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Synthesis of Modified Nucleosides for Use as Energy Transfer Probes in Genetic Analysis

By

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ABSTRACT

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SYNTHESIS OF MODIFIED NUCLEOSIDES FOR USE AS ENERGY TRANSFER PROBES IN GENETIC ANALYSIS

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Energy transfer probes are used for target recognition in genetic analysis assays. The development of a novel energy transfer linker utilising a modified nucleoside (uridine or cytidine) is described.

The nucleoside (uridine or cytidine) was modified by the addition of an ethoxy functionality to the 2'-hydroxyl position. The synthesis of the phosphoramidites of both nucleosides is outlined.

The nucleosides were incorporated into oligonucleotides, as phosphoramidites, and their performance as energy transfer linkers was investigated.

The energy transfer linker was incorporated into both Hybeacon and Taqman assays. The linker's ability to function as an energy transfer probe was assessed.

There is a good possibilty of success with the Hybeacon assay as the energy transfer linker shows better discrimination between wild type and mutant sequences. Success with either of the above systems will lead to a commercially viable product.

Contents

Abbreviations

Å	Angstrom unit (=10^{-10} m)
A	adenine
(A)	acceptor dye
ABI	Applied Biosystems
Aq	aqueous
Ar	aryl
ARMS	amplification refractory mutation system
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BOM	benzyloxymethyl
bp	base pair
*t*Bu	tertiary butyl
C	cytosine
^{13}C	carbon [NMR]
$CDCl_3$	deuterated chloroform
cDNA	copy deoxyribonucleic acid
CPG	controlled pore glass
δ	chemical shift
(D)	donor dye
DAB	diaminobenzamide tetrahydrochoride
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
DCM	dichloromethane
DDQ	2,3-dichloro-5,6-dicyanobenzoquinone
DDQH	2,3-dichloro-5,6-dicyanohydroquinone
DEPT	distortionless enhancement by phase transfer
DIG	digoxygenin
DIPEA	diisopropylethylamine
DME	ethyleneglycol dimethyl ether

DMF	*N*,*N*-dimethylformamide
DMSO	dimethylsulphoxide
DMT	4,4'-dimethoxytrityl
DNA	deoxyribonucleic acid
DNP	dinitrophenyl
dNTP	deoxynucleotide triphosphate
ε	molar extinction cefficient
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme linked immuno sorbant assay
F	fluorophore
FAM	5(6)carboxyfluorescein
FISH	fluorescence *in situ* hybridisation
Fmoc	fluorenylmethyloxycarbonyl
FRET	fluorescence resonance energy transfer
G	guanine
^{1}H	proton [NMR]
HEG	hexaethyleneglycol
HEO	hexaethylene oxide
HEX	hexachlorinated fluorescein
HPLC	high performance liquid chromatography
HMDS	hexamethyldisiloxane
Hz	Hertz
kb	1000 base pairs
LGC	Laboratory of the Government Chemist
MALDI	matrix assisted laser desorption
MeNPOC	α-methyl-2-nitropiperonyloxycarbonyl
MeOH	methanol
mer	nucleotides in length
mRNA	messenger ribonucleic acid

ms	mass spectroscopy
NBT	nitroblue tetrazolium
NMR	nuclear magnetic resonance
OD	optical density
OLA	oligonucleotide ligation assay
PC	personal computer
PCR	polymerase chain reaction
PMB	*ρ*-methoxybenzyl
PMT	photomultiplier tube
ppm	parts per million
Q	quencher
RNA	ribonucleic acid
ROX	carboxy-6-rhodamine
RP	reverse phase
RT-PCR	real time polymerase chain reaction
SAP	shrimp alkaline phosphatase
SNP	single nucleotide polymorphism
T	thymine
TBAF	tetrabutylammonium fluoride
TBDMS	*t*-butyldimethylsilyl
TCA	trichloroacetic acid
TET	tetrachlorinated fluorescein
THF	tetrahydrofuran
TIPS	1,1,3,3-tetraisopropyldisiloxane
TLC	thin layer chromatography
Tris	tris(hydroxymethyl)aminomethane
UV	ultraviolet
Vis	visible

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Chapter 1

Introduction

1.0 INTRODUCTION

1.1 DNA Structure and Function

1.1.1 DNA Primary Structure[1]

One of the definitions of life is the ability of an organism to reproduce. In order for this to happen it needs a blueprint that can easily be copied very accurately. In all living organisms the information is contained in one of two high molecular weight polymers, deoxyribonucleic acid (DNA) or ribonucleic acid (RNA).

DNA consists of four chemical units each containing three separate parts: a phosphate group, 2-deoxy-D-ribose and either a purine or pyrimidine base (thymine, cytosine, guanine or adenine).

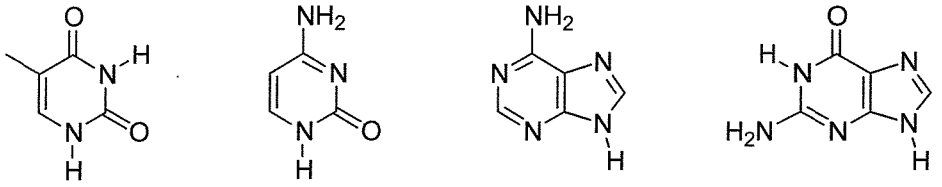


Fig 1.1.1.1 :- Structure of the DNA bases

The chemical unit containing all three of these parts is called a nucleotide, the structure containing the base and the deoxyribose sugar is called a nucleoside.

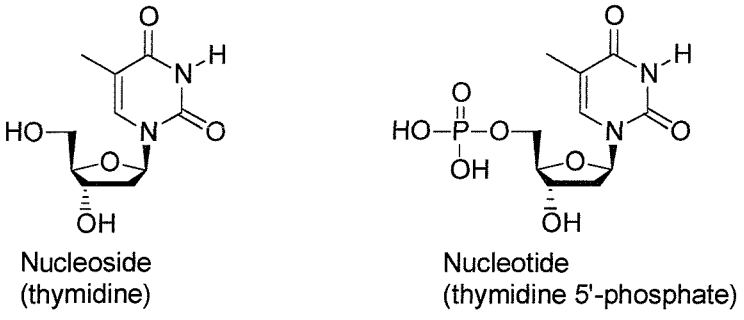


Fig 1.1.1.2 :- Structure of a DNA Nucleoside and Nucleotide

The structures of the four nucleotides were discovered by Klein and Thannhauser in 1935 by enzymatically cleaving DNA. The nucleotides form a polymer through the phosphate groups which join the 5' hydroxyl group of one nucleotide to the 3' hydroxyl group of the next. The phosphodiester linkage gives the DNA directionality.

1.1.2 DNA SECONDARY STRUCTURE

The characteristic secondary structure of DNA is known as the "double helix". The structure was first deduced by James Watson and Francis Crick in 1953,[2-3] making use of X-ray diffraction data obtained by Rosalind Franklin[4-5] and Maurice Wilkins,[6-7] and information contained in Chargaff's rules.

The structure consists of two separate, antiparallel strands of DNA wound around each other forming a helix. The coiling produces a right handed double helix with the negatively charged sugar-phosphate chain forming the external backbone.

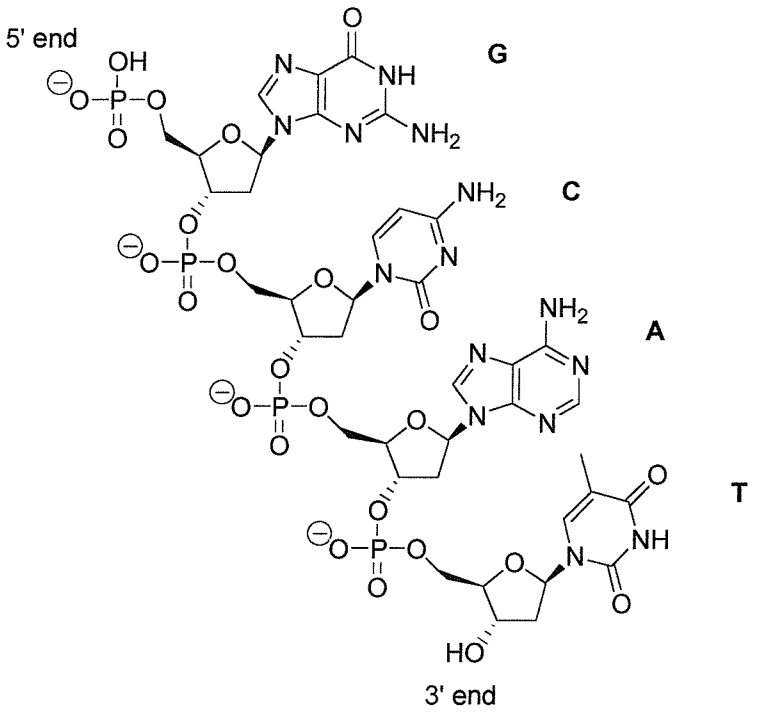


Fig 1.1.2.1 :- DNA Chain Showing the Sugar-Phosphate Backbone

The heterocyclic bases, being hydrophobic, form the core of the helix. They stack one above the other providing considerable stability to the helix. The stacking leads to the

formation of the major and minor grooves that follow the coiled path of the molecule. The two strands are held together by hydrogen bonds formed between the two bases on either helix, about 10 to 11 of these 'base pairs' make up one turn of the helix.

Under the influence of early work by Avery, MacLeod and McCarty,[8] Erwin Chargaff in the early 1950's published rules relating to the ratio of the four bases in samples of DNA. He found that the amount of adenine in a sample always equalled the amount of thymine and the amount of guanine equalled that of cytosine.[9] The rule supports the structure deduced by Watson and Crick, where adenine always binds with thymine and cytosine always pairs with guanine. The two strands of DNA are complementary so the sequence of one always defines that of the other.

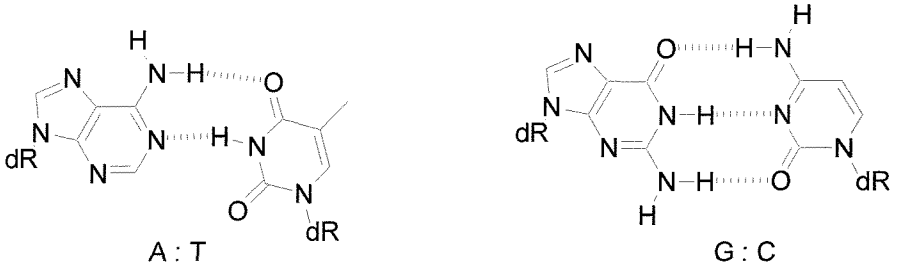


Fig 1.1.2.2 :- Hydrogen Bonding Between the Bases

1.2 The Human Genome[10-11]

The human genome consists of 3 x 10^9 base pairs (b.p.) and is stored wrapped around proteins called histones arranged into 23 discrete chromosome pairs. 3-5% of DNA contained in the chromosomes codes for the 30-40,000 genes, each with it's own unique function. The other 95% is contained in stretches of the DNA called introns and has no discernible function. Genes produce proteins, some of which are enzymes, and others structural proteins.

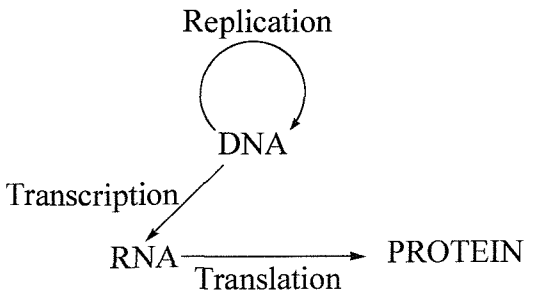


Fig 1.2 :- The Central Dogma of Molecular Biology

To produce a protein, the DNA coding sequence is copied by an enzyme called RNA polymerase. The copy, complementary to the original DNA coding sequence is made out of a form of RNA called messenger RNA (mRNA). The mRNA is transported outside the nucleus to the cytoplasm and, using a macromolecular protein-RNA complex called a ribosome, is translated into the protein. The sequence of the four bases in the mRNA determines the order of amino acids that are used to manufacture a protein. The bases are split into units of 3 bases called codons, each codon is then translated into a specific amino acid. There is a level of redundancy in the genetic code as most amino acids have more than one corresponding codon.

1.3 Genetic Disorders[12-13]

Every time a cell divides, the DNA in the nucleus is duplicated in 2 identical exact copies so that both of the two new cells have a full copy of the genome. Although the copying process is very accurate, some errors are made. It is estimated that somewhere in the region of 30 mutations will occur in an individual genome within a lifetime. These mutations can be beneficial, for example leading to a protein with improved resistance to a pathogen; however they can also be detrimental to the organism. These effects can be manifested as a disease, e.g. cancer, where a fault in several genes, a multifactorial defect, causes a cell to divide continuously. As they are usually due to somatic mutation, e.g. after exposure to radiation or chemical carcinogens, these defects tend not to be passed down a generation to become an inherited disease. The other class of genetic defects comes from modifications of the germ cells, leading to inherited genetic conditions. There are four classes of genetic disorder: autosomal dominant, autosomal recessive, X-linked and mitochondrial. Mitochondrial disorders

happen when a mutation occurs in the mitochondria of a cell. They are very rare so will not be discussed.

Every human has two copies of every chromosome, one inherited from each parent. These 22 chromosome pairs are called autosomes. The only exceptions to this are the sex chromosomes, X and Y. The female genome has two copies of X, and the male genome has one copy each of X and Y.

In the case of a recessive disorder, the progeny have to inherit two faulty copies of a gene for any symptoms to manifest themselves, although if they inherit one copy then they will become a carrier. With a dominant disorder, a fault with just one copy of a gene is enough to cause a disorder to manifest itself. The progeny of such a parent have a fifty percent chance of inheriting the condition. Most dominant disorders manifest themselves in later life, as without the possibility of becoming a carrier, a person has to be able to survive to childbearing age.

The difference between dominant and recessive conditions is due to the product for which the faulty gene codes. In a recessive disorder the gene product is generally an enzyme. Enzymes are present in the body in excess, so a 50% reduction in enzyme concentration will probably result in a satisfactory level of the enzyme. In a dominant disorder the product is likely to be a protein involved in cell structure. Any fault with the structural folding of the protein can result in failure of essential pathways in the cell, leading to cell death, (apoptosis).

X-linked chromosomal disorders only afflict males as they only inherit one copy of the X chromosome. If there is a fault within the chromosome, the child will inherit the disorder.

1.4 Genetic Testing[14,15,16,17]

Testing a human DNA sample for either forensic investigation or genetic analysis is a relatively new science, around 15 years old. The worldwide market for these applications is huge, totalling many millions of pounds a year just in the medical

diagnostics sector. It is hoped that as the technology becomes more refined, new applications will be found to avoid fatal adverse drug reactions, for instance.

A test involves taking a sample of DNA from the individual, usually a blood or tissue sample, and comparing it at a particular locus with that of a gene sequence known to cause the disorder being investigated.

A piece of synthetic DNA, an oligonucleotide, whose sequence is complementary to either the wild type or mutant gene is incubated with the gene. If the gene and probe are complementary then the two will hybridise (join) together. The probe is labelled, using one of the many techniques discussed in the following sections, and its hybridisation to the gene of interest can be detected.

Researchers interested in the particular disease under investigation have up to now analysed certain specific gene sequences. However with the recent release by the Human Genome Project of the draft sequence of the human genome, the speed with which these genes are sequenced has increased dramatically.

1.5 Isotopic Classical Labelling Methods

The first DNA probes were labelled using radioactive methods. The labels used were generally radioactive deoxyribonucleoside triphosphates incorporated directly into nucleic acid molecules by enzymatic methods. The methods employed include nick translation,[18] random priming,[19-20] polymerase chain reaction[21-22] and 3' end modification by terminal deoxynucleotidyl transferase.[23-24]

Labelling at the 5' end is carried out by phosphorylation using γ^{32}P-ATP and T4 polynucleotide kinase. The choice of labelling strategy is determined by the nature of the probe, DNA or RNA, and whether it is double stranded or single-stranded. The choice of isotope depends on the nature of the probe, the method of incorporation and the sensitivity and stability required.

An early example of the use of a radiolabelled DNA probe was described by Southern in 1975.[25] The technique he describes, known as Southern Blotting, allows the mapping of DNA fragments relative to restriction endonuclease sites. DNA is cut up into fragments by restriction enzymes; the single stranded DNA fragments are separated electrophoretically in denaturing conditions and immobilised onto a nitrocellulose membrane by blotting. ^{32}P labelled probes specific to sites onto the target nucleic acid sequence are introduced. After hybridisation and stringent washing, any band containing complementary sequences can be visualised by autoradiography. Quantification of the amount of a specific DNA or RNA present in a sample can be deduced by a similar technique known as dot blotting.[26] Here the nucleic acid contained in the sample is immobilised onto a membrane as a dot (or slot), hybridised with the probe, carefully washed, and then the signal intensity of the probe remaining on the membrane is measured. The reading directly represents the amount of target sequence present in the sample.

Radiolabelling has the advantage of very high sensitivity, for example ^{125}I has a lower detection limit of 10^{-18} molar (attomolar). Radiolabels are also easily incorporated. This technique has the following disadvantages:

- Hazardous handling, regulated by the Home Office
- Short half-life, short shelf life
- Expensive to purchase
- Limited signal emissions
- Time consuming methodology, due to numerous washing steps and long exposure times
- Waste disposal is expensive

1.6 Non Isotopic Labelling

As radiolabels are hazardous there is a need for safer, easier methods of labelling oligonucleotides and DNA. Non-covalent and non specific labelling of nucleic acids can be used, e.g. the intercalation of ethidium bromide[27] in double stranded DNA, however covalent labelling has proved to be more popular and versatile. Some of the methods employed today involve colorimetric,[28-29] chemiluminescent,[30]

bioluminescent[31] or fluorescent reporter groups.[32-33] The use of non-isotopic labels has allowed the simultaneous visualisation of several DNA probes in one experiment.[34] It has also allowed the development of efficient methods of DNA sequencing, where each nucleotide is labelled with a different fluorophore.[35]

The label used can be any molecule that can be attached to a protein or nucleic acid and is capable of producing a detectable chemical change. The ideal non-isotopic label would have the following characteristics:

- Cheap and simple to synthesise
- Stable to long term storage
- Easily modified to introduce other functionality
- Harmless
- Easy disposal
- Simple and inexpensive labelling procedure
- Stable to the conditions of oligonucleotide synthesis and deprotection if added by way of a phosphoramidite, with no effect on the aqueous solubility of the oligonucleotide, and on oligonucleotide hybridisation
- Detectable at low concentration using simple instrumentation

The label consists of three parts, the signal emitting moiety, a spacer and the reactive group.

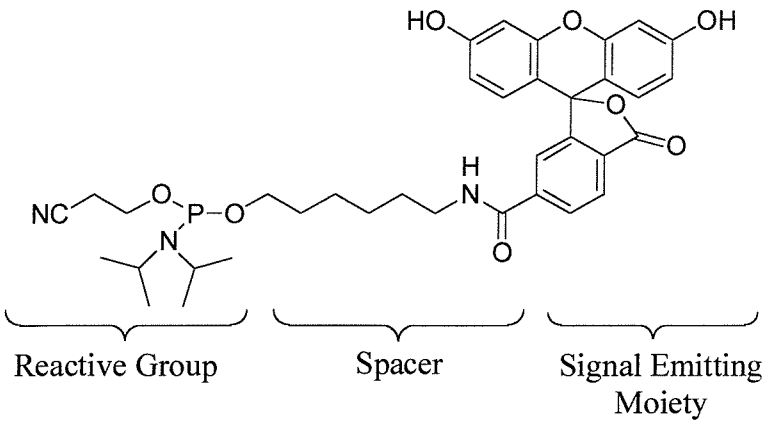


Fig 1.6 :- Fluorescein Unit Introduced by way of a Phosphoramidite

1.6.1 The Signal Moiety

Two strategies are used; direct labelling and indirect labelling. Examples of direct labels are chemiluminescent labels or enzymes such as alkaline phosphatase bound to the oligonucleotide probe.[36] For direct labels to be effective the molecule has to display a satisfactory quantum yield in aqueous solution or turnover a signal generating substrate. Indirect labelling strategies employ enzymes or haptens (small molecules to which antibodies can be raised)[37] as the label, with the signal generated sequentially. Enzymes used include horseradish peroxidase[38] and alkaline phosphatase.

1.6.2 The Spacer

The spacer's function is to keep the label a certain distance away from the oligonucleotide to prevent interference in the hybridisation.[39] Many aliphatic and aromatic molecules have been used. By varying the type of molecule used it is possible to alter the hydrophobicity and hydrophilicity of the signal group.

1.6.3 The Reactive Group

The reactive group can be any molecule capable of reacting with a nucleic acid, under mild conditions, to form a covalent linkage. Examples of the most common groups used are described:

1. Free amino groups on DNA or oligonucleotides can couple with the *N*-hydroxysuccinimide ester, activated carboxyl groups or isothiocyanate found on luminescent dyes yielding amide bonds ((a and b), fig 1.6.3)[40,41,42,43,44]
2. The labels can be synthesised as a phosphoramidite and be incorporated into DNA during automated DNA synthesis ((c), fig 1.6.3)[29,32,45]

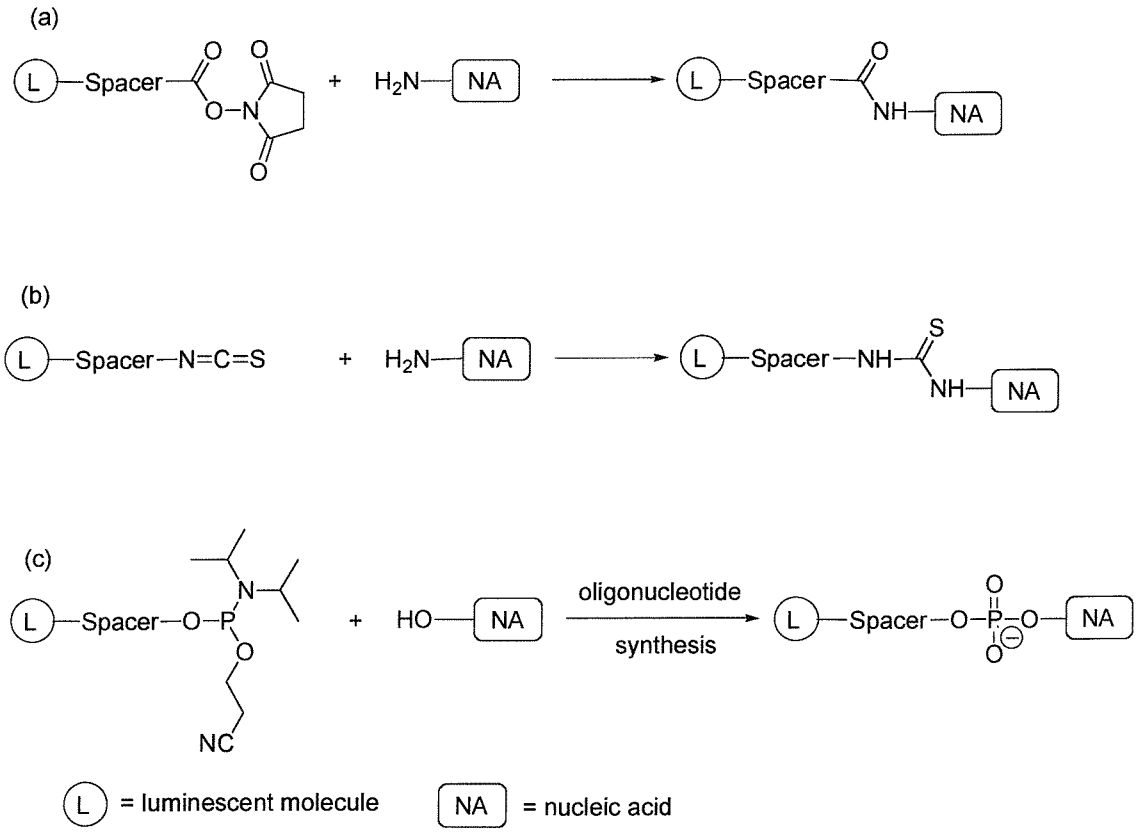


Fig 1.6.3 :- Common Label Incorporation Techniques

1.7 Indirect Labelling

Indirect labelling involves attaching a hapten to a DNA probe. The most commonly used labelling group is biotin, which can be incorporated into the DNA probe enzymically, photochemically, by reaction with amino-functionalised oligonucleotides or by incorporation of biotinylated phosphoramidites during solid-phase synthesis. The label is detected by Enzyme-Linked Immuno Sorbant Assay methodology, (ELISA).[17] In the classical ELISA method, the target and hapten bound probe complex is incubated with an antibody-enzyme conjugate, which binds to the hapten. A suitable substrate is added to produce a colorimetric or chemiluminescent signal. A more sensitive, though time consuming method is to incubate the target probe complex with a primary antibody directed against the hapten. The mixture is then incubated a second time with an antibody-enzyme conjugate, directed against the first antibody. The enzyme substrate is then added to produce the signal.

