-dissolved in toluene (2 cm^3) and precipitated from light petroleum (65 cm^3; −65 °C) under vigorous stirring. The precipitate was collected by filtration as a solid, re-dissolved, and coevaporated with anhydrous acetonitrile (3 × 5 cm^3) to give compound **5b** as a pale yellow foam (243 mg, 64%), $\delta_P(CDCl_3)$ 149.5 and 150.6.

Oligonucleotide synthesis

All oligonucleotides were synthesized on a Pharmacia Gene Assembler Special® synthesizer. Solid supports on a 0.2 μmol scale were obtained from Pharmacia or Cruachem. The amidite solution volume applied for all couplings was 75 μl. The commercial amidites were used in 0.1 M concentration with 2 min coupling time. The modified *arabino*-uridine building block was used in 0.1–0.2 M concentrations and the coupling time was extended to 12 min or 2 × 12 min as described in the Results and discussion section. The levulinyl protection group was removed by treatment of the solid support either with a 0.5 M solution of hydrazine hydrate in pyridine–acetic acid (4:1, v/v) for 90 min or with a 0.5 M solution of hydrazine hydrate in a buffer consisting of pyridine–acetic acid–water (4:3:0.35, v/v/v). The branching step was performed with a 0.1 M amidite solution using 12 min or 2 × 12 min coupling time. For all modified oligonucleotides the DMT group of the latest incorporated nucleotide was left on for purification, but for the unmodified references it was removed as the last step on the synthesizer. The oligonucleotides were deprotected and cleaved from solid support by incubation in 32% aq. ammonia at room temperature for 72 h. All oligonucleotides were desalted through size-exclusion chromatography (NAP™-10-columns, Sephadex® G-25 medium, Pharmacia). Purification of the modified oligonucleotides was achieved by use of disposable reversed-phase columns (COP™-columns, Cruachem; the procedure includes detritylation). The procedures were carried out according to manufacturer's protocols.

Determination of T_m

Mps (T_m) were determined as described earlier.[4]

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