In the case of biotin,[46] the detection is made using strepavidin or avidin, which are proteins that bind very tightly to biotin. The main drawback with using biotin is that it occurs at high levels in certain tissues, making it unsuitable for some applications due to large background signals.

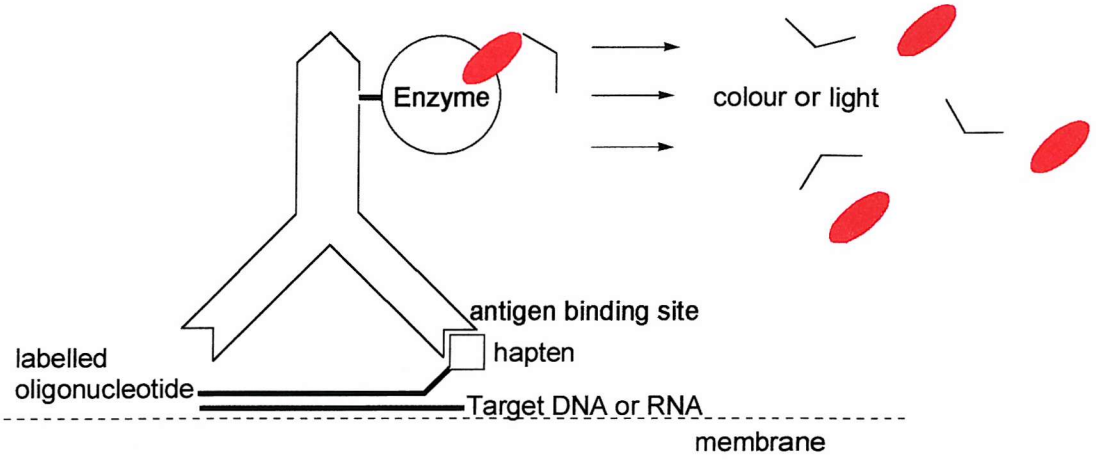


Fig 1.7 :- The ELISA Assay

Other haptens used are digoxygenin (DIG) combined with anti-DIG antibodies and dinitrophenyl (DNP) twinned with anti-DNP antibodies conjugated to alkaline phosphatase.[47-48]

1.8 Direct Labelling

In direct labelling, the reporter group is bound directly to the nucleic acid. The advantage is that much less time is needed to produce a signal.[49]

1.8.1 Radioactive Labelling

Although radiolabels can be utilised as signalling systems in the new genomic tests developed in the past decade, the problems inherent with radiolabelling still exist (Page 6).

1.8.2 Enzyme Labelling

Oligonucleotides can be conjugated with enzymes directly. The oligonucleotide is detected by adding an enzyme substrate, leading to a colorimetric or chemiluminescent change. Enzyme labels offer very high sensitivity with a very low background and very rapid visualisation. Enzymes typically used are alkaline phosphatase[36,50] and horseradish peroxidase[51] whose substrates are respectively 5-bromo-4-chloro-3-indolyl phosphate/ nitroblue tetrazolium (BCIP/ NBT) and diaminobenzamide tetrahydrochloride (DAB). The disadvantage with enzyme labels is that the synthesis and purification of oligonucleotide-conjugates is time consuming and the conjugates themselves are unsuitable for use in techniques such as PCR.

1.8.3 Chemiluminescent Labels

With a chemiluminescent label, the emitted light from the molecule comes from a chemical reaction in which a substrate undergoes a reaction to form a product with the emission of a photon.

$$\text{Chemiluminescent Label} + \text{Reactant} \xrightarrow[\text{catalyst}]{} P^* \longrightarrow P + h\nu$$

As the emission of light comes from a chemical change to the molecule, each individual molecule can only produce one photon. Compared to fluorescent systems, they are inefficient. Normally the chemiluminescent label is an enzyme substrate and several substrate molecules can be turned over by one enzyme molecule, e.g. luminol used as a substrate for horseradish peroxidase with streptavidin.

1.8.4 Properties of Fluorescence

1.8.4.1 Fluorescence[52-53]

Fluorescence is the property of some atoms and molecules to absorb light of a particular wavelength (excitation wavelength) and after a short period (fluorescence lifetime) to re-emit the light at a slightly longer wavelength (emission wavelength).

The absorption of the photonic energy by a fluorophore occurs between a number of closely spaced vibrational excited states within the molecule. The arrangement of these states is shown by the Jablonski diagram (1953).[52] In fig 1.8.4.1 the fluorophore absorbs energy from a photon placing it in an excited state (S_2). Some energy is lost within the molecule, a process called internal conversion. The molecule, now at an excited state (S_1) loses a photon and fluoresces to reach the ground state (S_0). It is also possible to populate (S_1) directly. The process occurs in a period of a few nanoseconds.

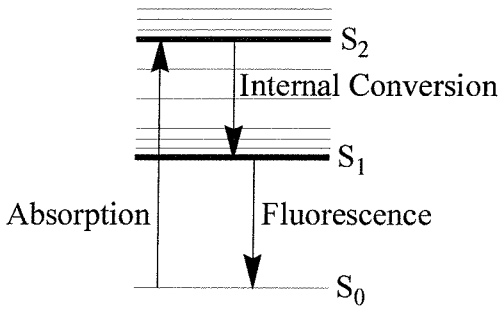


Fig 1.8.4.1 :- Jablonski Diagram

The difference in energy between the absorption and emission wavelengths is called the Stokes shift after G.G. Stokes who observed the phenomenon in 1852.[54] The Stokes shift allows fluorescence to be a sensitive analytical tool, allowing a small number of emitted photons to be detected against a much larger background of emitted photons.

The quantum yield, Q of a fluorophore is a measure of its efficiency. It is an expression of the ratio of the number of photons emitted to the photons absorbed. The closer to 1, the higher the efficiency is.

1.8.4.2 Fluorescence Quenching[55-56]

Fluorescence quenching refers to any process where the fluorophore interacts with another molecule in such a way that its fluorescence intensity is reduced.

There are two main types of quenching; collisional, sometimes known as dynamic quenching and static quenching. In both types of quenching the quencher needs to be in contact with the fluorophore for the decrease in fluorescence intensity to occur.

In collisional quenching a molecule must come into contact with the fluorophore during the lifetime of the excited state. Contact causes the fluorophore to return to the ground state without emission of a photon. Some examples of collisional quenchers include molecular oxygen, hydrogen peroxide, acrylamide, nitromethane and certain polyaromatic systems.

For static quenching to occur, the fluorophore has to form a complex with another molecule. The complex, called an exciplex is non-fluorescent. One example of such an exciplex is that of anthracene and diethylaniline. The environment surrounding the exciplex is important, as in non polar solvents, some complexes are actually fluorescent.

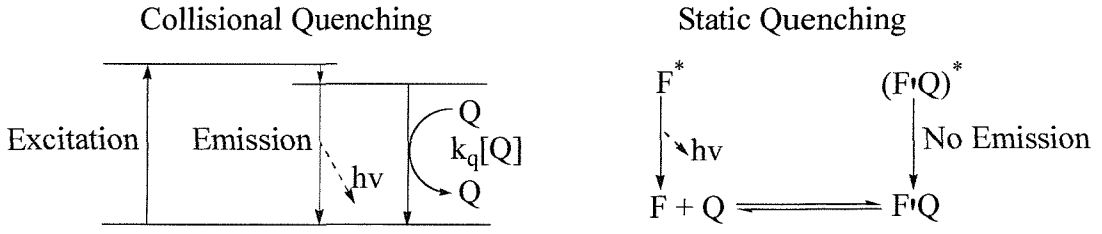


Fig 1.8.4.2 :-The Two Quenching Pathways

1.8.4.3 Fluorescence Resonance Energy Transfer (FRET)[57,58,59,60]

FRET is the transfer of excited state energy from a donor (D) to an acceptor (A). The transfer itself is an interaction between the dipoles of the donor and acceptor molecules and as such is a non-radiative transfer involving no release and reabsorption of a photon.

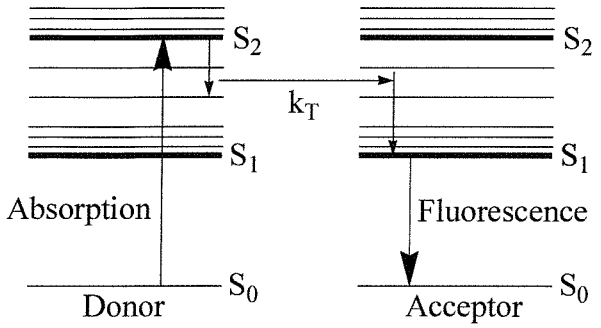


Fig 1.8.4.3.1 :- Jablonski Diagram Showing the Process of Energy Transfer

The equations that govern the energy transfer were derived in the late 1940's by Förster. The first equation shown describes how the rate of energy transfer from a donor to an acceptor relates to the distance between them.[57]

$$k_T = \frac{1}{\tau_d}\left(\frac{R_0}{r}\right)^6$$

k_T: rate of energy transfer

τ_d: lifetime of donor in absence of acceptor

r: distance between donor and acceptor

R_0: Förster distance (distance at which the efficiency of energy transfer is equal to 50%)

The Förster distance tends to be between 10-70 Å depending on the donor and acceptor used, and as energy transfer at a specific distance is always constant, FRET can be used as a spectroscopic ruler. The 10-70Å distance is important as the diameter of most proteins falls within this range.[61] The two dyes are attached to different amino acid

residues and the amount of energy transfer between them allows the distance of the two residues from each other to be calculated.

Another frequently measured parameter is that of the efficiency of energy transfer (E). E is a measure of the proportion of photons absorbed by the donor which are transferred to the acceptor. It can be equated to the quantum yield of the donor-acceptor pair. The equation is shown below.

$$E = \frac{k_T}{\tau_d^{-1} + k_T}$$

E: efficiency of energy transfer

k_T: rate of energy transfer

τ_d: lifetime of donor in absence of acceptor

The equation can also be derived directly from distance measurements:

$$E = \frac{R_0^6}{R_0^6 + r^6}$$

E: efficiency of energy transfer

R_0: Förster distance (distance at which the efficiency of energy transfer is equal to 50%)

r: distance between donor and acceptor

Efficient energy transfer is only obtained between two dyes if the emission spectrum of the donor overlaps with the absorption spectrum of the acceptor. If the overlap is sufficient then it is possible to excite the donor dye and only get emission from the acceptor dye. The reason that fluorophores can be used as signals in oligonucleotide probes is due to the Stokes shift (Page 13). With a single fluorophore the Stokes shift is quite small (~25nm), meaning that the signal to noise ratio can be quite low. Using an energy transfer system, Stokes shifts as large as 110nm can be obtained, vastly increasing the sensitivity of the technique.

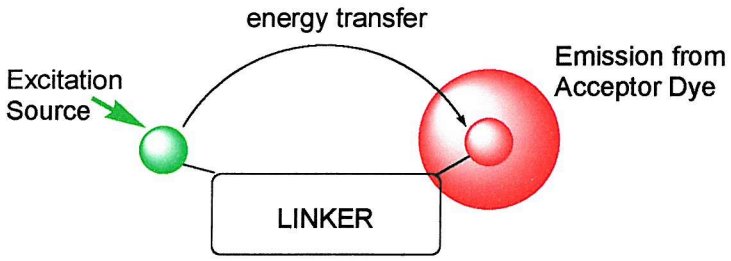


Fig 1.8.4.3.2 :- Energy Transfer System

Energy transfer can also be used as an alternative quenching technique.[62] If two fluorescent dyes, e.g. fluorescein and methyl red, are placed a distance from each other no fluorescence is observed. The lack of fluorescence is not due to a normal quenching interaction but to energy transfer occurring between the two dyes, a process termed Förster quenching. Energy absorbed by the donor dye (fluorescein) is being transferred by non-radiative processes to the second acceptor dye (methyl red). Methyl red emits in the infra red region of the spectrum, but its emission is not observed by the spectrometer.

1.8.4.4 Fluorescence Spectrometer[63]

A fluorimeter is designed to deliver excitation energy to a fluorescing species, and separate the weaker emitted light from the excitation light source.[64]

A fluorescence spectrometer consists of the following elements:

- An excitation source
- Wavelength selection device (monochromator)
- Sample cell
- Detector

The most common excitation source is a high pressure xenon arc lamp. A xenon lamp is favoured as it produces fairly constant light from 270-700nm. Mercury arc lamps can also be used but the intensity is concentrated in discrete lines. Mercury lamps are only useful if these lines correspond to the excitation wavelength of the fluorophores.

The monochromators used are likely to be diffraction gratings rather than prisms. A good diffraction grating produces low stray light levels and is chosen for the ability to detect low light levels. The best gratings are produced by holographic methods as imperfections are rare.

The detector used in nearly all fluorimeters is a photomultiplier tube (PMT). PMT's are able to detect individual photons by the production of a current, proportional to the light. A PMT consists of a photocathode; a thin film of metal at a high negative potential (-1000 to -2000V), and a series of dynodes which act as amplification stages.

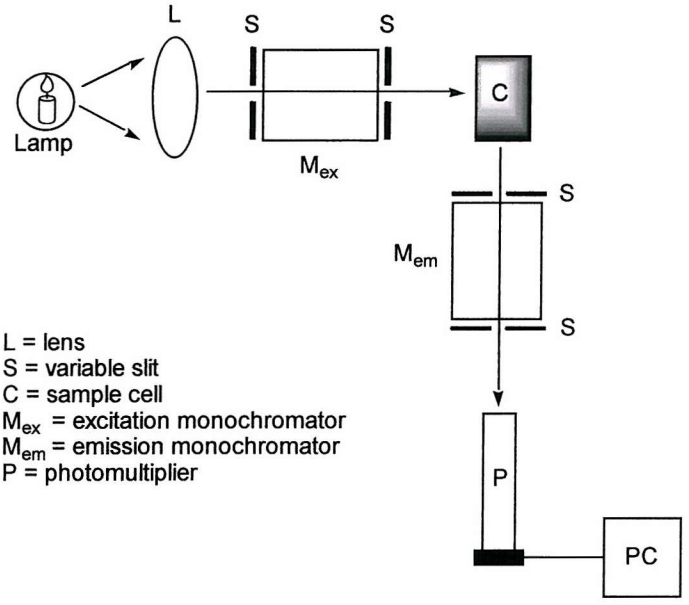


Fig 1.8.4.4 :- A Fluorescence Spectrometer

1.8.5 Fluorescent Labels

Fluorophores absorb light at one wavelength and emit it at a slightly longer wavelength with lower energy. Historically, the high sensitivities offered by fluorescence have not been realised due to light scattering, background effects and quenching, but with improvement in instrumentation the sensitivity of the technique is improving.

$$F \xrightarrow[\text{Light}]{} F^* \longrightarrow F + h\nu$$

There are many commercially available fluorophores with diverse properties. The most commonly used are 6-carboxyfluorescein (FAM)[32,49,65,66], its tetra and hexachlorinated forms, TET and HEX and carboxy-X-rhodamine (ROX).[67] The fluorescein derivatives are very cheap and have a good variety of absorption and emission wavelengths allowing the detection of more than one dye in an experiment.[68] They suffer from photo bleaching however, a phenomenon in which the light absorbed by the dye causes a change in its structure making it become non-fluorescent. In addition, fluorescein based dyes have broad emission peaks meaning the sensitivity of multiple probe systems is dramatically reduced; the dyes are also pH sensitive.[69]

New dyes have now been brought into the market which dramatically reduce these problems, two of the most important groups are the CyeDyes and the BODIPY dyes.

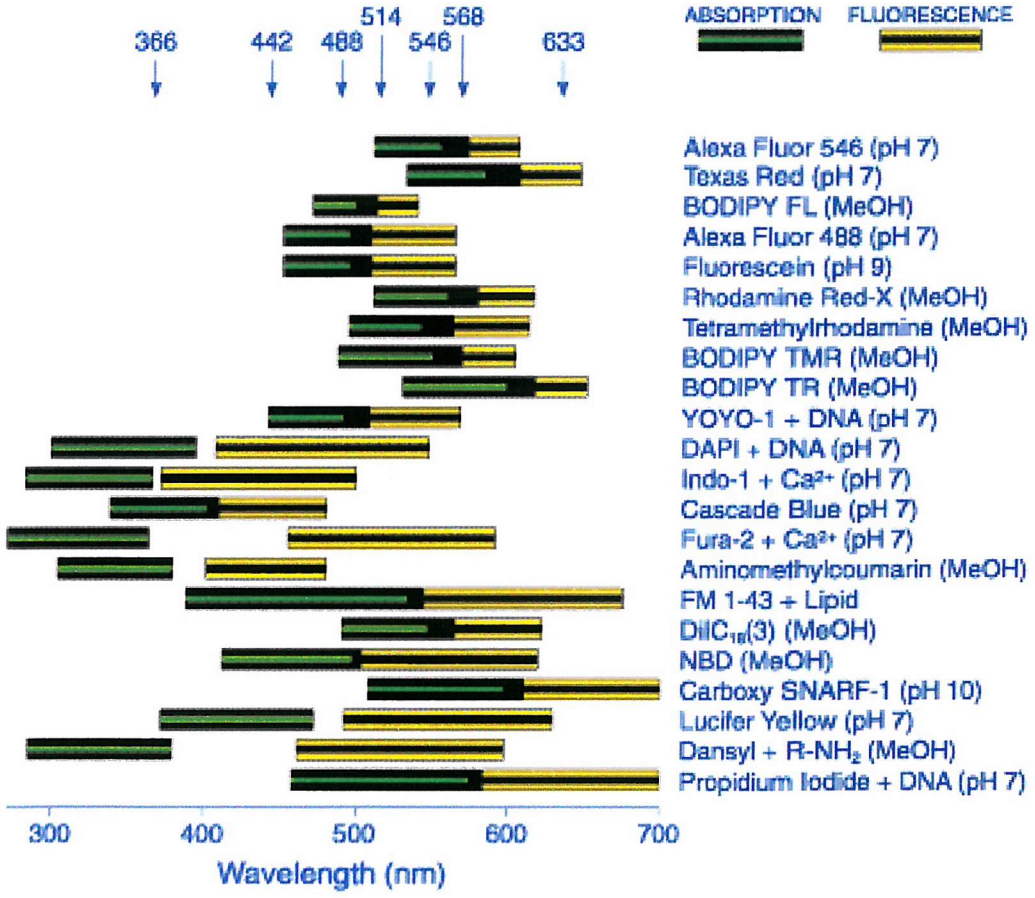
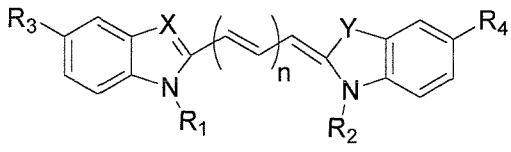


Fig 1.8.5 :- Excitation and Emission Maxima of Various Fluorophores

1.8.6 Cye Dyes

Sold under licence by Amersham the Cye dyes[70] are a covalent labelling system that emit in the far-red region of the visible spectrum. They have properties that are much improved over fluorescein based dyes, including:

- High extinction coefficients.
- High quantum yields.
- Excellent photostability.
- Excellent pH stability



X, Y = S, O, NR, $C(CH_3)_2$

n = 1, 2, 3.....

R_1 - R_4 = substituent

Fig 1.8.6 :- General Structure of a CyeDye

The spectral properties of the dyes, for example the excitation and emission maxima can be tuned by selecting the appropiate heterocyclic element (X and Y) and altering the length of the polymethine chain (n). The substituents R_1-R_4 can also be varied to alter the overall charge, reactivity and solubility of the molecule.

1.8.7 Lanthanide Dyes

Lanthanides form chelates that are highly fluorescent with large Stokes shifts and extremely long lifetimes.[71,72,73] DNA can be directly labelled with lanthanide chelates by incorporation of labelled deoxynucleoside triphosphosphates via nick translation, random priming, PCR and ruthenium phosphoramidites if using oligonucleotides. Hurskainen *et al.*[74] describe a method using the amino group on cytidine, which undergoes a transamination reaction in the presence of sodium bisulfite diamine. The free primary amine groups react with an isothiocyanate derivative of a europium

chelate. The method leads to multiple labelling of the DNA, which can affect the efficiency of hybridisation by altering the melting temperature of the probe-target duplex. It has been reported that the optimum system contains four to eight europium chelates per hundred bases. The sensitivity of these chelates is comparable to the detection limits achieved with radioisotopes.

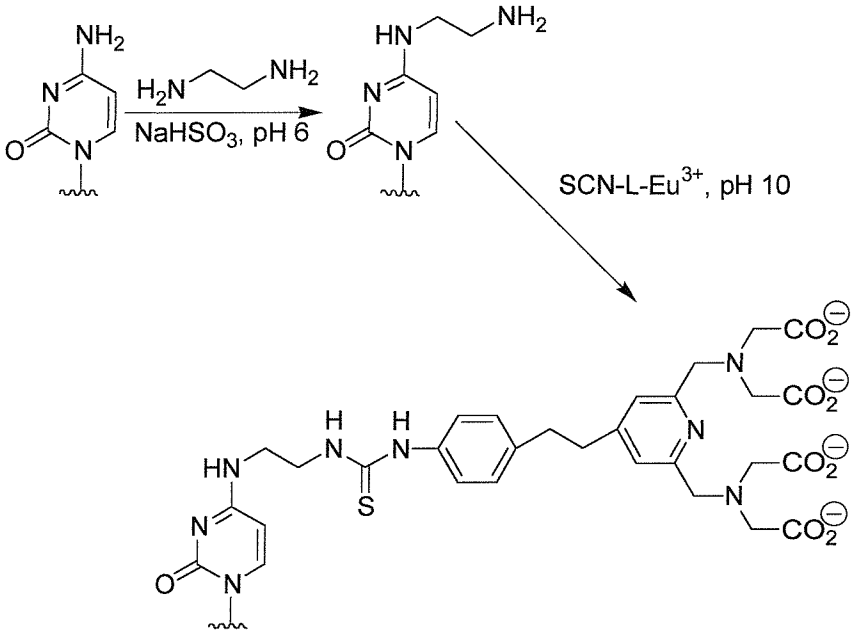


Fig 1.8.7 :- Labelling of Cytosine with a Europium Chelate

1.8.8 Quantum Dots[75]

Quantum dots are nanocrystalline particles of CdSe of a certain radius. They are coated with an inorganic shell, most commonly ZnS. Invented in the mid 1990's quantum dots have very broad absorption spectra and a narrow emission band. They can be tuned to cover a broad spectral range from ~500-~650nm depending on the size of the CdSe particle. They are also pH stable and do not suffer from photobleaching. Quantum dots are quite expensive and the synthesis and purification is time consuming. The dots are large compared to fluorophores limiting their use in certain analytical techniques.

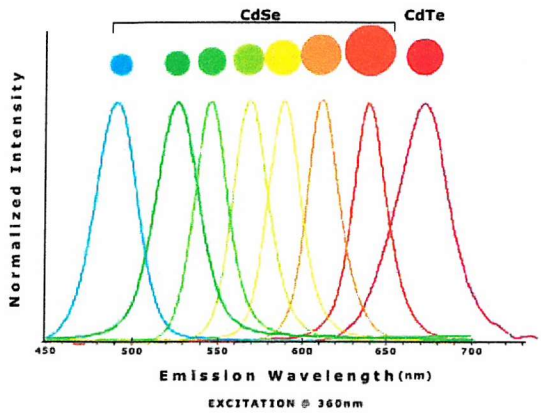


Fig 1.8.8 :- Emission Profile of Quantum Dots

1.8.9 Nanoparticle Probes[76-77]

Metal nanoparticles can be designed to scatter light of different wavelengths according to their surface plasmon resonance, the ability of a small cluster of certain atoms to emit light when excited due to the collective oscillation of electrons in the atom cluster induced by an interacting electromagnetic field. To be of use in genomic analysis the surface of the particle is functionalised with oligonucleotides of a distinct sequence. The nanoparticle can then function as a hybridisation probe (Page 27). Nanoparticles have the advantage of inexpensive fabrication costs and are not photobleached.

1.9 Biological Techniques Used in Genetic Analysis

1.9.1 Polymerase Chain Reaction

PCR is a technique used to amplify a sequence of DNA. The technique involves taking two oligonucleotide primers, between 17-30 nucleotides in length, which are designed to flank the sequence to be amplified. PCR has three steps:

- Denaturisation
- Annealing
- Elongation

The sample is heated to 95°C causing the double stranded DNA target to denature. The mixture is next cooled to 55°C allowing the two primers to anneal to the target

strand then heated to 72°C allowing the DNA polymerase present to extend the target region of the DNA. After three cycles, two double stranded molecules comprising precisely the target region in double stranded form have been synthesised. The following cycles produce an exponential accumulation of target DNA so that after 22 cycles there has been a 10^6 fold amplification of the target region of DNA.

In the original PCR method,[78] Klenow DNA polymerase was used. The heat used in the experiment denatured the DNA polymerase so a fresh aliquot of DNA polymerase needed to be added after each cycle. To avoid the risk of cross contamination a heat stable polymerase was used. Isolated from the bacteria *Thermus aquaticus* the enzyme, *Taq* polymerase has a half life of over 2 hours at 95°C making it ideal for PCR.[79-80] Performing the extension at high temperatures leads to a better specificity of primer annealing. The disadvantage is that *Taq* polymerase lacks a 3'→5' proof reading exonuclease function leading to copying errors in the amplification. Other heat-stable polymerases have been used to overcome these problems.[81]

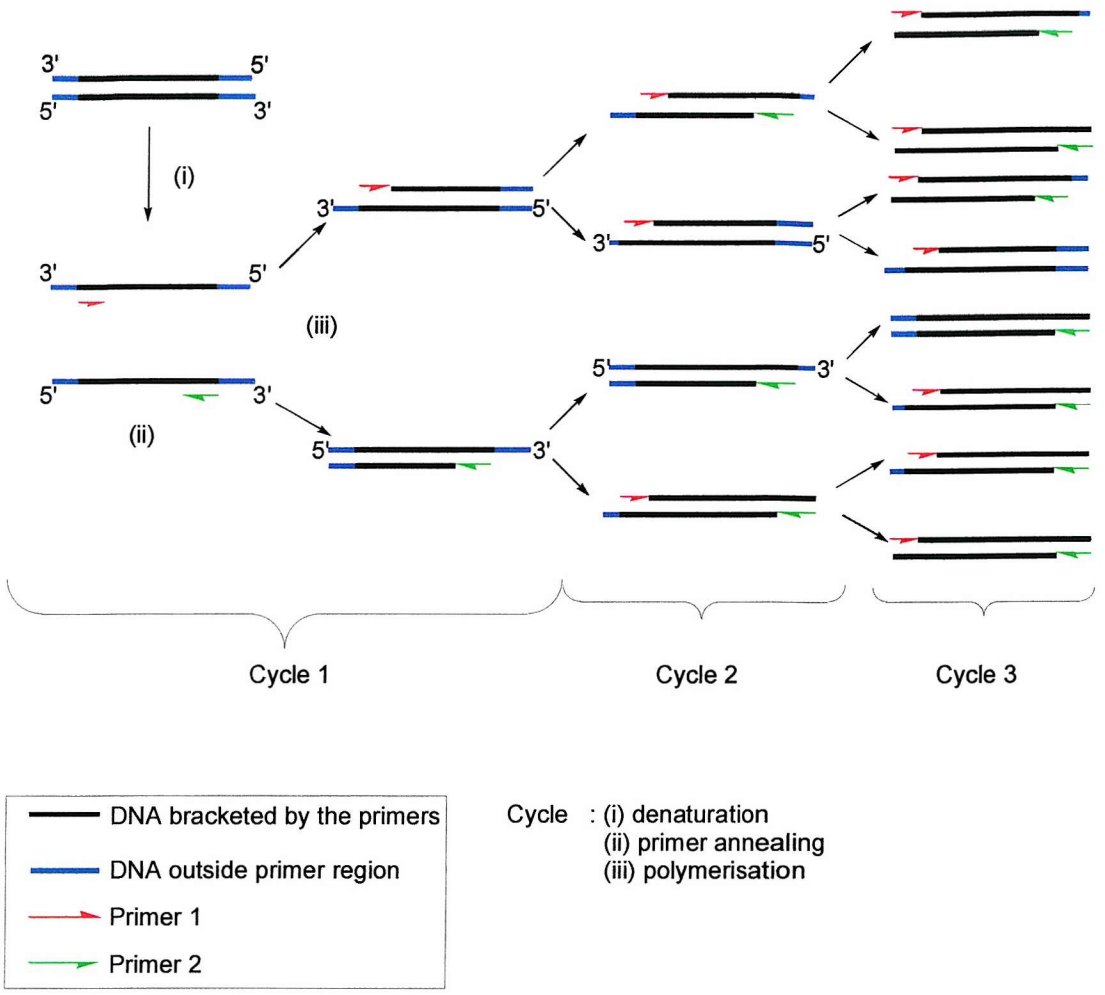


Fig 1.9.1 :- Three Cycles of a PCR Reaction

1.9.2 Hot Start PCR

Hot start PCR was designed to alleviate problems associated with background PCR signals, e.g. primer dimer and non-specific priming. The initial procedure was manual. The reaction mixture is assembled lacking an essential component; magnesium ions or DNA polymerase for the initial heating cycle, so no amplification occurs. The tube is then opened and the missing ingredient added, allowing the PCR to proceed as normal. The manual method introduces a risk of cross contamination so new technologies where the experiment does not need to be opened to air have been developed. One of these involves the use of an enzyme, which binds to the polymerase inhibiting it. The enzyme is denatured by heat allowing it to then become active. The polymerase can

also be fixed in wax, which melts at a high temperature releasing the active polymerase.

1.9.3 Fluorescence In Situ Hybridisation (FISH)[82]

Fluorescence in situ hybridisation, or FISH is a powerful tool in genetic analysis allowing identification of the presence and location of cellular DNA or RNA within morphological preserved chromosome preparations with sensitivity and ease. The technique uses a probe, either directly or indirectly labelled, which binds to a target sequence within a fixed cell or tissue culture. The label allows the location of the probe within the sample to be identified.

In situ hybridisation was introduced by Gall and Pardue in 1969.[83] Initially the technique used radioactively labelled RNA probes, however isotopic probes produced very high backgrounds caused by the isotopic label reacting with the sensitive photographic emulsion.[84] The high background limited the ability to precisely locate the target sequence. In response, a number of non-isotopically labelled probes[85] were developed enabling quick, simple and precise localisation of the target sequences. One of the first non-isotopic probes developed used biotin as a label. The biotin was detectable by fluorescently labelled avidin, enzyme linked reactions or gold particles.

The probe used is generally 200bp to 1kb long. Longer probes increase non specific binding and lead to a higher background, however if the probe is too short then they become difficult to detect due to insufficient hybridisation. The target sequence needs to be accessible to the probe and must not be degraded by nucleases in situ.

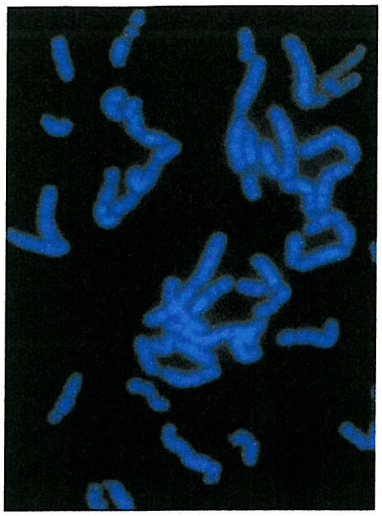


Fig 1.9.3 :- Human metaphase chromosomes stained with DAPI (Image from Violette Paragas and Jeff Pollack, Molecular Probes, Inc.)

1.10 Practical Examples of Probe Systems[86]

There are many techniques discussed in the literature for the detection of specific DNA sequences. The techniques used can be split into two main types: homogeneous and heterogeneous. Heterogeneous assays such as radiolabelling rely on manipulating a sample through more than one procedure and present more disadvantages over newer and more efficient homogeneous assays. Homogeneous assays are advantageous because:

- Less user operations required
- Absence of downstream analysis reducing the 'time to result'
- Reduction of risk of cross contamination by use of closed tubes
- Semi-quantitative output, as opposed to qualitative
- Observed in real-time

The assays can be split into hybridisation, enzymatic and microarray techniques.

1.10.1 Hybridisation Techniques

Alleles can be distinguished by hybridising complementary oligonucleotide sequences. As both alleles are very similar it is important to design the experiment to reduce the cross talk inherent with hybridisation probes. Visualisation systems used tend to be based around a fluorescence/quencher system although mass spectroscopy can be used.

1.10.1.1 Molecular Beacons[87,88,89,90]

Molecular beacons, invented by Tyagi and Kramer are a new technique designed to eliminate the removal step of unhybridised probes from solution and the washing steps implicated in previous hybridisation probe design.

The probe comprises a stem and loop structure. The loop portion of the molecule is a probe sequence that is complementary to a predetermined sequence in a target nucleic acid. The stem comprises two complementary arm sequences that can anneal together. The arm sequences are unrelated to the target sequence. To one of the arm sequences is attached a fluorophore, and to the other a quencher moiety. At room temperature the beacon configuration keeps the fluorophore and quencher close together so that energy received by the fluorophore is transferred to the quencher and is dissipated as heat, rather than being emitted as light. In the presence of the target molecule the probe forms a hybrid that is longer and more stable than that formed by the arm sequences. The beacon undergoes a spontaneous conformational change forcing the arm sequences apart. The quencher is now held apart from the fluorophore and excitation of the fluorophore leads to a fluorescent signal being produced.

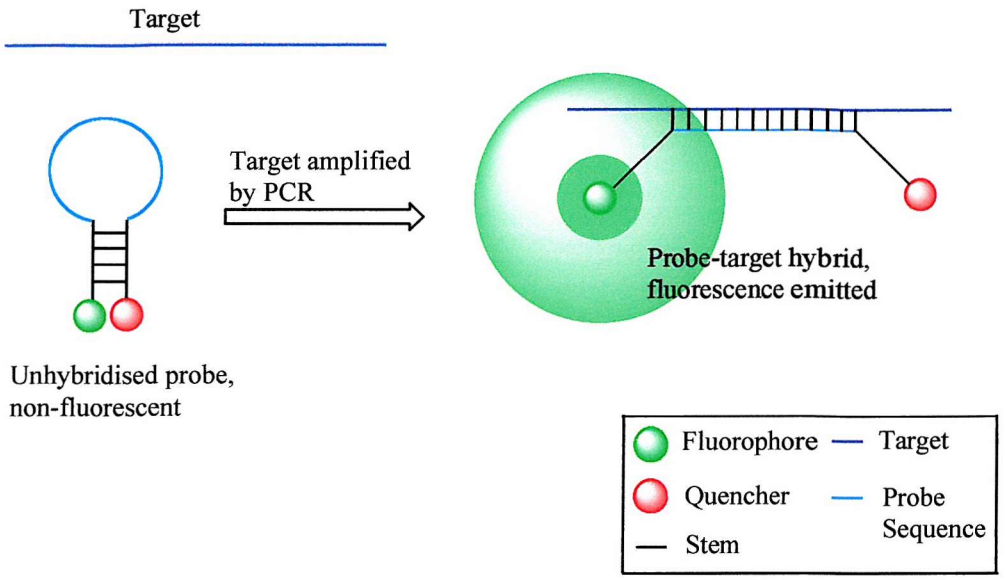


Fig 1.10.1.1 :- A Molecular Beacon

It is also possible to use molecular beacons for real time PCR (RT-PCR). The beacon is introduced to a tube containing the target, both PCR primers and the *Taq* polymerase. The PCR is cycled and the fluorescence produced is measured at each room temperature phase. A plot of fluorescence intensity vs the number of cycles is obtained from the experiment. The advantage of RT-PCR is that a small amount of target is able to be detected in a homogeneous assay.

1.10.1.2 Acridine Quenchers[91]

Acridine quencher probes are hybridisation probes. The probe sequence contains both a fluorophore (6-carboxy fluorescein) and an acridine moiety (9-amino-6-chloro-2-methoxyacridine). The fluorophore and acridine are held close together in the unhybridised probe and due to energy transfer between the two the probe is essentially non-fluorescent. When a complementary target is introduced the acridine interacts with the double stranded DNA of the probe target hybrid and no longer quenches the fluorophore. A fluorescent signal is then produced that can be measured.

Acridine quencher probes are devoid of probe secondary structure, meaning that shorter probes can be used with enhanced mismatch discrimination. The acridine also stabilises the probe target hybrid leading to a greater discrimination between the bound and unbound probe.

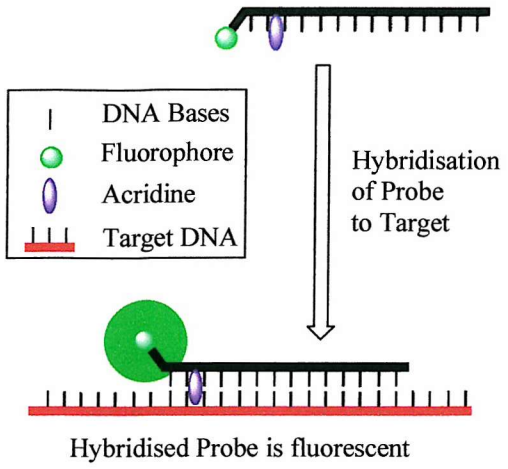


Fig 1.10.1.2 :- An Acridine Probe

1.10.1.3 Hybeacons (LGC)[92]

Developed in 2000 by LGC, hybeacons are a novel type of hybridisation probe. The probe consists of a single stranded oligonucleotide 15-30 nucleotides long. Previously the probe contained both a fluorophore and quencher moiety affixed to modified nucleosides, held a certain distance from each other. When a complementary target is introduced the probe and target anneal. The formation of double stranded DNA changes the angular orientation of the fluorophore and quencher and an increase in fluoresence is observed. The distance between the pair can be 1 to 6 nucleotides. At a distance of 6 nucleotides the increase of fluorescence between probe and probe/target is about 3 fold. Subsequent experiments have shown in probes without a quencher an increase in fluorescence is still observed when a double stranded DNA probe-target complex is formed. The reason for the increase in fluorescence is unclear.

As there is no secondary structure with the probe as opposed to other hybridisation probes, e.g. molecular beacons the annealing between the probe and target strands is much quicker and more efficient.

1.10.1.4 Sunrise Primers[93]

An amplification system has been developed called SUNRISE™. The basis is the incorporation of fluorescence energy-transfer labelled primers into PCR products. These primers have specific target sequences at the 3' end and hairpin structures at the 5' end that are non fluorescent prior to PCR. Incorporation of the primer during PCR causes the hairpin to be disrupted and fluorescence is detectable. The fluorescent signal intensifies with the increasing number of PCR cycles as more primers are incorporated.

Following this, a Universal SUNRISE™ Primer was reported to allow the incorporation of an identical hairpin primer into any target nucleic acid. An initial PCR step incorporates a primer that carries an oligonucleotide tail (15 bases) that is complementary to the sequence of the universal hairpin primer. Following incorporation of the tail, the hairpin primer takes over and is unwound in the same manner as before.

Sunrise primers have been successfully applied to a closed tube amplification detection format to distinguish between the normal (WG4) and mutant (RG4) alleles of the β(3)-adrenergic receptor gene.

1.10.1.5 Scorpions Primers[66,94]

Scorpions primers are used in a specific detection method in which the amplicon produced is probed specifically with an internal oligonucleotide leading to a change in fluorescence if a target sequence is present. The unimolecular binding interaction is favoured over bimolecular probes (e.g. molecular beacons). Considerations with bimolecular interactions are:

- Intra-strand folding can mask the probe binding sites making amplicon detection inefficient
- Inter strand competition is noticed late in PCR with the double stranded amplicon competing with the probe for binding

In Scorpions the probe element is attached to the 5'-end of one PCR primer, *via* a linker which blocks copying of the 5'-end. The probe hybridises to a different section of the amplicon than the primer. The probe can therefore bind to its target when the target site has been incorporated into the same molecule by extension of the tailed primer.

The advantages of the scorpions technique are:

- The binding interaction is unimolecular
- The probe/target binding is thermodynamically favoured over secondary structures
- The probe/target interaction is kinetically favoured over re-annealing of 2 strands of the PCR amplicon, 1 amplicon yields 1 fluorescent signal

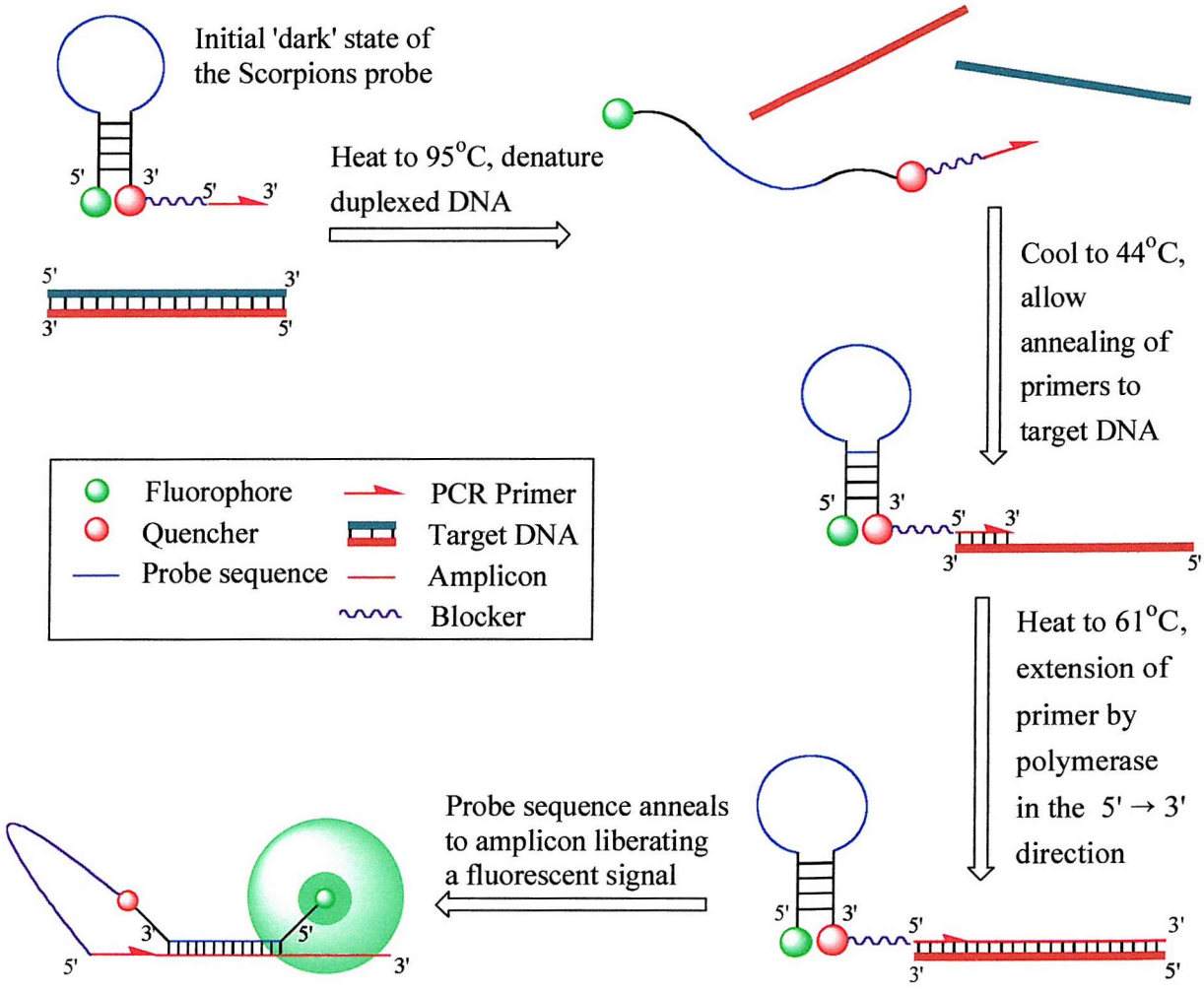


Fig 1.10.1.5 :- Scorpions Primers

1.10.1.6 Good Assay[95]

Matrix-assisted laser desorption/ionisation mass spectroscopy (MALDI-MS) has revolutionised the instrumental detection of biomolecules.

Several methods of genetic analysis have utilised MALDI-MS as a detection method but all have the disadvantage of relying heavily on purification procedures before diagnosis can be made. The GOOD assay developed in 2000 by Sauer and co workers[96] can sensitively pick SNP's after PCR without purification.

The sample to be analysed is amplified by PCR. Any excess dNTPs present are degraded using shrimp alkaline phosphatase (SAP) and a primer containing a charge tag near the 3'-end is introduced. Another round of PCR is carried out using α-S-dNTPs to form an oligonucleotide containing a phosphorothioate backbone. Any DNA with a convential phosphate backbone is degraded using a 5'-phosphodiesterase leaving the phosphorothioate oligonucleotide complete with the mass tag. The oligonucleotide is alkylated with MeI to neutralise the DNA and samples are introduced directly onto the MALDI-MS without purification. Peaks corresponding to the wild type or mutant sequence can be observed

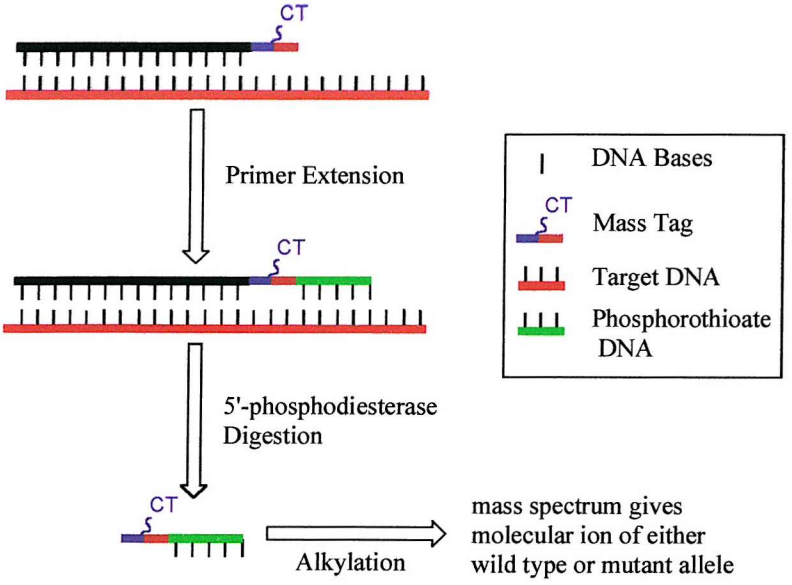


Fig 1.10.1.6 :- The GOOD Assay

1.10.2 Enzymatic Methods

Adding an enzymatic step to a genomic test improves the fidelity. In all the methods a hybridisation event is followed by the intervention of an enzyme. Many enzymes are employed including DNA polymerase and DNA ligases.

1.10.2.1 Amplification Refractory Mutation System (ARMS)

Invented in 1989 by Newton and co workers, ARMS was one of the first techniques established to use PCR in a homogeneous genomic assay.[97] The SNP to be probed for is designed to fall at the 3'-end of the PCR primer binding sequence. A single b.p. mismatch at the 3'-end of the primer will prevent the primer being extended and consequently no PCR amplification of the target sequence will occur in the case of a mutant.

Two primers are synthesised, one complementary to the mutant, the other to the wild type gene. Two aliquots of the sample are taken and a primer introduced to each. PCR is then performed. Detection in the original system relied on gel electrophoresis, a heterogeneous technique. Newer variations of ARMS however utilise fluorescent labels through labelling each primer with a different fluorophore.[98] The primer that sucessfully binds to the target sequence can then be detected by the wavelength of fluorescence emitted. Taqman can also be used.

1.10.2.2 Taqman[99,100,101,102]

Taqman, a technique developed in 1991 uses the inherent 5'→3' exonuclease activity of *Taq* DNA polymerase allowing the assay to provide probe detection concurrently with target amplification and as such is a real-time PCR method.

Taq polymerase, in common with most DNA polymerases possesses an exonuclease activity allowing it to correct replication mistakes. In the case of *Taq* it cleaves 5' terminal nucleotides in double stranded DNA releasing both mono and oligonucleotides, a process called nick translation.

If a probe is introduced on the 3'-side of the enzyme-DNA binding site, the probe will be cleaved. It is possible to introduce a means of detecting whether the probe has been cleaved, or is intact. Early Taqman experiments used radiolabelling, but due to the disadvantages inherent in isotope labelling, fluorescence is now used. The probe is designed with a fluorophore and quencher attached to modified nucleotides either side of a *Taq* polymerase cleavage site. When the fluorophore and quencher are kept close together in the Taqman probe no fluorescence is observed (Page 14, Page 28). If after PCR, the amplicon and probe are complementary then the two will anneal together. *Taq* polymerase can now degrade the double stranded DNA present (the Taqman probe) and in the process cleave the fluorophore from the quencher. Now free in solution, the fluorophore is no longer quenched and excitation yields a fluorescent signal.

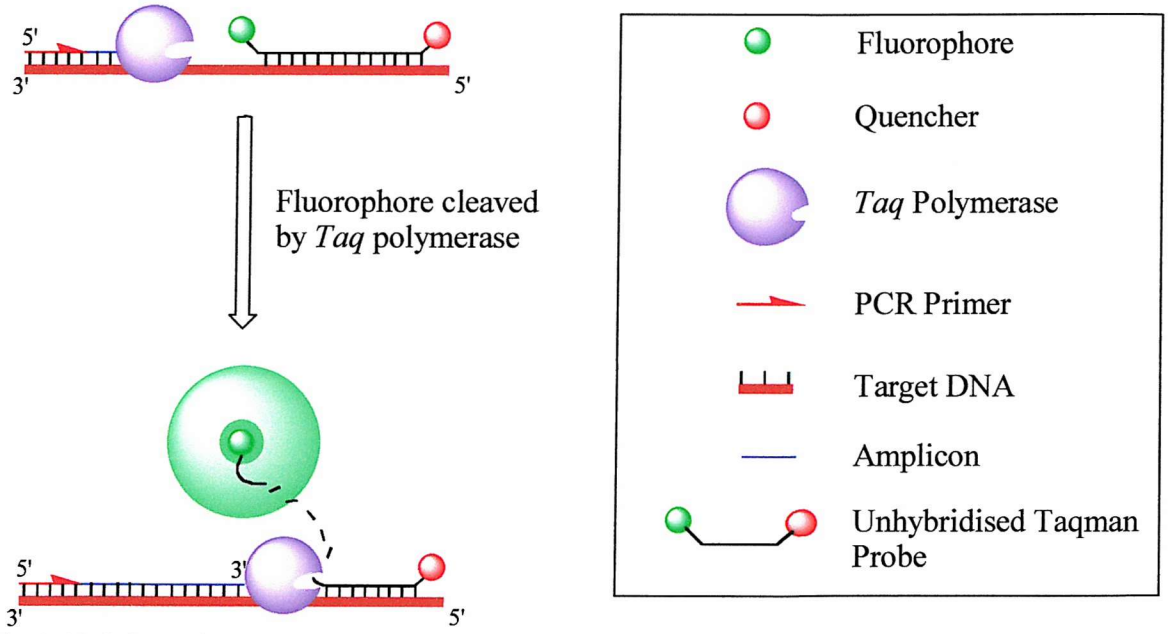


Fig 1.10.2.2 :- The Taqman Assay

1.10.2.3 Oligonucleotide Ligation Assay (OLA)[103,104,105,106,107]

OLA uses the properties of DNA ligase as a detection technique. In the assay two oligonucleotides adjacent to each other are ligated enzymatically by a DNA ligase, when the bases next to the ligation position are fully complementary to the template strand. The ligation can be detected in a variety of ways; in the past fluorescence, UV melting, gel electrophoresis and ELISA have all been used.

With gel electrophoresis the 5' probe is labelled with a polyhexaethylene oxide (HEO) tail while the 3' probe is labelled with 6-carboxyfluorescein. If ligation takes place then the ligated product being larger will run at an increased rate down the gel. Detection of the bands is performed by irradiating with UV and recording the fluorescein emission.

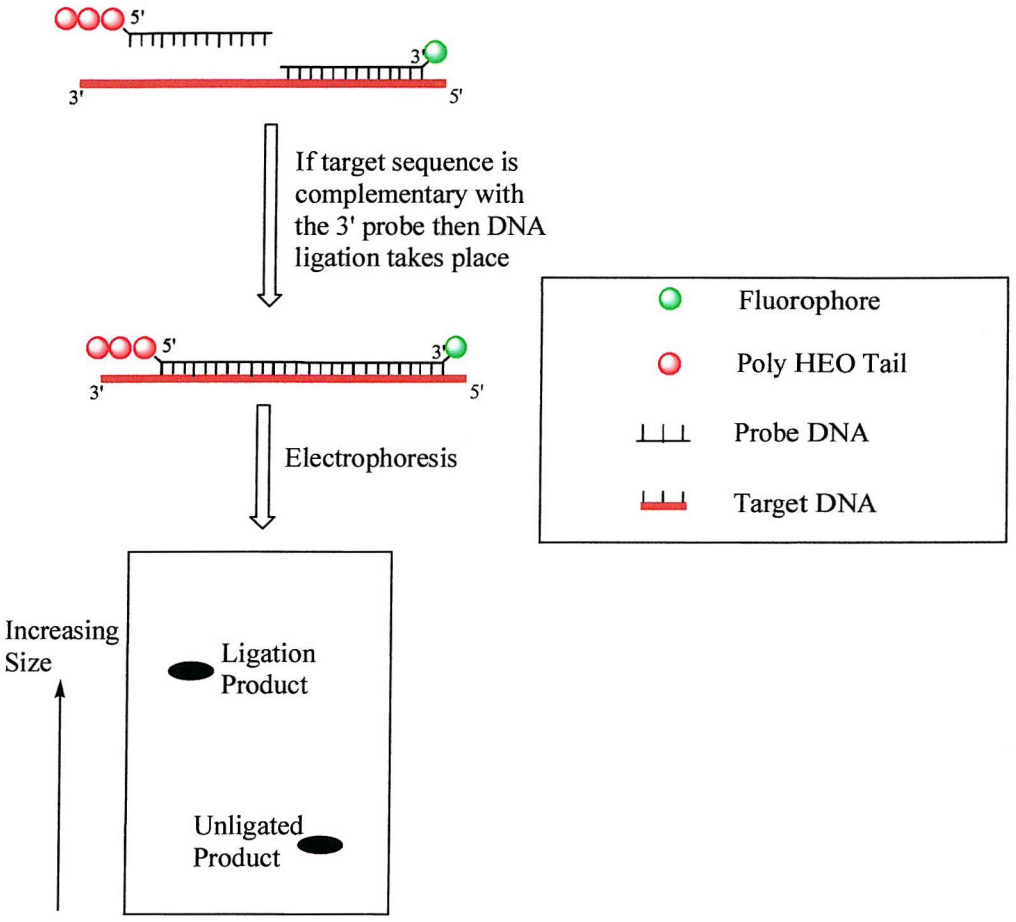


Fig 1.10.2.3 :- The OLA Assay

1.10.2.4 Invader Assay (Flap Endonuclease)[108,109,110]

Third wave technologies Inc. have developed an isothermal, quantative assay that requires no PCR and generates amplified signals upon probe-target hybridisation. Termed 'Invader Assay' the system is comprised of two probes that hybridise adjacent to each other on a target nucleic acid. The probes are designed so that the 3'-end of the upstream Invader probe overlaps the 5'-end of the downstream, labelled, signal probe.

The invasion of the signal probe-target duplex region causes the displacement (by at least one base) of a portion of the 5' signal probe, creating a branched structure with a single stranded flap. The junction between the flap and the partially invaded duplex is recognised and cleaved by the Cleavase™ enzyme.

Detection can be effected by direct gel analysis, FRET or mass spectrometry. The relatively high temperature of the assay causes the cleaved signal probe to dissociate and another intact signal probe to anneal, whilst the invader probe remains bound to the target. Providing the signal probe is in excess there will be an amplification of signal. It has been shown, using Human cytomegalovirus genome as a target, that cleavage generates a signal that is proportional to the number of copies of target DNA present. Carry-over contamination, false positives and background signals are all alleviated as signal is only produced in the presence of a target.

Chapter 2

The Design of an Energy Transfer Linker

2.0 The Design of an Energy Transfer Linker

2.0.1 Introduction

The preceeding chapter introduced various reagents, techniques and strategies employed in the design of systems to probe DNA sequences. On page 17 a system for linking two fluorescent dyes together to enable energy transfer to occur was described. There is a need for novel energy transfer linkers in genetic analysis as many of the probe systems described in chapter 1 utilise this technique. The following chapters aim to record the work carried out in the design, synthesis and testing of new energy transfer linkers and probes.

2.0.2 Limitations in the Use of the Roche LightCycler for Genetic Analysis

Most genetic diseases can be caused by multiple single nucleotide polymorphisms (SNPs), so the ability to detect more than one mutation simultaneously in a test is valuable. Oligonucleotide probes, designed to detect several mutations are labelled with different fluorophores. Each fluorophore absorbs and emits light at discrete wavelengths, so the emission wavelength of the probe-target complex will change depending on the nature of fluorophore used in a particular probe. This technique is called multiplexing. It is difficult to carry out multiplexing experiments using currently available equipment as platforms such as the Roche LightCycler can only excite at one wavelength. As all fluorophores have broadly similar Stokes shifts, only one dye can be used for each experiment. Energy transfer allows the same donor dye to be linked to many different acceptors so that multiplexing can take place, as acceptors that emit from 530nm to above 600nm can be excited from a single donor.

2.0.3 The Design of an Energy Transfer Probe

There are different methods of designing energy transfer probes for use in genetic analysis. The simplest method is to synthesise two probe oligonucleotides, one labelled at the 5'-end, the other at the 3'-end.[111,112] When these probes hybridise to

the target, the dyes are brought within close proximity allowing energy transfer to occur. In SNP detection one of the probes can be synthesised with two or more slightly different sequences. One sequence recognises the wild type target and the other the mutant. Each of the two probes is labelled with a different dye allowing multiplexing to be carried out (fig 2.0.3.1).

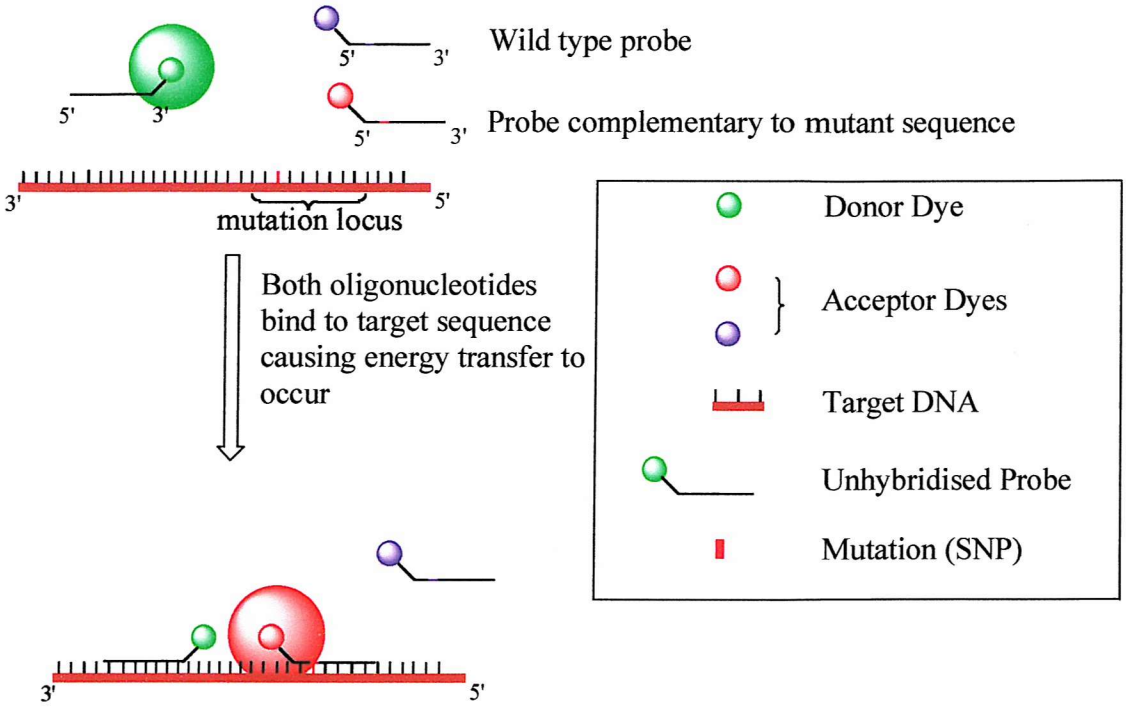


Fig 2.0.3.1 :- A Simple Energy Transfer Probe Assay

The binding of two oligonucleotides to a target sequence simultaneously is not as easy to achieve as the binding of a single oligonucleotide to the target.

A second way of carrying out energy transfer by using hybridisation probes is to use a single dye-labelled probe in the presence of an intercalating dye as the FRET donor. The dye will only intercalate into double stranded DNA, so only when probe and target hybridise will a fluorescent signal be observed (fig 2.0.3.2). Intercalators can stabilise DNA duplexes so the ability of such a system to discriminate between single base pair mismatches maybe reduced.

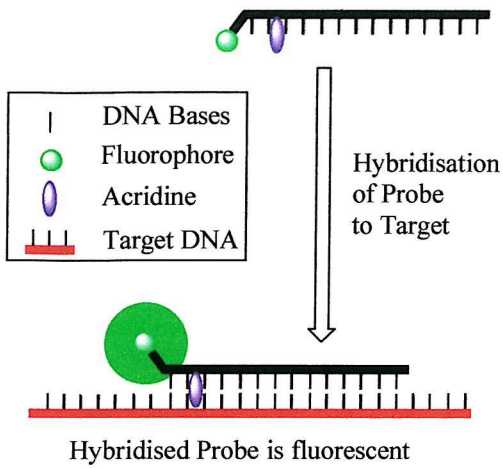


Fig 2.0.3.2 :- Energy Transfer Probe Using An Intercalating Dye

A third way of achieving efficient energy transfer is to join two dyes together by means of a linker.[113,114] The linker can be coupled onto one end of a molecular beacon probe (fig 2.0.3.3).

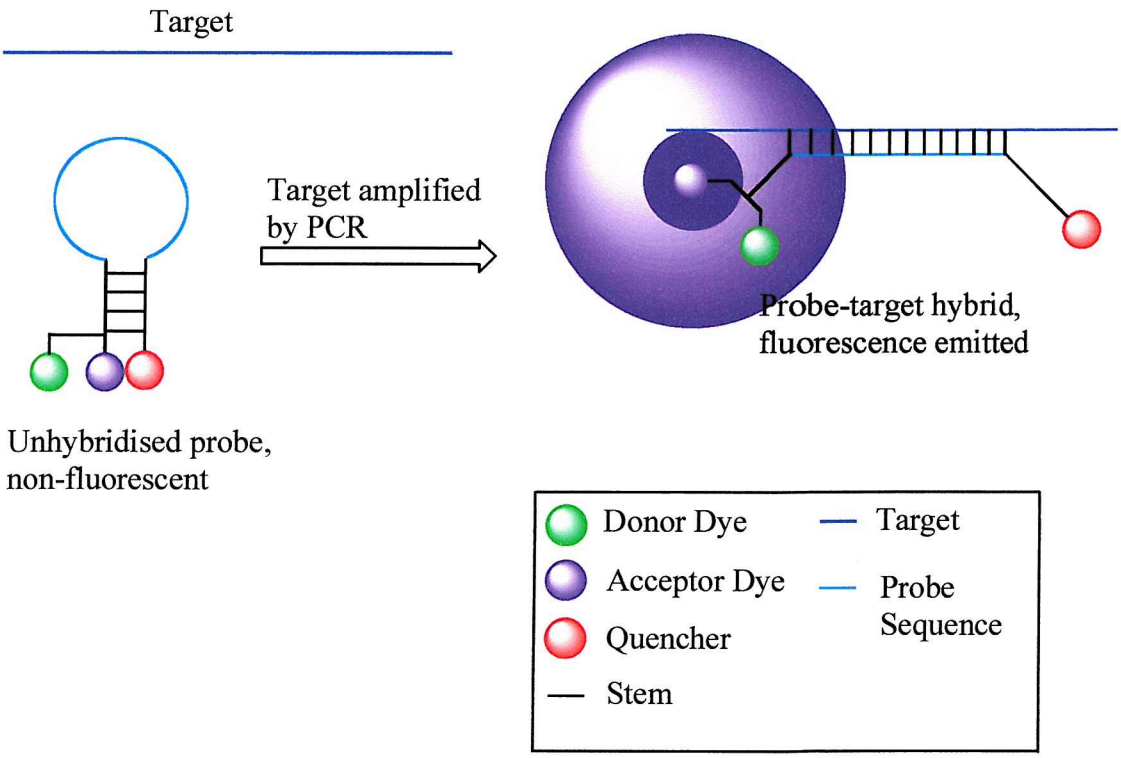


Fig 2.0.3.3 :- A Molecular Beacon Probe Utilising an Energy Transfer Linker

As energy transfer is both a distance and orientation dependent phenomenon the linker has to be designed carefully. The linker must possess certain qualities. It must:

- Not interfere with the fluorescence properties of the dyes
- Be rigid
- Keep the dyes in a certain orientation
- Have enough functionalisation to allow both dyes to be attached and a spacer to link the dyes to the oligonucleotide

One such linker, developed by ABI is used in BigDye terminator chemistry.[115] A modified fluorescein with additional methylamine functionality is attached *via* a phenyl linkage to a second fluorescent dye. The carboxylic acid of the fluorescein is used to attach the dyes to the oligonucleotide (fig 2.0.3.4).

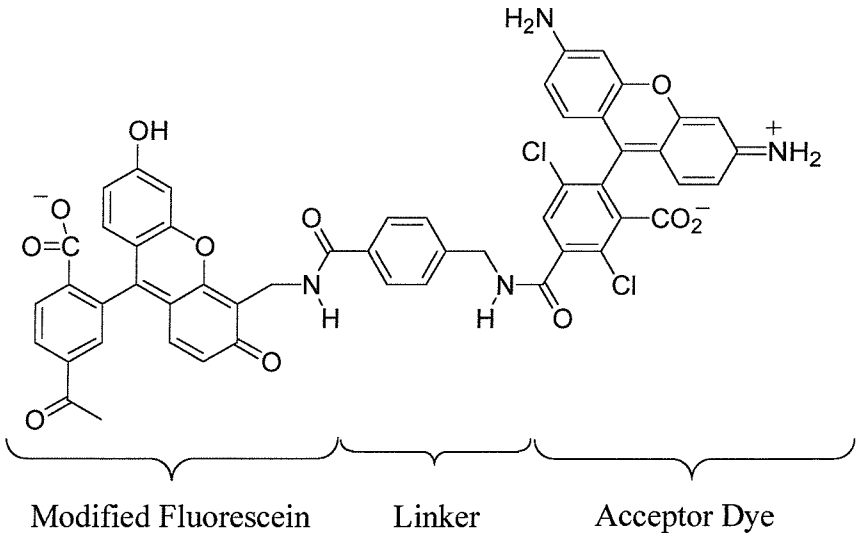


Fig 2.0.3.4 :- An ABI BigDye Terminator

The distance at which energy transfer is 50% efficient, the Förster distance[57,58,59,60] varies depending on the dyes used but on average is between 10-100Å (normally around 50Å). Various groups have used two different approaches to accomplish the task of keeping both dyes the correct distance and orientation from each other. The first approach uses the rigidity of aromatic or allylic systems to keep the dyes apart and the second makes use of nucleoside analogues. Of the two designs, linkers assembled from an aromatic or allylic backbone are far simpler to synthesise, so it was decided to base the design of the energy transfer linker on these structures.

2.1 A Biphenyl Energy Transfer Linker

2.1.1 Introduction

We decided to modify the design that ABI used for their BigDye terminator molecule and use a biphenyl group instead of just a phenyl linkage. A biphenyl compound was chosen for two reasons:

1. The design was simple and could be assembled using the Suzuki Coupling reaction in one synthetic step
2. The biphenyl structure had not been patented for this application, so in principle a patent could be filed

The modified fluorescein used in the ABI structure was to be used to functionalise the energy transfer system to an oligonucleotide as the proposed design called for the biphenyl ring to be functionalised with a benzoic acid group and benzylamine moiety attached to the two free para positions.

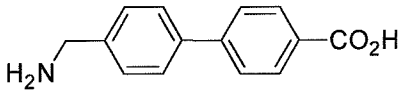


Fig 2.1.1.1 :- Structure of the Biphenyl Energy Transfer Linker Chosen

Two dyes can be attached to the linker in the form of phosphoramidites. Fig 2.1.1.2 shows the structure of a phosphoramidite dye.

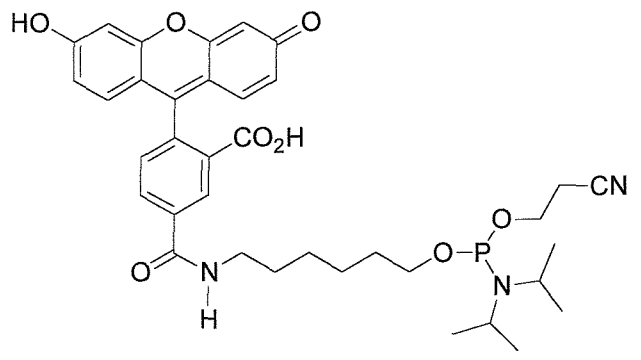


Fig 2.1.1.2 :- Structure of Fluorescein as a Phosphoramidite

2.1.2 The Suzuki Reaction

In 1981 Suzuki suggested a method for the cross coupling of organoboranes or boronic acids with organic halides. The catalytic cycle[116] described is shown in fig 2.1.2.

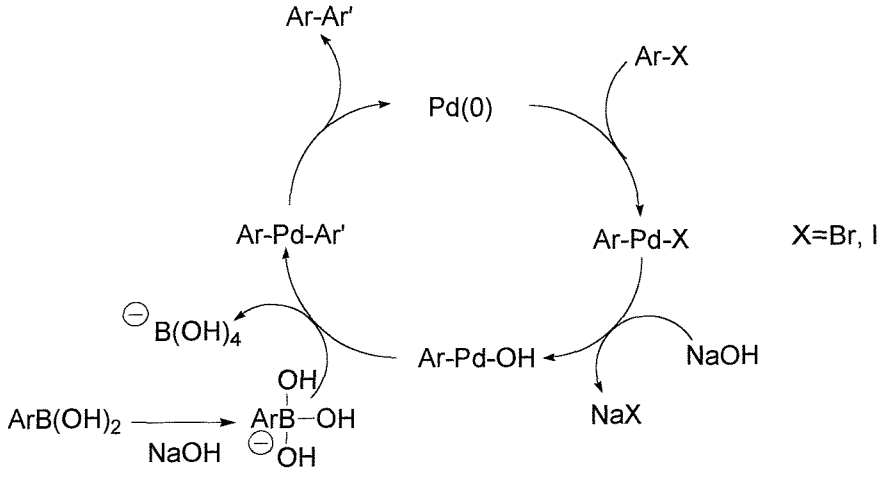


Fig 2.1.2 :- Catalytic Cycle of Suzuki Coupling

In the cycle, the oxidative addition of the organic halide is followed by the metathetical displacement of the halide ion from (Ar-Pd-X) by a base to give the organopalladium alkoxide (Ar-Pd-OR) or organopalladium hydroxide (Ar-Pd-OH). The organopalladium hydroxide species is believed to be more reactive than the organopalladium halide allowing the transmetallation to occur.

The transmetallation step is not very well understood. One theory, described by Stille[117] is that the transmetallation reaction takes place by an electrophilic substitution mechanism, the organopalladium hydroxide acting as the electrophile.

Since the discovery in 1981 by Suzuki & coworkers of the formation of these biphenyl analogues, the methodology has been used consistently in organic synthesis.

2.1.3 Attempted Synthesis of a Biphenyl Linker[118,119]

The synthesis via the Suzuki coupling of the biphenyl linker shown on page 41 was attempted. The reagents used were 4-carboxybenzeneboronic acid and 4-bromobenzylamine hydrochloride (see fig 2.1.3.1).

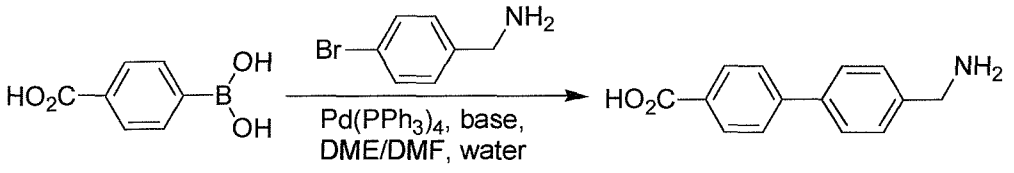


Fig 2.1.3.1 :- The Attempted Suzuki Reaction

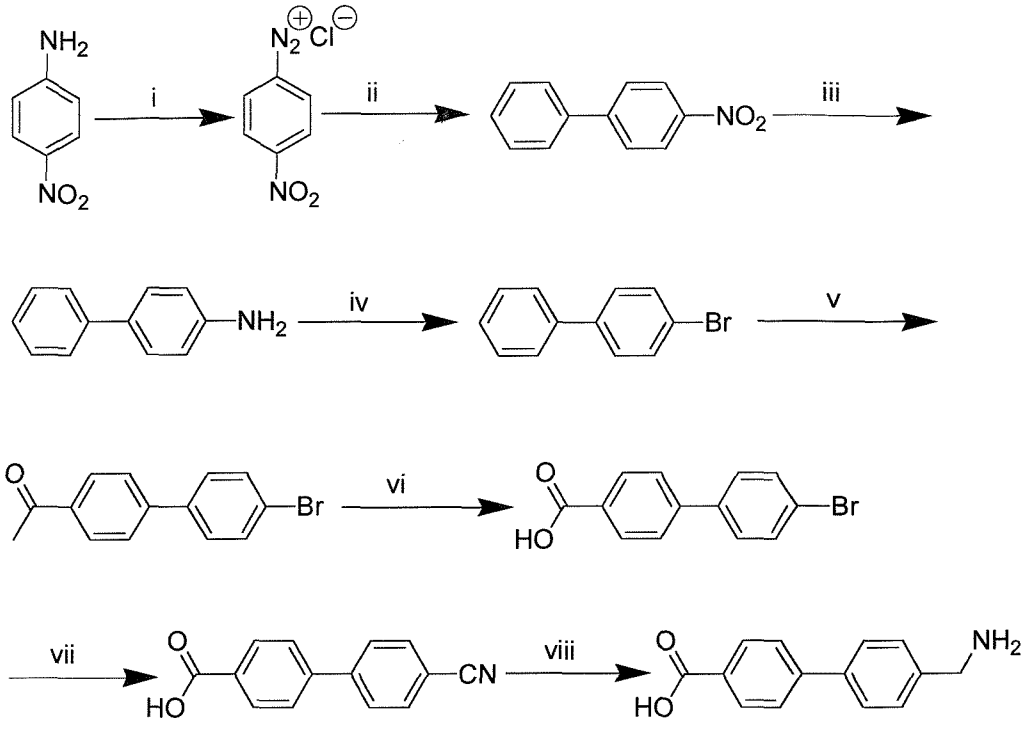
Various conditions were used but no product could be isolated. The tetrakistriphenylphosphine palladium(0) was washed with methanol and dried over phosphorus pentoxide overnight but the reaction still proved unsuccessful. Three different bases were used in the reaction:

1. Sodium carbonate (as a solid and a 2M solution)
2. Barium hydroxide
3. Sodium hydroxide

Altering the nature of the base had no effect on the reaction. All of the initial experiments were attempted in DME:water (5:1). The literature suggested that using a higher percentage of water gave better yields in compounds containing a benzoic acid moiety. However an experiment using DME:water (2:1) also proved unsuccessful. Suzuki couplings have also been reported using DMF as a solvent, so experiments utilising DMF:water (5:1) and DMF:water (2:1) were tried, both of which proved unsuccessful. TLC showed that both starting materials were being consumed and a product was formed. The product was extracted using DCM, washed with brine, dried over sodium sulfate and the solvent removed *in vacuo*. An NMR of the solid formed showed only evidence of the unreacted starting materials. We thought that the problem could be with the isolation of the product. As the product contains both a negatively

charged carboxylic acid group and a positively charged amine functionality the compound could be a zwitterion, making isolation very difficult. Because of the difficulty in synthesising this linker it was decided to look at other designs.

Literature searches uncovered a new pathway to the biphenyl linker.[120,121] The new route involves the formation of the biphenyl with a nitrile group at the 4 position as opposed to the benzylamine. The nitrile can then be reduced according to Tsukinoki's[121] procedure using a Raney Ni-Al alloy to give the corresponding primary amine (fig 2.1.3.2). The route seems to be very complicated and rather hazardous, making use of both copper cyanide, sodium cyanide and benzene as reagents. Because of these reasons and the fact that an alternative successful linker design was found, no further time was devoted to the synthesis of the biphenyl linker.



Reagents and conditions: i, HCl, $NaNO_2$, ii, benzene, NaOH, iii, reduction, iv, halogenation, v, $AlCl_3$, nitrobenzene, acetyl chloride, vi, Br_2, NaOH, water, vii, CuCN, NaCN, DMF, pyridine, viii, Raney Ni-Al, KOH, water

Fig 2.1.3.2 :- Proposed Synthesis of the Biphenyl Linker

2.2 Modified 1'-*O*-methylribose Linker

2.2.1 Introduction

The failure to synthesise an energy transfer linker based on a biphenyl system meant a different approach was needed.

In his review Wojczewski[115] discusses various labelling strategies for attaching dyes to oligonucleotides. A dye can be attached directly to the 5' position, either by use of a phosphoramidite dye (Page 42) or by the use of a 5'-amino modifier, added to the oligonucleotide as a β-cyanoethyl phosphoramidite (fig 2.2.1.1).

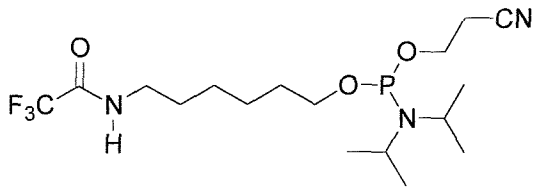


Fig 2.2.1.1 :- A 5'-Amino Modifier

A dye can also be attached to a modified base, the base is functionalised with an amine, alkyl, alkenyl or alkynyl linker (fig 2.2.1.2).

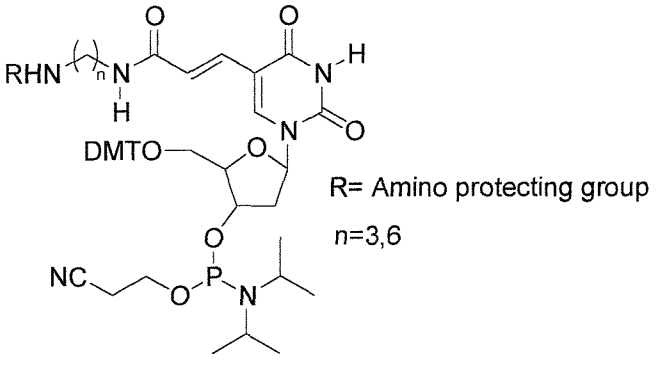


Fig 2.2.1.2 :- An Alkylamino dU Phosphoramidite

It is possible to add a fluorescent dye to the 3' position of the oligonucleotide leaving the 5'-end unmodified. The 3' modification can be used with Taqman probes (Page 34, Page 96). The modification is attached using a controlled pore glass (CPG) support and contains two functional groups, a protected amine allowing dye coupling and a dimethoxytrityl protected hydroxyl group allowing the modification to be introduced to an oligonucleotide (fig 2.2.1.3).

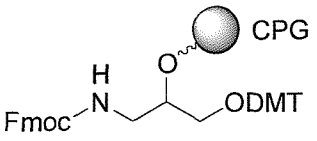


Fig 2.2.1.3 :- Aminoalkyl CPG for Use in Oligonucleotide Synthesis

Cuenoud[122] has also reported a modification that could be used to introduce a dye to the 2' position of the nucleoside.

To produce an energy transfer linker a combination of these strategies has to be employed as two dyes must be joined onto one molecule. Base modifications can destabilise DNA duplexes, so the 2'-*O*-modification, described in Cuenoud's paper (fig 2.2.1.4) was chosen as the basis for further work.

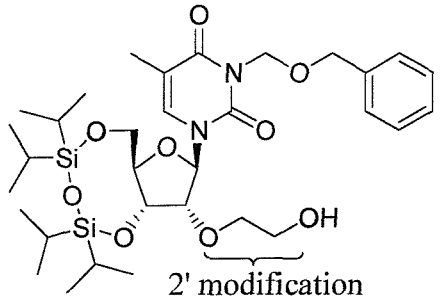
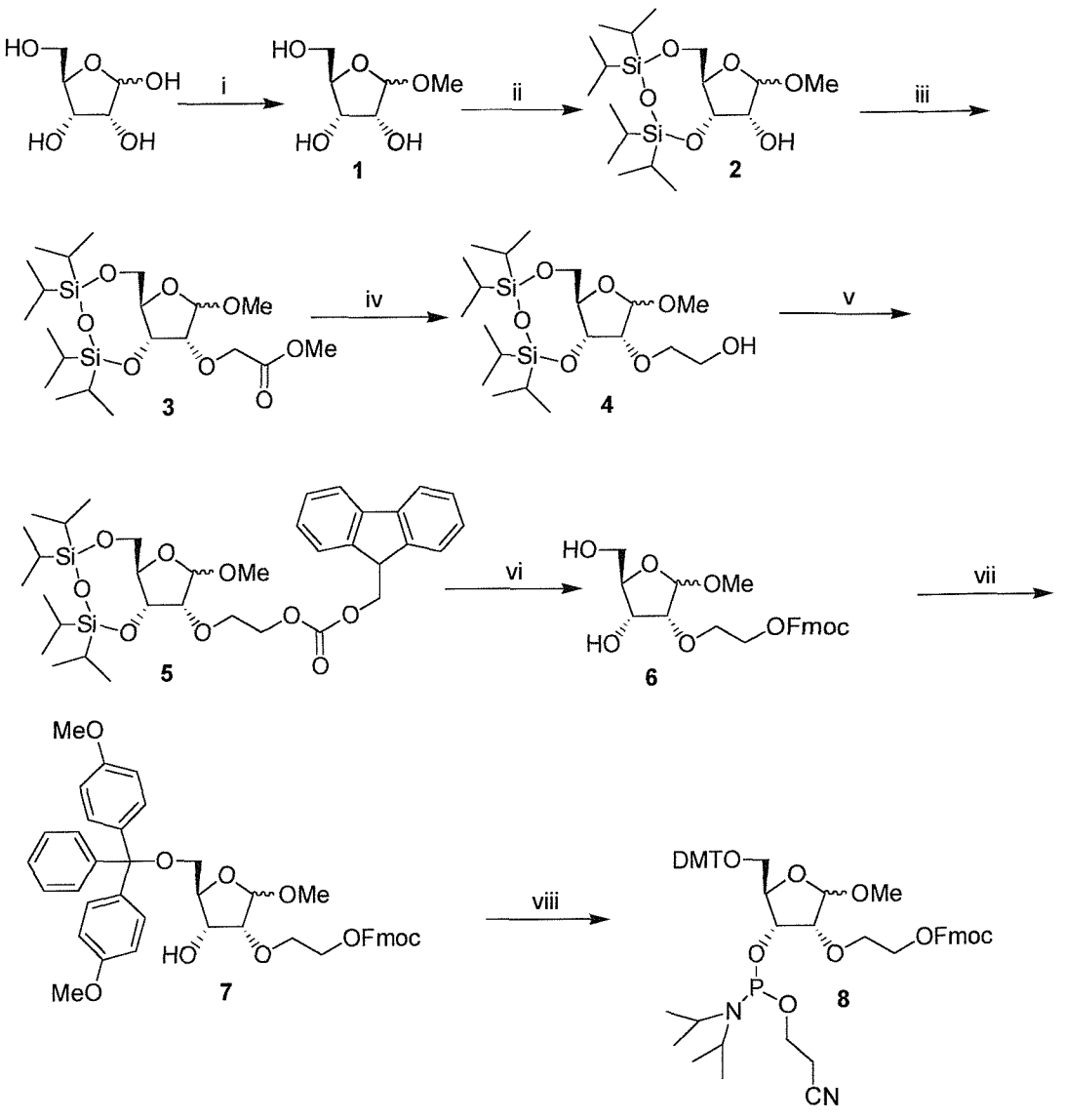


Fig 2.2.1.4 :- 2'-*O*-Modification Reported by Cuenoud

The second dye could be joined to the oligonucleotide at either the 5' or 3'-end depending upon the structure and function of the oligonucleotide required. Ideally the dye would be added to the 5' end of the sequence as modified nucleosides generally give lower coupling efficiencies in automated DNA synthesis than natural nucleotides. DNA is synthesised from the 3' to 5' terminus, so if the modification was attached near to the 3' terminus and gave very low coupling efficiencies then the overall oligonucleotide yield would be low. The initial scaffold chosen was a 1'-*O*-methylribose analogue. The synthesis of ribose analogues is simpler than nucleosides and the lack of a base does not alter the energy transfer efficiency.

2.2.2 Synthesis of the 1'-*O*-Methylribose Analogue



Reagents and conditions: i, MeOH, HCl; ii, 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane, pyridine, *47% over 2 steps*; iii, methyl bromoacetate, NaH, DMF, *35%*; iv, $LiBH_4$, MeOH:THF (1:4), *76%*;, v, FmocCl, pyridine, *92%*; vi, HF-pyridine,THF, *69%*; vii, dimethoxytrityl chloride, pyridine, *72%*; viii, 2-cyanoethyl diisopropylchlorophosphoramidite, DIPEA,THF, *68%*

Fig 2.2.2 :- Reaction Scheme for the 1'-*O*-Methylribose Analogue

Reactions ii-iv were carried out by Cuenoud[122] in 1998 on 5-methyluridine. No problem was encountered using the 1,1,3,3-tetraisopropyldisiloxane reagent, however when the alkylation using methyl bromoacetate was first attempted, a yield of 30% was obtained. Cuenoud's paper does not give a detailed method, so the author was

contacted and kindly sent a full experimental method. Even by following this method the highest yield obtained was 35%.

The fluorenylmethyloxycarbonyl (Fmoc) group was used to protect the alcohol function attached to the 2' position. There are precedents in the literature for the Fmoc group being used in DNA synthesis and the deprotection conditions of 10 minutes in 20% piperidine are mild enough for it to be removed without separating the oligonucleotide from the solid support. Assembling the apparatus together with the weighed starting materials and drying them all in a desiccator over phosphorus pentoxide overnight improved initial low yields. A respectable yield of 72% was thus obtained.

2.2.3 Problems with TIPS deprotection

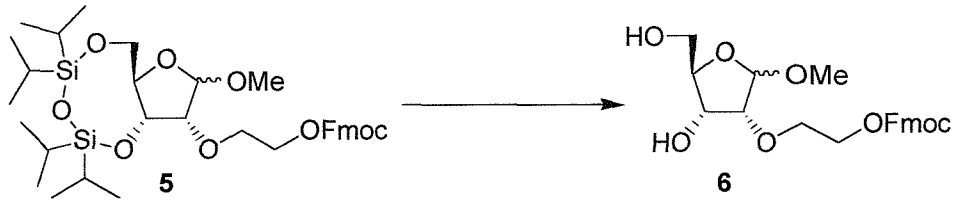


Fig 2.2.3.1 :- 1,1,3,3-Tetraisopropyldisiloxyl Deprotection

The 1,1,3,3-tetraisopropyldisiloxyl group (TIPS) is a silicon-based 1,3 diol protecting group. It's length means that it is particularly effective at protecting 3' and 5' alcohols. As is normal with silyl ethers, it is removed with a buffered source of fluoride. The most common reagent used is tetrabutylammonium fluoride (TBAF). When TBAF was used to deprotect the TIPS group from the molecule the base labile fluorenylmethyloxycarbonyl (Fmoc) group was also removed. To prevent the deprotection of the Fmoc group, a milder source of fluoride was needed. Various commercially available reagents were tried (fig 2.2.3.2).

Group removed by reagent	Fluoride Reagent Used			
	TBAF	TEA. 3HF[123]	CsF	HF/pyridine
TIPS Group	YES	YES	YES	YES
Fmoc Group	YES	YES	YES	NO

Fig 2.2.3.2 :- Conditions Used for TIPS Group Deprotection

Only HF-pyridine selectively removes the TIPS group, so HF-pyridine was used for the reaction giving the desired product in a yield of 69%. The synthesis of the 1'-*O*-methyl ribose monomer was successful, partially following methods reported by Cuenoud. The use of HF-pyridine instead of TBAF allowed the 1,1,3,3-tetraisopropyldisiloxane group to be removed selectively without the deprotection of the fluorenylmethoxycarbonyl group.

2.3 Fluorescence and Energy Transfer Efficiency Analyses

It was important to prepare some oligonucleotides containing the modified monomer to measure the efficiency of energy transfer between the donor and acceptor dye. At this stage no specific probe systems were prepared, only standard oligonucleotides.

A short oligonucleotide sequence of six T residues, oligonucleotide **38** containing the modified ribose was prepared. The modified ribose monomer was functionalised with two phosphoramidite dyes; hexachlorofluorescein (HEX) and fluorescein (FAM). The FAM was added to the 5' position and HEX was attached to the 2' position (fig 2.3.2).

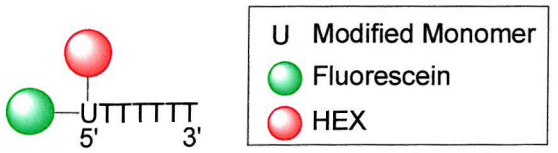


Fig 2.3.1 :- Sequence of Oligonucleotide **38**

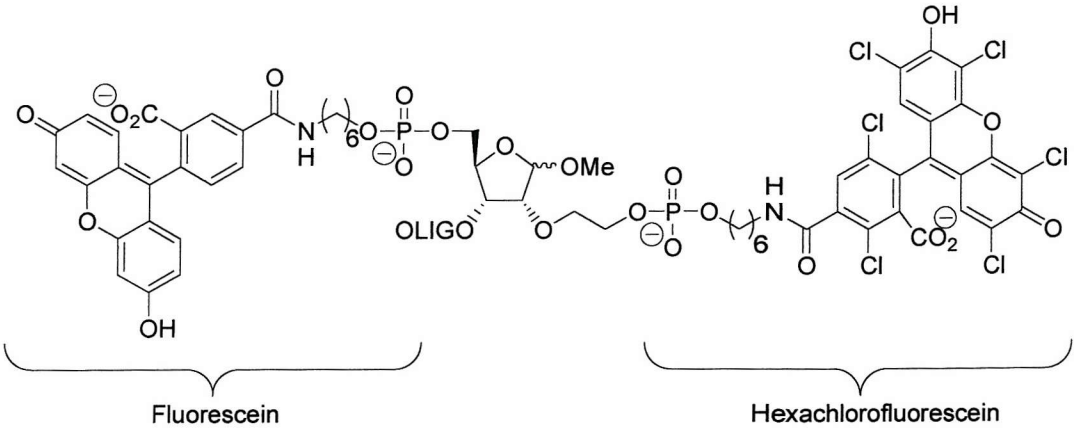


Fig 2.3.2 :- Monomer used in Oligonucleotide **38** Showing the Attachment of the Two Phosphoramidite Dyes

On reverse phase HPLC the oligonucleotide was a single major peak, shown by mass spectroscopy to be the correct product. Three minor peaks due to failure sequences and unlabelled dye were also present (fig 2.3.3). The oligonucleotide was purified prior to fluorescence studies.

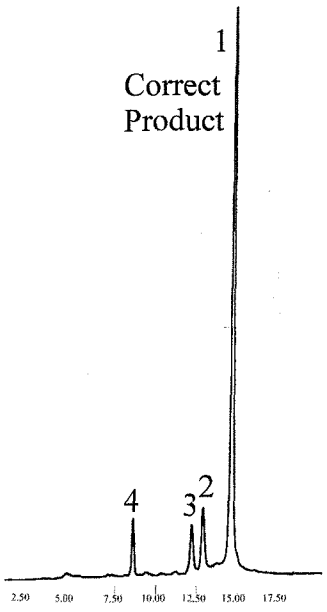


Fig 2.3.3 :- HPLC Trace of Oligonucleotide **38**

The oligonucleotide, diluted in pH 7.0 phosphate buffer was excited at 495nm in a Perkin Elmer fluorimeter and an emission spectrum from 510 to 750nm was recorded (fig 2.3.4). A small band at 520nm, the emission wavelength of FAM, can be seen, however the major peak is at 555nm, the emission wavelength of HEX. These results show that nearly all the energy from the FAM was being transferred to the HEX.

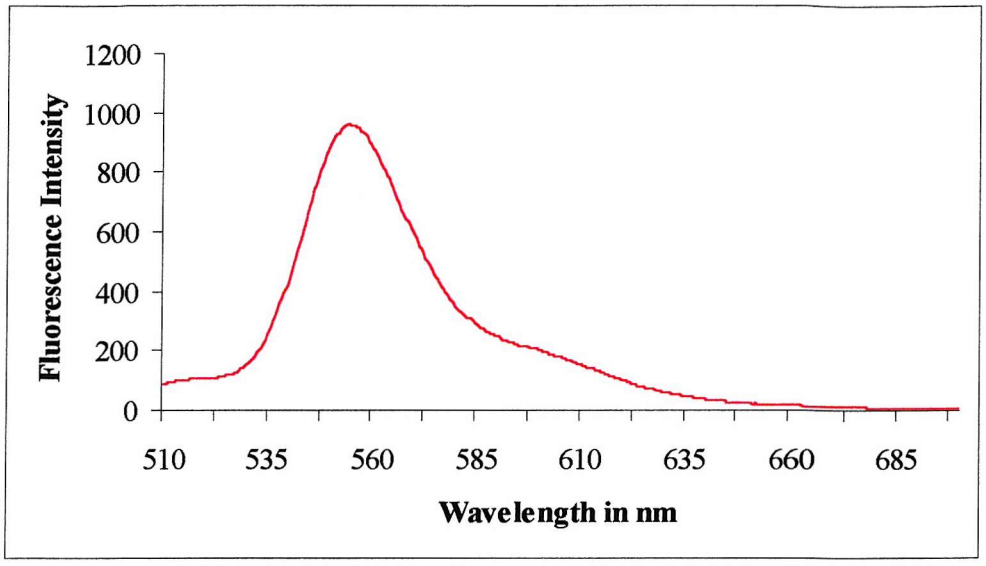


Fig 2.3.4 :- Fluorescence Emission Spectrum of Oligonucleotide **38**

A second oligonucleotide containing both FAM and rhodamine red (ROX), oligonucleotide **39** was prepared. It is important that energy transfer can be achieved between FAM and ROX as the LightCycler monitors three emission frequencies efficiently; 520nm, 640nm and 705nm (called channel 1, 2 and 3 respectively). FAM can be monitored on channel 1, ROX on channel 2 and a third dye, for example Cy5 on channel 3.

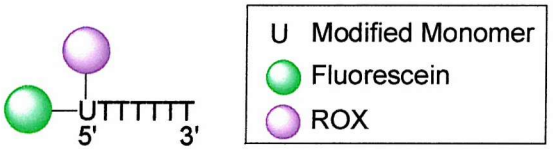


Fig 2.3.5 :- Sequence of Oligonucleotide **39**

ROX absorbs at 575nm and emits at 602nm. Energy transfer between FAM and ROX would mean that a much larger Stokes shift of 107nm would be observed making the process much more sensitive.

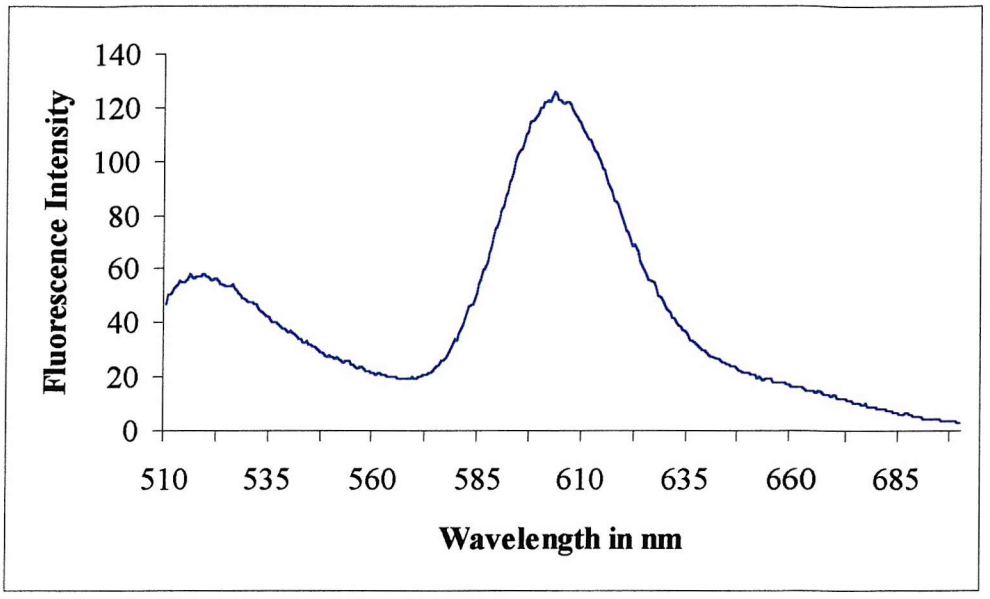


Fig 2.3.6:- Fluorescence Emission Spectrum of Oligonucleotide **39**

Fig 2.3.6 shows quite clearly that energy transfer is occurring between FAM and ROX. The oligonucleotide was excited at 495nm. Usually an excitation pulse at such a low wavelength would have no effect on an isolated ROX dye but with the FRET system, the fluorescence intensity of the 610nm emission peak was twice as high as the 520nm emission peak. The emission signal at 520nm, observed from the FAM shows that energy transfer across the much larger wavelength gap is less efficient. The ROX signal obtained however is still at least at a two fold greater intensity than the FAM signal.

The spectra in figs 2.3.4 and 2.3.6 show that energy transfer is taking place both between FAM and HEX and between FAM and ROX. The next experiment carried out was to compare the fluorescence obtained from oligonucleotides containing just one fluorescent dye. Oligonucleotides containing FAM only and HEX only were used for these experiments. This was carried out to:

1. Give a sense of the increase in intensity observed with the FRET system

2. Check whether energy transfer occurred between the dyes free in solution, important to find out whether the energy transfer was intra or intermolecular.

Energy transfer was not observed between free FAM and HEX at a concentration of 0.01μmol/ml of each oligonucleotide showing that the energy transfer process between the two dyes in the doubly-labelled modified monomer was intramolecular. A twofold difference in fluorescence intensity was observed between a free HEX excited at 495nm and HEX attached to the monomer excited at 495nm through FAM, thus demonstrating that FRET between the two dyes was efficient.

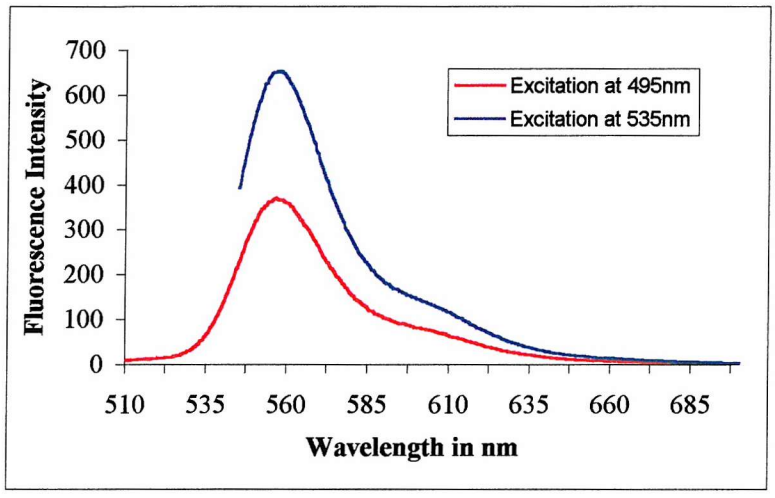


Fig 2.3.7 :- Fluorescence Emission Spectrum of Oligonucleotide **38** Excited at 495 and 535nm

To test the efficiency of the energy transfer, the modified monomer was excited at both 495nm and 535nm. If energy transfer between the dyes is efficient, then, as energy transfer is more efficient than photonic absorption, the excitation of the FRET system at 495nm might give a higher intensity than excitation at 535nm. As can be seen from fig 2.3.7, the opposite is true and excitation at 535nm gives approximately two fold more fluorescence than at 495nm. This can be explained if some of the energy transfer is being quenched. As both dyes are on alkyl chain spacers, it is possible for them to collide on either side of the monomer. If this happens, then the fluorescence will be quenched by internal mechanisms (Page 14).

Choosing oligonucleotides **38** and **39**, we have shown that energy transfer occurs between a donor and acceptor dye when they are affixed to the 5' and 2' position as phosphodiesters. However fig 2.3.7 shows that some internal quenching is occurring

between the two dyes preventing the maximum amount of fluorescence being obtained from the system. To prevent the internal quenching from taking place it was decided to introduce a spacer between the monomer and the dye at the 2' position. We decided to use hexaethyleneglycol (HEG) as the spacer as:

1. It can be purchased as a phosphoramidite allowing it to be added to the oligonucleotide on solid phase

2. In aqueous buffer the oxygen atoms in the backbone keep the structure very rigid and extended by hydrogen bonding to water

3. Each unit adds approximately 20Å to the spacing between the dyes

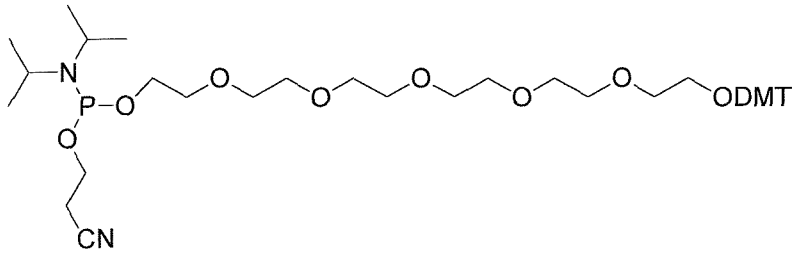


Fig 2.3.8 :- Structure of Hexaethyleneglycol (HEG) Introduced as a Phosphoramidite

Oligonucleotides **40**, **41** and **42** were prepared, containing 10 thymidine residues and the modified ribose monomer at the 5'-end.

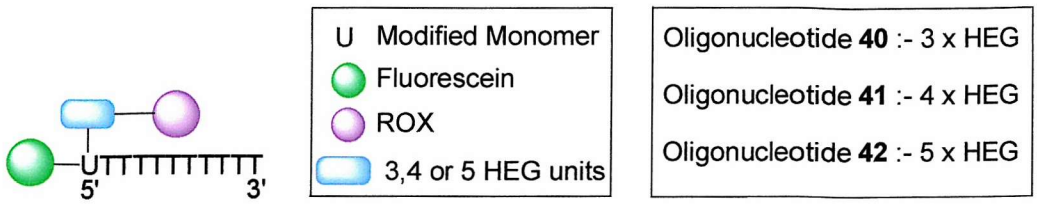


Fig 2.3.9 :- Sequence of Oligonucleotides **40**, **41** and **42**

The ribose monomer was coupled to a FAM dye at the 5' position and a varying number (3 to 5) of hexaethyleneglycol units attached at the 2' position followed by HEX. Fluorescence emission spectra, exciting at 495nm and monitoring the emission from 510 to 700nm were obtained.

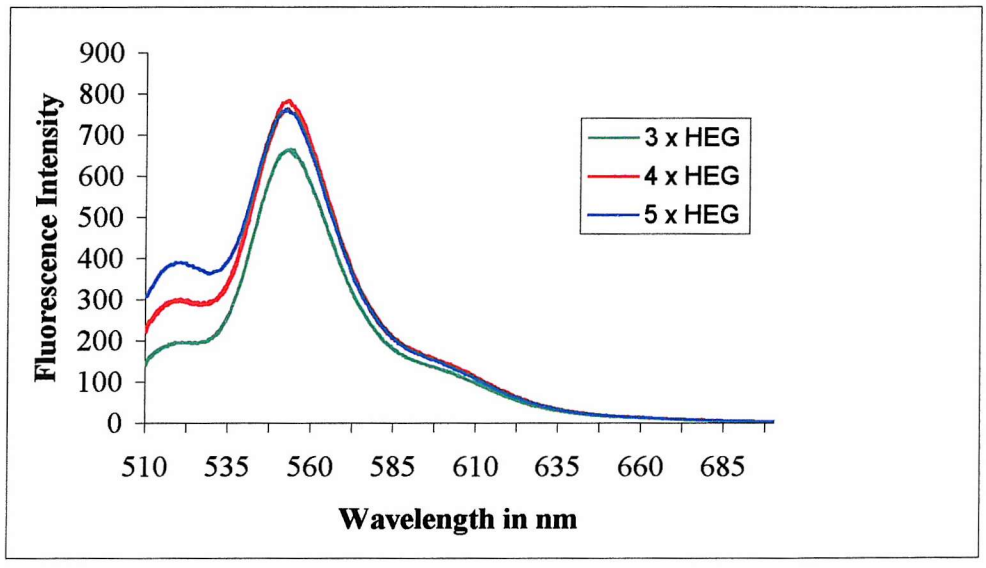


Fig 2.3.10 :- Effect of Number of HEG Spacers on Fluorescence Intensity Using Oligonucleotides **41**, **42** and **43**

As can be seen in fig 2.3.10 there is an increase in fluorescence intensity, when increasing the number of HEG spacers from three to four. With increased numbers of HEGs, the efficiency of energy transfer should decrease, giving a drop in fluorescence intensity. The observed increase seems to confirm the previous hypothesis, suggesting that the dyes are in fact closer together in space. As there is little difference in fluorescence intensity when the fifth HEG is added and also a small rise in the fluorescein emission signal, it seemed that the optimum number of spacers to use in the monomer was four.

All of the above fluorescence spectra were obtained with the oligonucleotides diluted in a pH 7.0 phosphate buffer. However for genetic analysis experiments involving PCR a 10x PCR buffer (Tris HCl, 6.67mM; EDTA, 0.033mM; NaCl, 10mM; $MgCl_2$, 0.67mM; $MnCl_2$, 0.67mM) would be needed.

The experiments were repeated with oligonucleotide **38** using the above PCR buffer instead of the phosphate buffer to check that the intensity would remain the same. Surprisingly the fluorescence intensities in the PCR buffer were approximately twice that of the phosphate buffer. The difference in intensities could be a function of one of two factors, salt concentration or pH. Experiments were carried out in which the pH of the phosphate buffer was altered from pH 6.0 to pH 7.9. These variations in pH had no effect on the intensity of the fluorescence (Fig 2.3.11). However carrying out the experiment in pure water gave a much smaller amount of fluorescence than either the phosphate buffer or the PCR buffer (Fig 2.3.12). As the PCR buffer had a higher salt concentration than the phosphate buffer it appears that increasing the level of salt increases the amount of fluorescence. One theory as to how the effect might work stems from the fact that in solution the oligonucleotide and linkers can curl up, the higher the salt levels in the solution the more rigid the structure. As FRET depends very much on the orientation of the dyes the more rigid orientation could place the dyes further apart from one another.

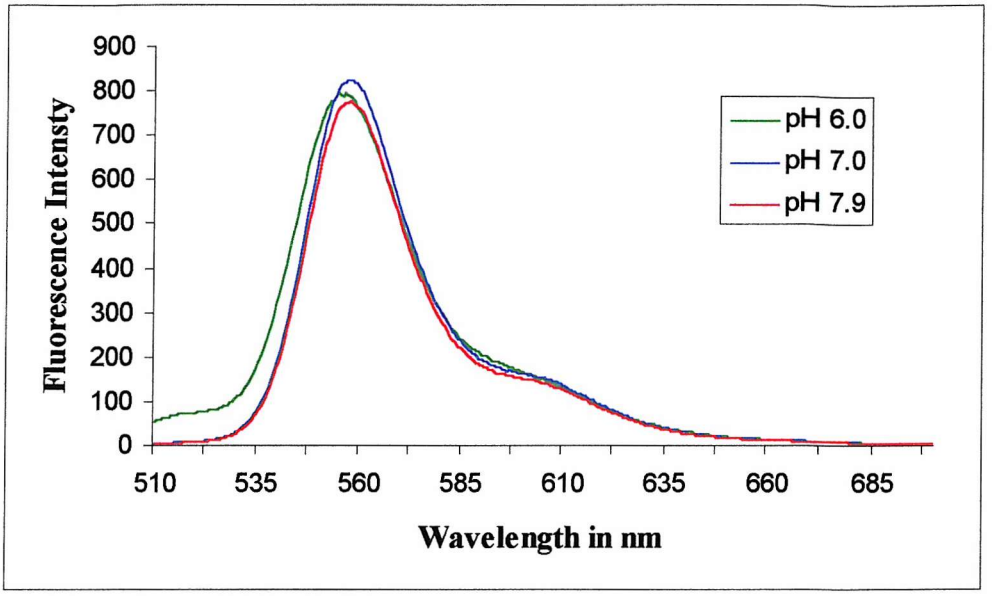


Fig 2.3.11 :- Fluorescence Emission Spectrum Showing the Effect of pH on Fluorescence Intensity Using Oligonucleotide **38**

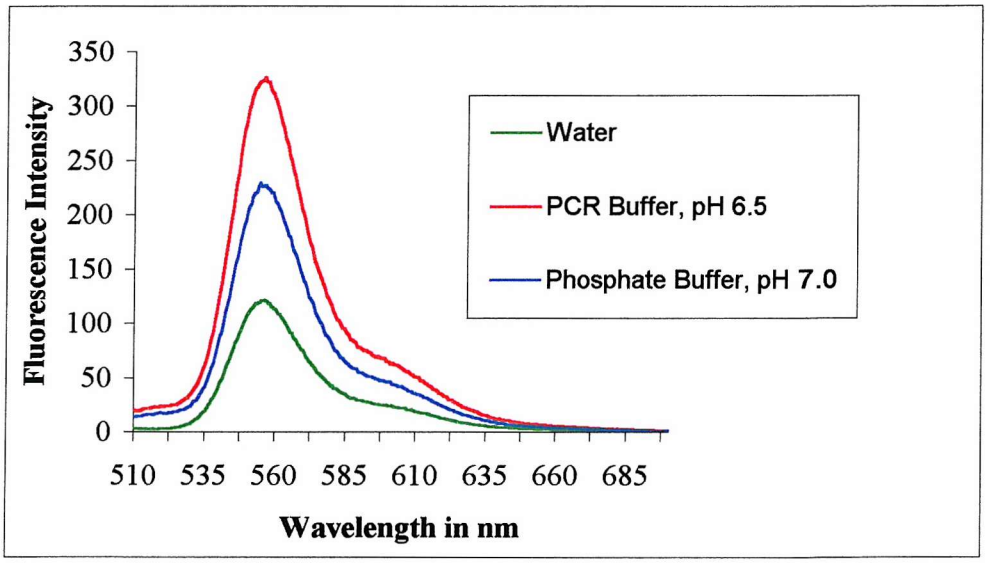


Fig 2.3.12 :- Fluorescence Emission Spectrum Showing the Effect of Buffer on Fluorescence Intensity Using Oligonucleotide **38**

2.4 Conclusion

In chapter 2 the design of an energy transfer linker was discussed. The initial design chosen, a biphenyl system, proved too difficult to synthesise so the synthesis of a modified ribose FRET monomer was attempted. The design chosen consisted of a 1'-*O*-methylribose with a hydroxyethyl modification at the 2' position. The synthesis of the modified 1'-*O*-methylribose proved straightforward, the only difficulty being the selective deprotection of the tetraisopropyldisiloxane group. The removal of the silicon protecting group was achieved using HF-pyridine in good yields without removing the fluorenylmethoxycarbonyl group.

Oligonucleotides were prepared containing two fluorescent dyes attached to the 5' and 2' position. Energy transfer was achieved between the dyes in the system but a degree of quenching was also observed. The quenching was thought to occur as the two dyes were too close together in space. A spacer unit, hexaethyleneglycol was employed to move the dyes further apart. Fluorescence intensity measurements showed that 4 or 5 HEG units provided a maximum amount of fluorescence in the FRET system examined.

Chapter 3

A Nucleosidic Linker

3.0 A Nucleosidic Linker

3.0.1 Introduction

The previous chapter discussed the design and synthesis of various types of energy transfer linkers. The synthesis of one of the designs, based on ribose was discussed at length and oligonucleotides containing dyes attached to the linker were prepared and tested. The energy transfer linker was shown to function efficiently.

In chapter 3 the synthesis of modified nucleoside linkers, containing the 2'-*O*-modification optimised in the ribose linker will be discussed. The incorporation of the linker into probe systems will also be discussed.

3.1 Introducing Dyes Into Oligonucleotides Using Modified Nucleosides

One of the main problems with introducing dyes not attached to nucleosides within an oligonucleotide, is that it can result in the destabilisation of the interaction between the probe and the targeted sequence, which can dramatically affect the selectivity of the probe. The ideal solution to this problem is to introduce the dye *via* a linker attached to a modified nucleoside so that the base pairing will not be affected.

Cuenoud and coworkers[122] employed 5-methyluridine (fig 3.1.1) in their synthesis but due to the expense of this nucleoside (£450/g) large-scale synthesis using 5-methyluridine was deemed impractical.

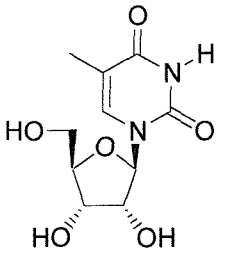
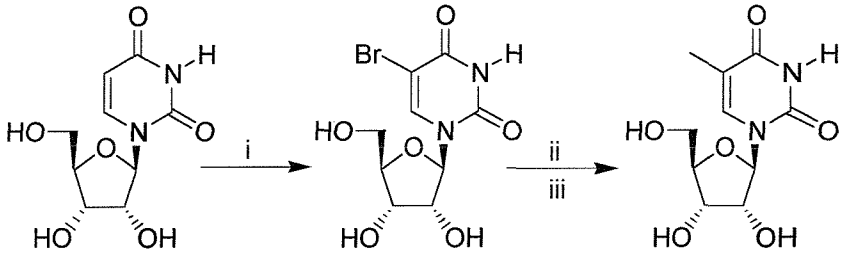


Fig 3.1.1 :- 5-Methyluridine

Two options were considered: to synthesise 5-methyluridine from uridine or to utilise a different nucleoside. The synthesis of 5-methyluridine, documented by Hirota,[124] involved the bromination of uridine in the 5 position followed by an alkylation using trimethylaluminium as the carbon source (fig 3.1.2). HMDS is used in the alkylation reaction as a solvent and to prepare the amount of nucleoside required would need over 6 litres of HMDS. Using this large amount of HMDS is impractical, as a very large reaction vessel would be needed. The reaction would also be expensive, HMDS costing £38 per 100ml.



Reagents and Conditions: i, Br_2, $CHCl_3$; ii, HMDS, $(NH_4)_2SO_4$;
iii, $PdCl_2$, Ph_3P, $AlMe_3$

Fig 3.1.2 :- Synthetic Scheme for the Preparation of 5-Methyluridine from Uridine

After deciding that it was uneconomic to synthesise 5-methyluridine, four other nucleosides were considered: guanine, adenine, cytidine and uridine. The manipulation of the purine ring is more difficult and the synthesis involves more steps, so it was decided to synthesise both a modified uridine and cytidine nucleoside.

3.2 Synthesis of Uridine Monomer Protected by Fmoc

The first synthesis undertaken was of a protected 2'-*O*-hydroxyethyluridine (fig 3.2.1).

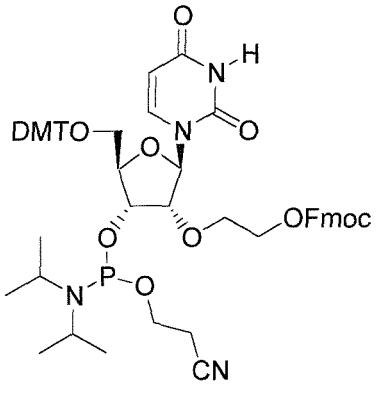
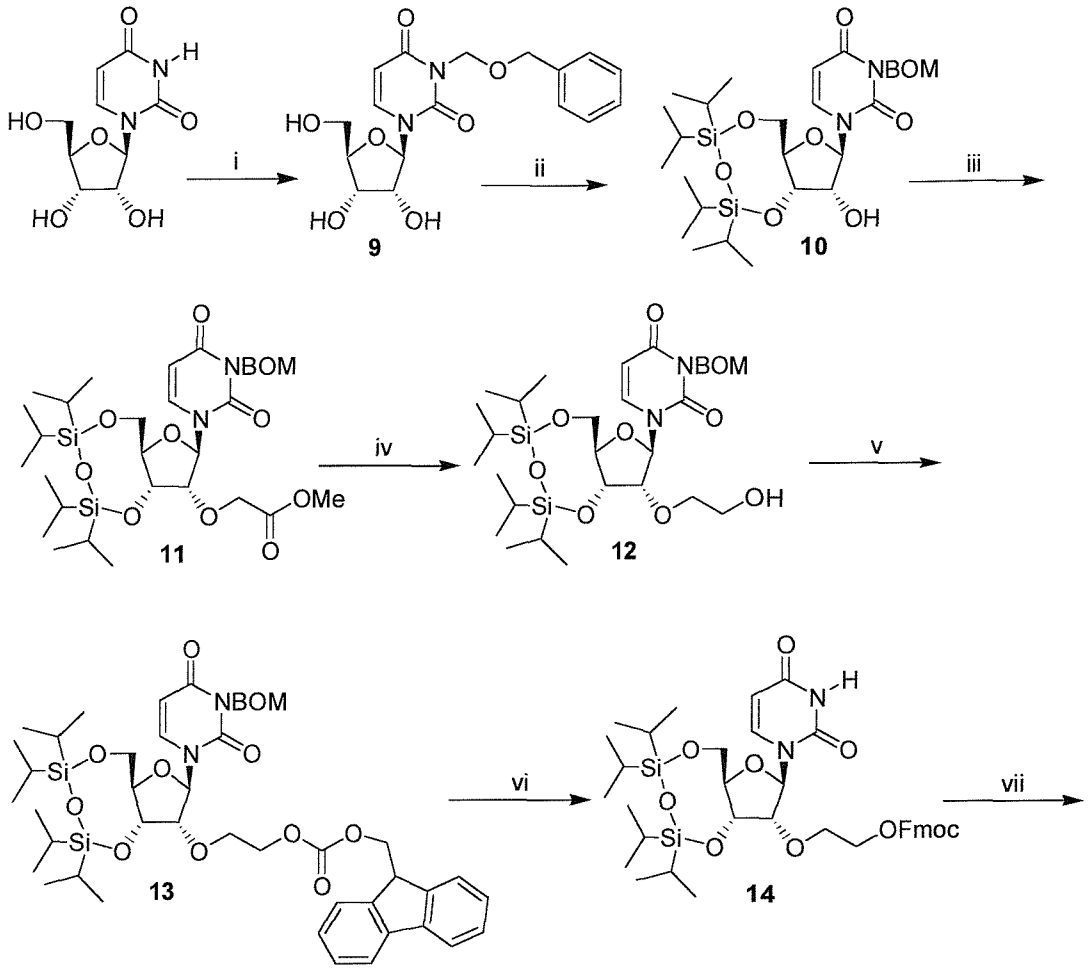


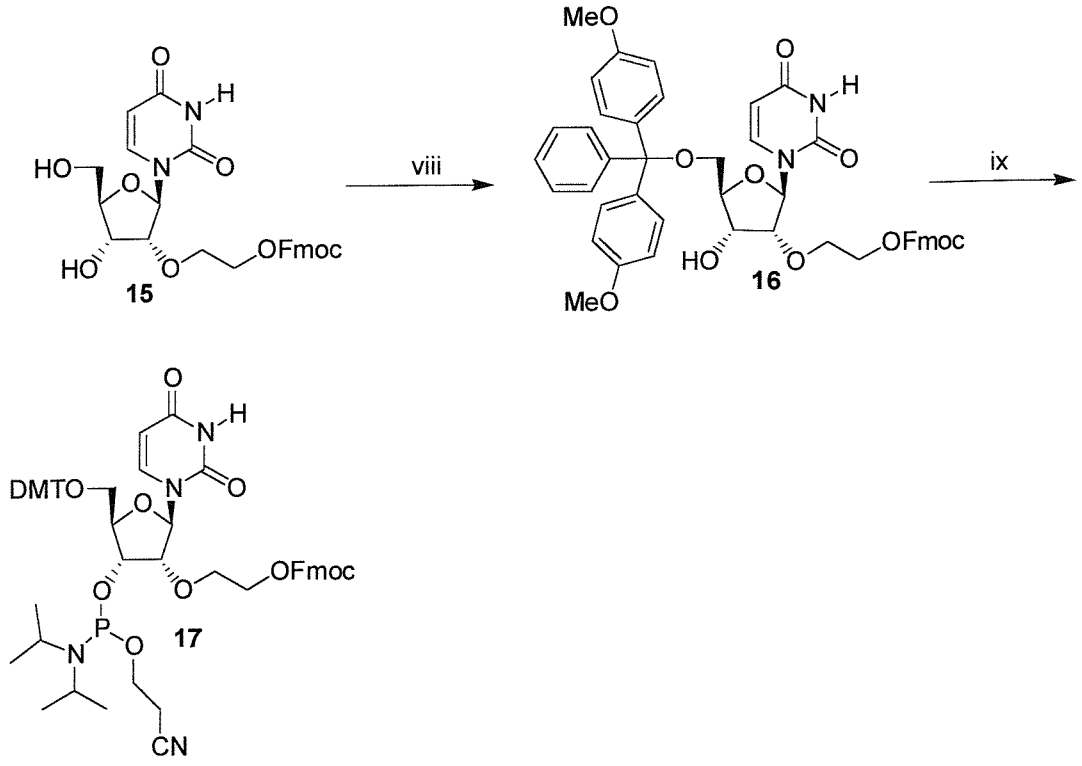
Fig 3.2.1 :- Modified Uridine Monomer

The synthetic pathway is described in fig 3.2.2.



Reagents and conditions: i, benzyl chloromethyl ether, DBU, DMF, *82%*; ii, 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane, imidazole, pyridine, *91%*; iii, methyl bromoacetate, NaH, DMF, *72%*; iv, $LiBH_4$, MeOH:THF (1:4), *28%*; v, FmocCl, pyridine, *86%*; vi, Pd/C (20wt%), 2M $HCl_{(aq)}$, THF:MeOH (1:1), $H_{2(g)}$, *62%*; vii, HF-pyridine, THF, *54%*

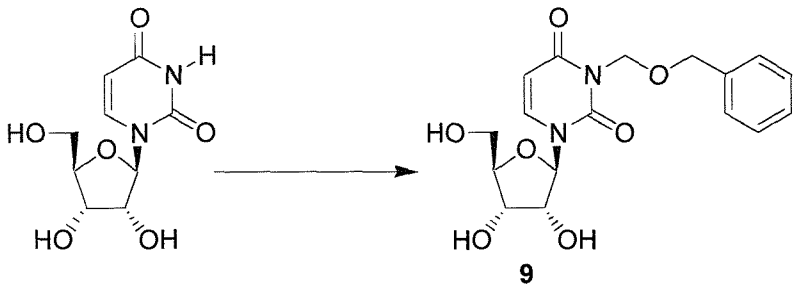
Fig 3.2.2 :- Synthesis of for Uridine Monomer



Reagents and conditions: viii, 4,4'-dimethoxytrityl chloride, pyridine, *80%*; ii, 2-cyanoethyldiisopropyl chlorophosphoramidite, DIPEA, THF, *72%*

Fig 3.2.2 :- Synthesis of Uridine Monomer

3.2.1 Alkylation of the N-3 Position



9

Fig 3.2.1.1 :- Protection of the N-3 Position

The first step of the synthesis is the protection of the N3 position by a benzyloxymethyl group. Following the reaction documented in Krecmerova's paper,[125] the product, was purified by recrystallisation from boiling methanol. When the reaction was carried out on a 500mg scale the purification proceeded as expected, affording the correct product as a white crystalline solid in greater than 90% yield. However upon scaling the reaction up to 10 grams the attempted recrystallisation failed, giving only an oil. If the oil, which contained only trace impurities by NMR, was taken onto the next reaction the yield of the silylated product **10** dropped from 90% to below 50%. One explanation for the decrease in yield could be that a trace of either DMF or DBU remained in product **9** and that the presence of one or both of these reagents prevent the tetraisopropyl disiloxane protection. The material was then purified by column chromatography, in a 5% methanol/DCM system. The oil obtained was then recrystallised from boiling methanol to give a white crystalline solid with an identical melting point to the batches of the compound previously obtained. Reaction of this purified product gave yields of greater than 90% of compound **10**.

3.2.2 Alkylation of the 2' hydroxyl Position

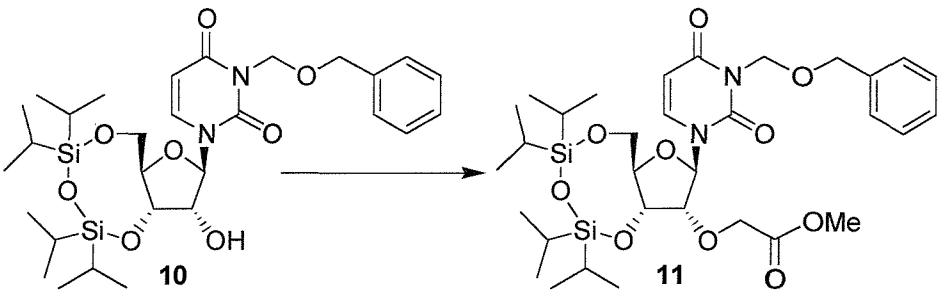


Fig 3.2.2.2 :- Alkylation of 2' Position

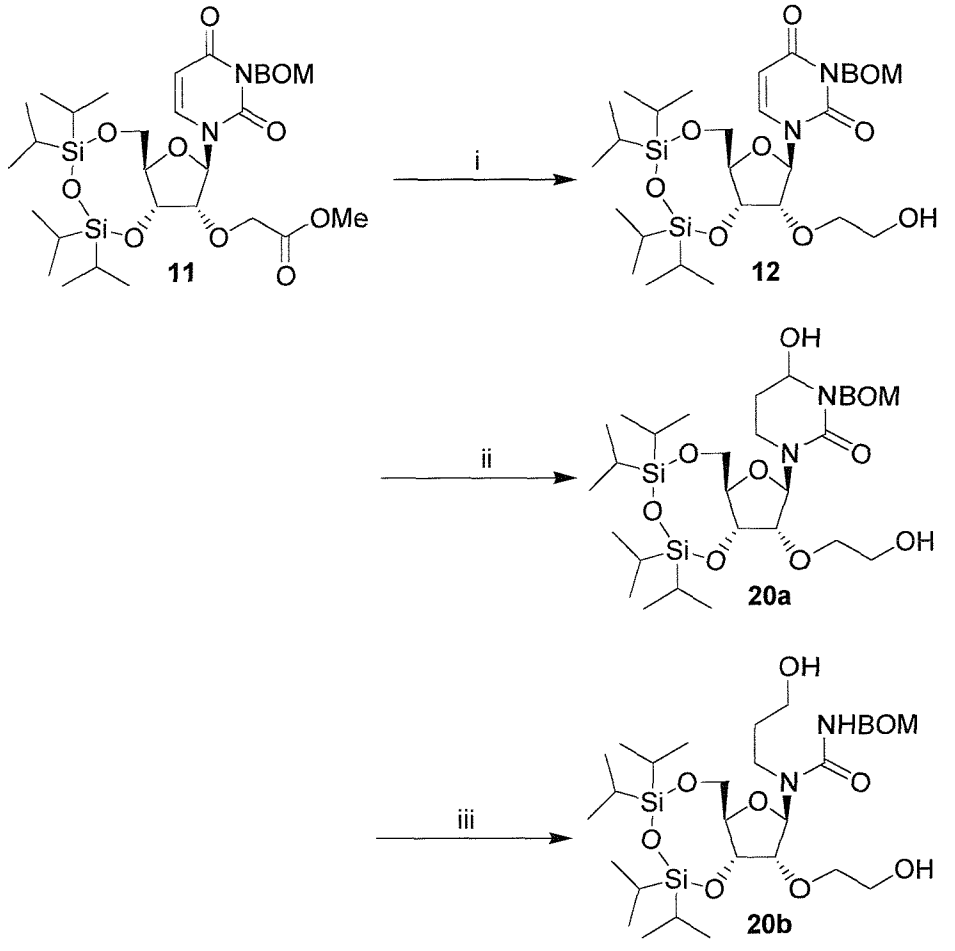
The alkylation of compound **10** using methyl bromoacetate proceeded well to give yields in excess of 70% of compound **11**. The reaction workup chosen dictated the extraction of compound **11** with ethyl acetate. The ethyl acetate was removed *in vacuo* using a diaphragm pump. Traces of DMF present were removed *in vacuo* at 50°C on a high vacuum pump. After half an hour under vacuum, a crystalline material was noticed around the neck of the flask and on the cold finger of the Büchi, analysis of the crystalline material showed it to be compound **11**. It was surmised that the temperature used to remove the DMF was too great allowing the product to be co-evaporated along with the DMF. The temperature of the water bath was lowered to room temperature and the product left under vacuum for no longer than 1 hour.

It was decided to take the product, now a dark yellow oil and recrystallise it. Cuenoud's conditions, hexane at -20°C overnight was tried but proved only partially successful. Approximately 10% of the compound was isolated as a white crystalline solid. The remainder of the compound could be purified by column chromatography.

In a new protocol, a flask was left *in vacuo* at room temperature on a high vacuum pump for 3 hours, then recrystallisation from boiling ethanol afforded a white crystalline solid in 72% yield, identified as compound **11**.

The synthesis of compounds **13** to **17** proceeded in good yields. Problems were encountered with the synthesis of compound **12** and these will be discussed in section 3.3. The phosphoramidite **17** was successfully used in oligonucleotide synthesis and its use as a FRET linker will be reported in section 3.5.

3.3 Over-reduction of Compound 11



Reagents and Conditions :- i, 4eq lithium borohydride(portionwise addition of 2x2eq), THF:MeOH (4:1), 5°C-20°C; ii, 6eq lithium borohydride, THF:MeOH (4:1), 5°C; iii 6eq lithium borohydride, THF:MeOH (4:1), 20°C (**20a** *22%* yield, **20b** *65%* yield)

Fig 3.3.1 :- Various Products Formed from the Reduction of Compound **11**

When the reduction of the ester group of compound **11** was attempted using identical conditions to those reported in the Cuenoud paper, the product obtained was very polar and did not have a UV chromophore. It is known that the C5-C6 double bond is fairly sensitive to reduction[126,127] so an assumption was made that over-reduction of the molecule had taken place. Different conditions for the reaction were investigated. Three variables were altered; the amount of lithium borohydride used, the reaction time and the reaction temperature.

Varying the amount of lithium borohydride proved unsuccessful, as if less than 4 equivalents of the reagent were used then the reaction of compound **11** to give compound **12** did not proceed to completion. The next variable to be altered was the temperature of the reaction. A series of experiments were carried out with 4 equivalents of lithium borohydride added to the reaction at different temperatures.

Effect of Temperature on Over-reduction of Compound **11**

Temperature Used	Reaction % Completion	Over reduction Product Present
20°C[(a)]	100%	YES (63% yield)
5°C, warmed to 20°C[(a)]	100%	YES (72% yield)
5°C[(a)]	~80%	YES
-20°C[(b)]	~50%	YES
-78°C[(c)]	~10%	YES

(a) :- 4eq of $LiBH_4$ used; (b) :- 4eq of $LiBH_4$ then 4eq added; (c) :- 10eq of $LiBH_4$ added

The initial experiment was carried out at 20°C and the reaction was complete within 15 minutes. The experiment gave two products; compound **12** in an 18% yield, and a second more polar product in a 63% yield, compound **20a/b.**

At 5°C, the lithium borohydride was added in 2 portions of 2 equivalents each, the reaction being stirred for 15 minutes at 5°C after each addition. After the second period of stirring the reaction was warmed to 20°C. The reaction was complete in 30 minutes. Although the yield of compound **12** was increased to 28% the major constituent of the reaction was still compound **20a/b**. The reaction took a period of 48 hours to go fully to completion if carried out at 5°C, the major constituent however was still compound **20a/b**. The temperature was lowered to -20°C (methanol/ice bath) and -78°C (acetone/solid CO_2 bath). In these reactions even after being left for 3 days starting material was still present. An additional 4 equivalents of lithium borohydride were added to the reaction at -20°C to convert any remaining starting material to product **12**. While some of the remaining starting material was consumed, compound **20a/b** was still formed in favour to compound **12**. At -78°C the problem was

exacerbated with the reaction proceeding only minimally to completion even though 10 equivalents of lithium borohydride were used. Compound **20a/b** still formed at these low temperatures, leading to the conclusion that compound **20a/b** is not a thermodynamic product.

The solution to the problem was to add the $LiBH_4$ at 5°C, slowly warm the mixture to 15°C, and then to work the reaction up after it had proceeded 50% to completion. At this stage the amount of compound **20a/b** was minimal with respect to compound **12** and the reisolated starting material could be taken through the reaction again. This allowed overall product recovery of 50-60% from 3 reaction cycles. Cuenoud[122] was successful in carrying out the reduction using 5-methyluridine so the lack of a methyl group at the 5 position dramatically affects the reactivity of the uridine.

3.3.1 Possible Structures of Compounds **20a** and **20b**

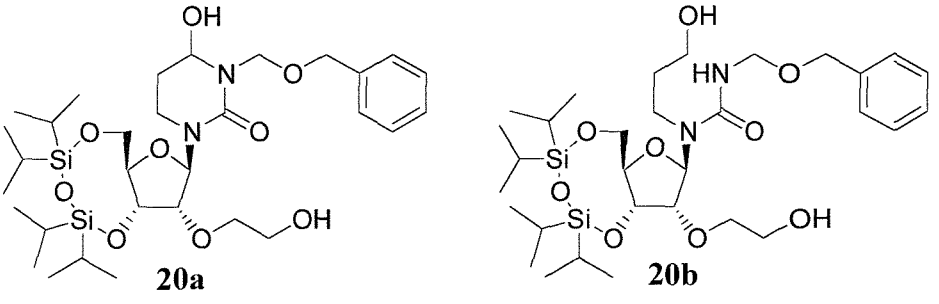


Fig 3.3.1.1:- Likely Structures of Compounds **20a** and **20b**

To isolate compound **20b** the reaction was performed in the most over-reducing conditions. The reaction was carried out at room temperature with 6 equivalents of lithium borohydride employed. The reaction was monitored by TLC, eluting with 10% methanol/DCM. By using a more polar elution system it was noted that two products were formed, tentatively assigned as compounds **20a** and **20b** with R_F's of 0.5 and 0.4 respectively. Both products were separated using flash column chromatography giving yields of 22% for the more polar compound, compound **20b** and 65% for the other less polar product, compound **20a**.

Low-resolution mass spectroscopy showed that compound **20b** had a molecular weight of 656gmol^{-1} which is compatible with the presence of the benzyloxymethyl group.

However more than the C5-C6 bond must have been reduced. A proton, carbon and DEPT NMR were performed. The DEPT 135 for compound **20b** (fig 3.3.1.3) shows evidence of 8 CH_2 groups. The starting material has 4 CH_2 functionalities. The reduction of the ester and the C5-C6 double bond would introduce another 3 CH_2 groups, meaning that the reduction has caused the formation of a further CH_2 group. There are 2 possible candidates where the extra group could be formed; the C4 ketone and the C1' position.

Type of Carbon Atom	No. of Carbon Type in NMR Experiment	
	Carbon	DEPT
Quaternary	2	—
CH/CH_3†	—	4
CH_2	—	8

† :- Number specified does not show benzyl or isopropyl groups.

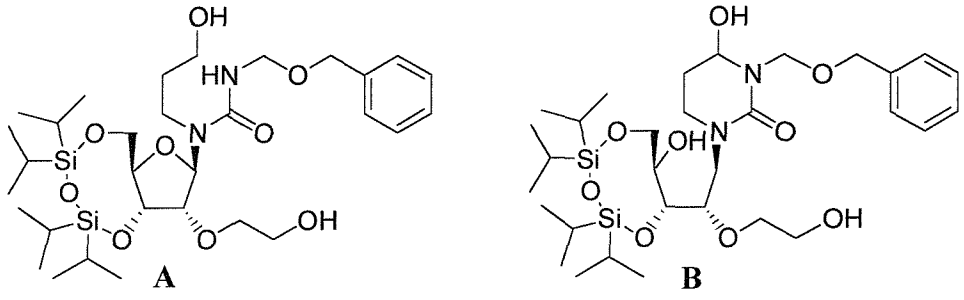


Fig 3.3.1.2 :- Possible Structures of Compound **20b** based on NMR evidence

It is difficult to tell purely from the NMR evidence which of the two structures drawn above is the correct one, although a 1H singlet at 5.32, where the C1' CH group is expected suggests the structure A is the correct compound. The C4 group in structure B would most likely not give a singlet.

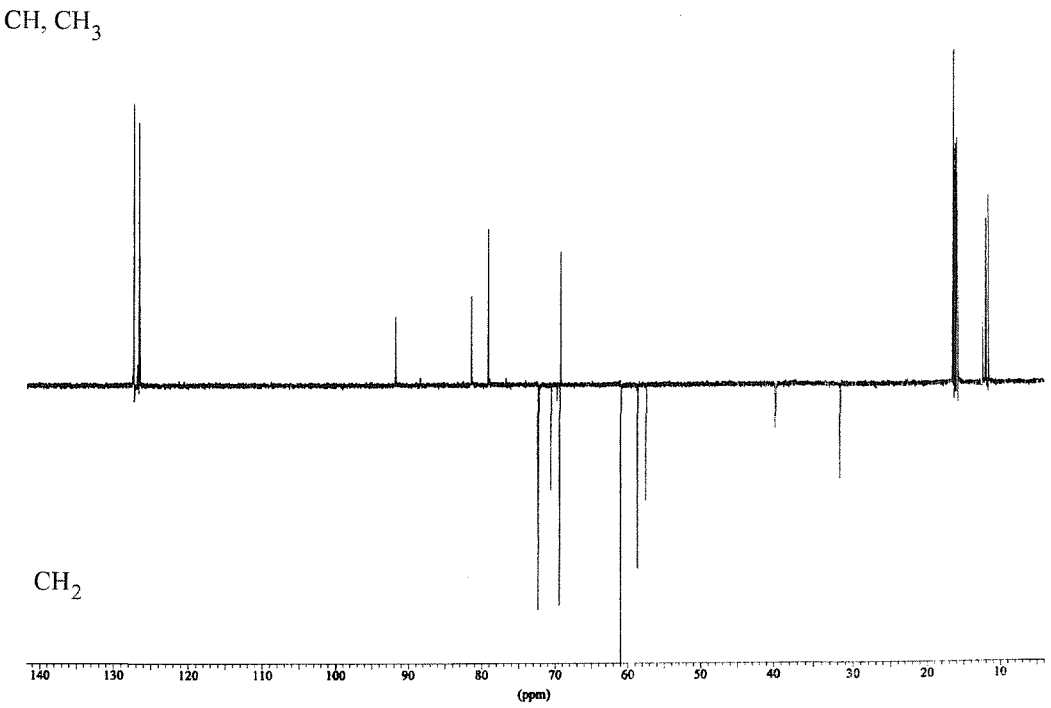


Fig 3.3.1.3 :- DEPT NMR of Compound **20b**

The major product of the over-reduction, corresponding to the less polar compound **20a** had a molecular weight of 654gmol^{-1}. Data from following experiments showed that compound **20a** is formed initially and is only slowly converted to the more polar compound **20b**. If the experiment was carried out at 5°C instead of room temperature then compound **20b** is not formed at all. It can be deduced that compound **20a** must be similar in composition to compound **20b** but, with the addition of 2 hydrogen atoms to fit the molecular weight of 654gmol^{-1}

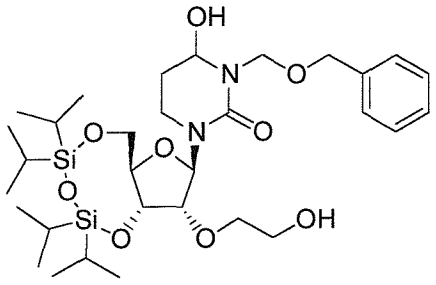


Fig 3.3.1.4 :- Proposed Structure of Compound **20a**

In summary, the reaction conditions used by Cuenoud for the reduction of 3',5'-1,1,3,3-tetraisopropyldisiloxan-1,3-diyl-3-benzyloxymethyl-5-methyluridine-2'-methylethanoate are not compatible for compound **11**, giving instead as major products two over reduced compounds, **20a** and **20b**. These have both been identified as tetrahydrouridine products with, in the case of compound **20b** the opening of the N3-C4 bond and reduction of the resulting C4 carboxyl group. The only way found to isolate compound **12** is to workup the reaction after half of the starting material has been consumed. Any reisolated starting material is taken through the reduction again.

3.4 Synthesis of the Uridine Monomer Protected by a Photolabile Group

Oligonucleotides containing the modified monomer were synthesised using automated DNA synthesis. Because the Fmoc protecting group was found to be partially labile during the capping step in automated DNA synthesis (section 3.5.1) a literature search has been carried out to identify a more stable protecting group. Any protecting group used has to be stable to acid as the trityl group on the oligonucleotide is removed by washing in 5% TCA in DCM. The deprotection conditions used should be fairly mild and be able to be carried out on the solid phase. Three protecting groups were identified as being suitable, these were:

1. The *t*-butyldimethylsilyl group (TBDMS)
2. The *p*-methoxybenzyl group (PMB)
3. The α-methyl-2-nitropiperonyloxycarbonyl group (MeNPOC)

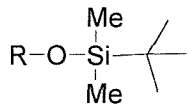


Fig 3.4.1:- A TBDMS Group

The TBDMS group[128,129] has been used in automated DNA synthesis before and is known to be stable to the conditions used. It can also be deprotected[130,131] on the solid phase easily, by simply washing away the TBAF after the completion of the deprotection. The problem with its use is the presence on the nucleoside of a tetraisopropyldisiloxane (TIPS) group. The TIPS group cannot be easily deprotected without also removing the TBDMS group and no simple alternative exists to the use of the TIPS group for 3',5' hydroxyl protection of the ribose.

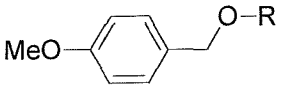


Fig 3.4.2:- A PMB Group

The PMB group has no history of being used in phosphoramidite DNA synthesis. The protecting group is introduced by forming the alkoxide of the alcohol, using sodium hydride in DMF or DMSO and introducing *p*-methoxybenzyl chloride in THF at room temperature. Providing the N-3 position is protected, the modified nucleoside is capable of standing up to these conditions. The protecting group is cleaved from the alcohol by addition of 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) in DCM:water (18:1).[132,133] The resin bound oligonucleotide should be stable to these conditions but the reaction leaves an insoluble precipitate of 2,3-dichloro-5,6-dicyanohydroquinone (DDQH). The precipitate could cause a problem for further additions of compounds to the oligonucleotide, if it is too coarse to be washed through the filters of the synthesis column.

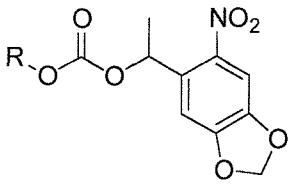
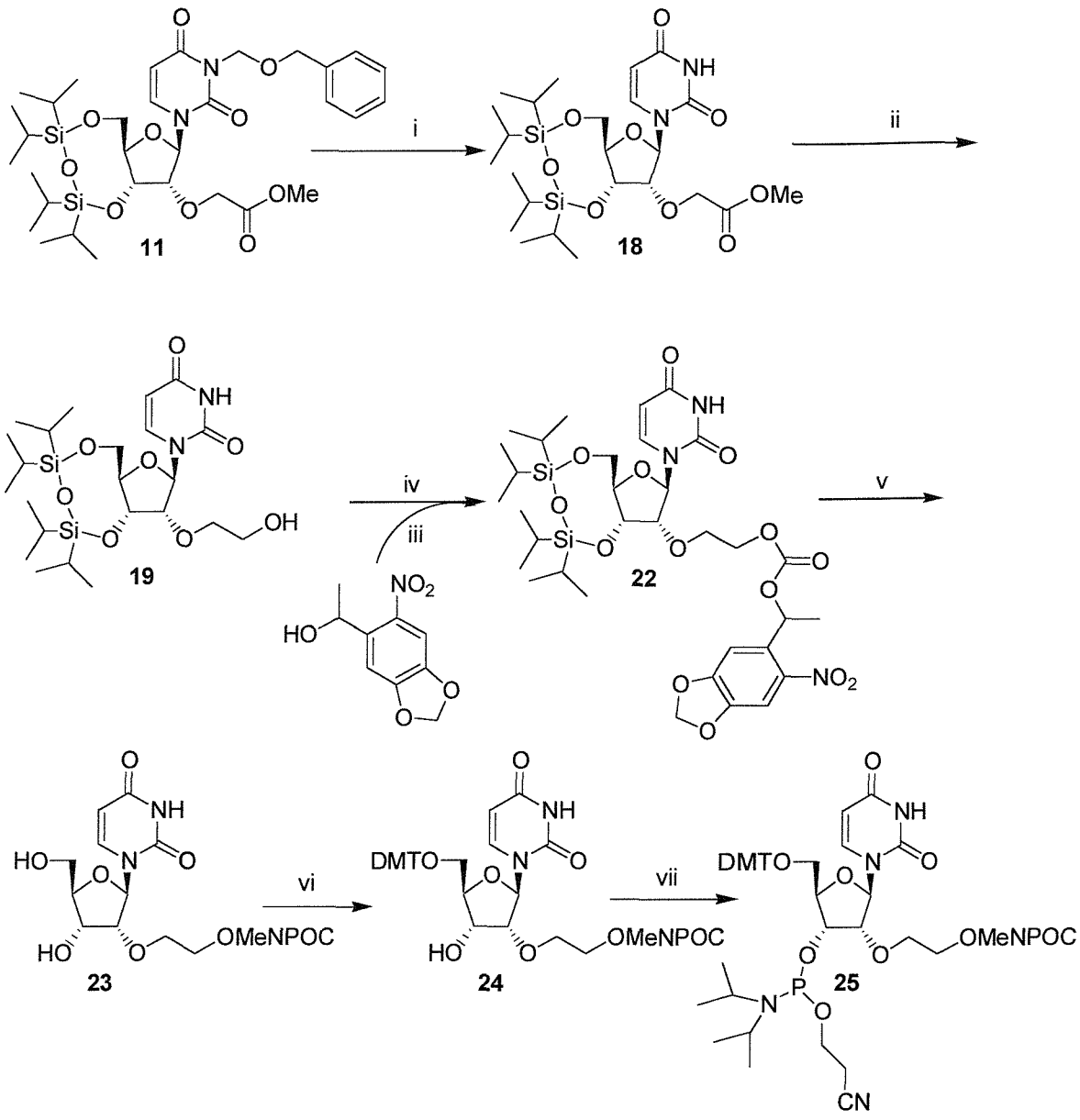


Fig 3.4.3:- A MeNPOC Photolabile Group

The MeNPOC group belongs to a class of compounds that are cleaved by UV light at 360nm and are known for their use as protecting groups for oligonucleotide synthesis on microarrays. Photocleavable linkers are used in microarrays[134,135,136,137,138] where they are utilised to produce a large number of short oligonucleotide sequences on a chip. A mask containing gaps, where labelling is to occur is placed over the chip. Light of a particular wavelength is shone on the chip and where there are gaps in the mask the photocleavable group is removed. A nucleotide is coupled and a different mask is applied, the procedure is repeated until the desired sequences are prepared. Photocleavable groups are also used as mass tags allowing mass spectrometry to be used as a tool in genetic analysis, to detect single nucleotide polymorphisms.[139] Various hybridisation probes linked to specific mass tags are synthesised. When a probe binds to its complementary target, any unhybridised DNA is removed. The mass tag is cleaved and identified by mass spectroscopy. The molecular weight of the tag allows the sequence of the particular probe to be identified.

The compound chosen as a protecting group for the modified nucleoside is a α-methyl-2-nitropiperonyloxycarbonyl group (MeNPOC). The structure is ideal as it is:

1. Cheap and simple to synthesise
2. Stable to acid
3. Mildly stable to base
4. Deprotected on the solid phase by irradiating the resin with UV light



Reagents and conditions: i, 20wt% Pd/C, THF:methanol (1:1), $H_{2(g)}$, *86%*; ii, $NaBH_4$, methanol, t-butanol, *76%*; iii, carbonyldiimidazole, nitromethane; iv, 1eq **19**, pyridine, *78%*; v, TBAF, THF, *67%*; vi, 4,4'-dimethoxytritylchloride, pyridine, *83%*; vii, 2-cyanoethyl diisopropylchlorophosphoramidite, DIPEA, THF, *84%*

Fig 3.4.4 :- Reaction Scheme for Photolabile Group Attachment

During the synthesis of the MeNPOC protected compound a new method of reducing the 2' ester to the alcohol was discovered. In a paper by Douglas and coworkers[140] a 2' ethyl ester on a uridine nucleoside was reduced to the corresponding alcohol using alcoholic sodium borohydride in a yield of 82%. The reaction conditions were used on compound **11** but led again to an over reduced product, **20c**. The NMR of the major

product obtained showed 1 CH_3 group, 6 CH_2 and 4 CH groups excluding the tetraisopropyl functionality. In addition the aromatic protons of the benzyloxymethyl group were not present. The structure suggested for compound **20c** is shown in fig 3.4.5.

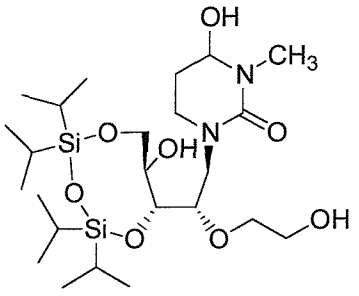
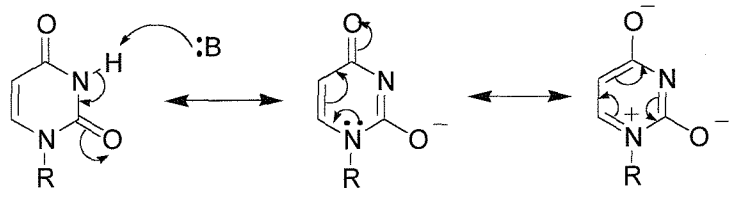


Fig 3.4.5 :- Proposed Structure of **20c**

In this compound the benzyloxymethyl group has been partially reduced to leave a methyl group attached to the N-3 position. The structure matches the NMR evidence and also has the correct mass, a low-resolution mass spectrum giving a molecular ion of 550gmol^{-1}. Douglas mentions that the protecting group that they employed, a 2,6-dichlorophenoxy moiety had to be removed to prevent the heterocyclic base being reduced. The benzyloxymethyl moiety was removed using $H_{2(g)}$ and palladium hydroxide and the reduction was attempted on the unprotected base; the reaction proved successful giving compound **19** in a 76% yield. Having obtained compound **19** the reduction was attempted on compound **11** using lithium borohydride, however the reaction failed giving a complex mixture of products, none of which have been isolated.

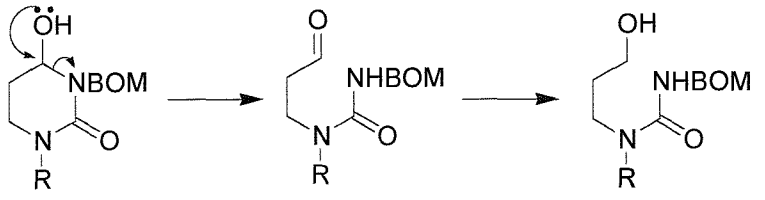
Some thought was put into why, with a protecting group present at the N-3 position an over reduced product is formed. The most convincing reason is one of resonance (fig 3.4.6). With the N-3 position unprotected in the presence of a base the proton of the nitrogen can be removed. The free electron pair can then donate into the heterocyclic ring causing it to become aromatic and stabilised. The extra stabilisation obtained prevents over reduction. If a protecting group is present then the stabilisation cannot occur leaving the double bond open to attack. With the double bond reduced the C-4 carbonyl then becomes prone to reduction, the C-2 being close to the N-1 nitrogen is

more stabilised and is not reduced. The lone pair of the C4 hydroxyl can then break the C4-N3 bond and the resulting aldehyde formed can be further reduced to an alcohol.



R :- Sugar

Fig 3.4.6 :- Proposed Stabilisation Mechanism of the Heterocyclic Base



R :- Sugar

Fig 3.4.7 :- Proposed Mechanism for the Breakage of the C4-N3 Bond

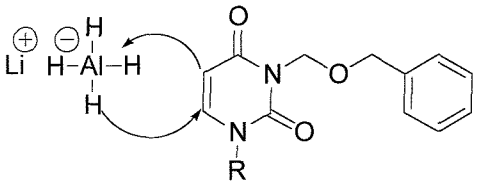


Fig 3.4.8 :- Mechanism of the Reduction of the C5-C6 Double Bond

The initial synthetic method of attaching the MeNPOC protecting group to the nucleoside was to form the chloroformate using a solution of 20% phosgene in toluene. The chloroformate was then reacted with the 2' ethoxy group, compound **12**, in pyridine for 48 hours leading to the protected compound in more than 70% yield. However no reaction was observed upon trying the reaction using compound **19**. The BOM group obviously acts as an activator in the reaction although how is unknown. A large amount of phosgene was needed to produce enough of the chloroformate for large-scale synthesis, so literature work was carried out to try and find an alternative reaction that would successfully couple the MeNPOC group as an alcohol to the

nucleoside without the need for the potentially hazardous phosgene step. The reaction that was used was found in a paper by Pirrung[141] who took methylnitropiperonyl alcohol and treated it with Rapoport's[142] methylated carbonyldiimidazole reagent. In the paper the intermediate carbonate imidazoliumide was added to a solution of thymidine in pyridine resulting in a 40% yield of the 5' MeNPOC protected thymidine. Compound **19** under the same conditions gave compound **22** in 78% yields with a total reaction time of under 12 hours. No further problems were reported with the reaction scheme, the tetraisopropyldisiloxane deprotection, tritylation and resultant phosphitylation all giving yields of over 65%.

In summary, the Fmoc protecting group was removed prematurely during automated DNA synthesis when the modified nucleoside was attached in the middle of an oligonucleotide or near the 3' terminus. The deprotection was found to be caused by a capping reagent used in the synthesis (section 3.5.1).

A new protecting group, removed using UV light, called a MeNPOC group was substituted. During the synthesis of this new compound a novel way to reduce the 2'-*O*-methyl ester in good yields was discovered.

3.5 Incorporation of the Fmoc Protected Monomer Into Oligonucleotides

3.5.1 Synthesis of Oligonucleotides for UV Thermal Melting

As mentioned in section 3.1 the advantage of producing a monomer with an unmodified base attached is that the melting temperature of any oligonucleotide produced should be similar to that without a modification. The 2' hydroxyl positions attached groups in the minor groove of DNA so it is possible that any dye affixed to the 2' position will create steric hindrance in the DNA strand. Any steric hindrance will lower the melting temperature of the oligonucleotide and make it less efficient at discriminating between complementary and mutant sequences.

1. A synthesis of three probes, containing the monomer attached to different positions in the oligonucleotide was attempted, oligonucleotides **43**, **44** and **45**.

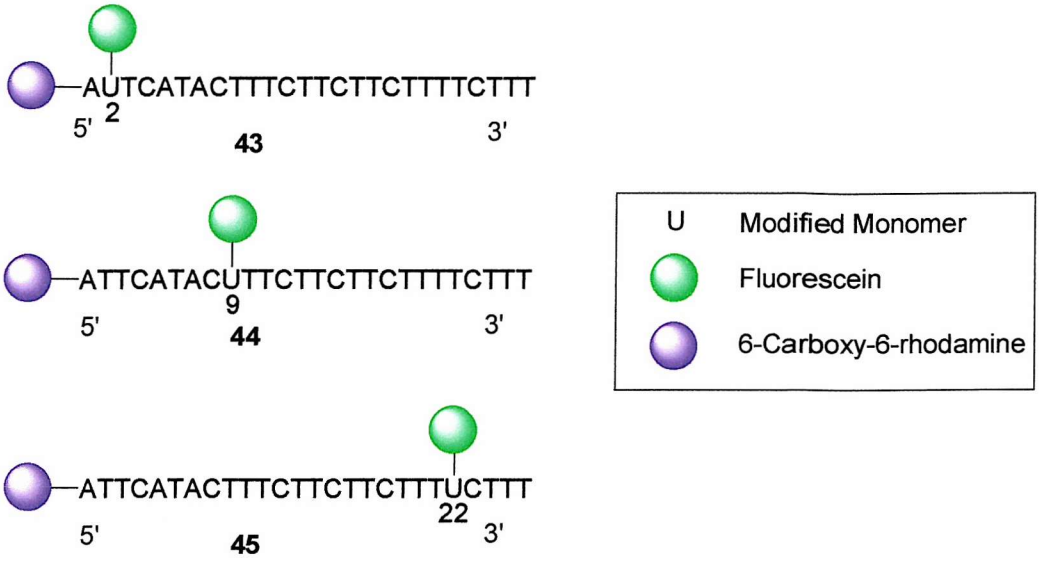


Fig 3.5.1.1:- Sequences of Oligonucleotides **43**, **44**, **45**

All the oligonucleotides contained a ROX joined to the 5' hydroxyl of the 5' DNA residue and a fluorescein introduced during the synthesis on the 2' modification on the monomer. The coupling efficiency for the monomer was good with all couplings proceeding above 98%. HPLC purification of the oligonucleotides gave one peak for each. Mass spectroscopic analysis of these peaks showed that for oligonucleotides **43** and **44,** molecular ions of 9063gmol^{-1}, corresponding to a successful synthesis were obtained. However a molecular ion of 8525gmol^{-1} was obtained for oligonucleotide **45**.

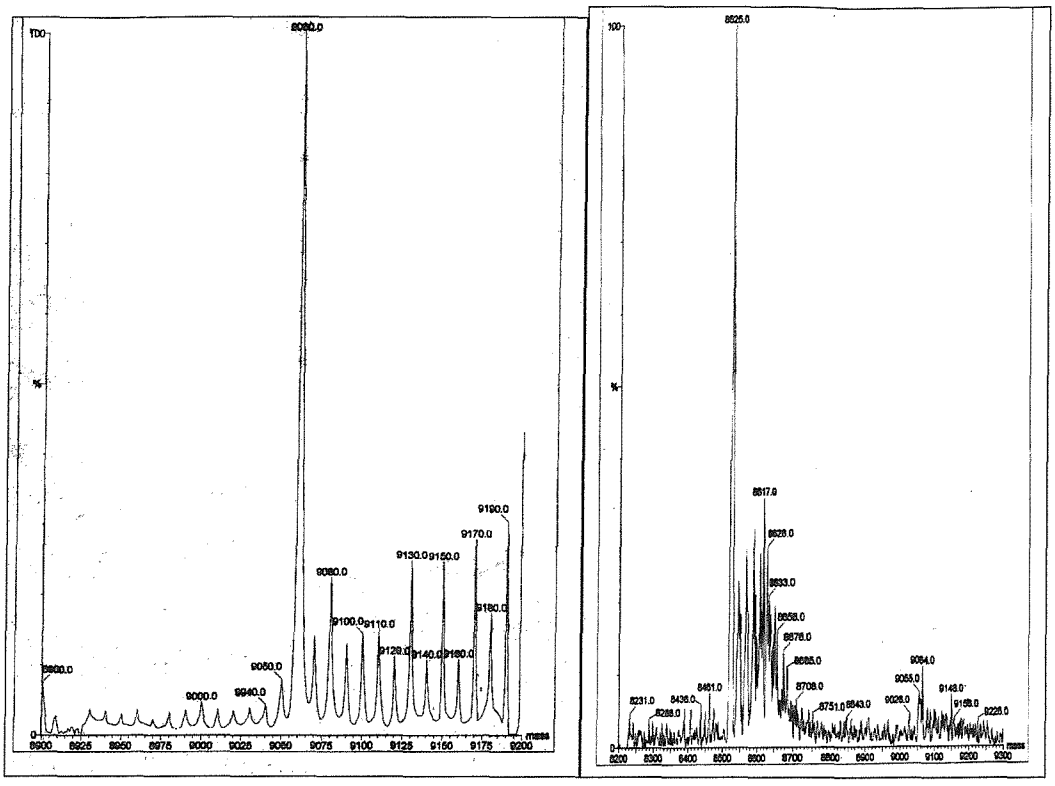


Fig 3.5.1.2 :- Mass Spectra of Oligonucleotides **43** and **45**

The mass of 8525gmol^{-1} corresponds to the mass of the oligonucleotide without the fluorescein phosphoramidite dye. The most likely explanation for the failed labelling is that the Fmoc protecting group was removed during oligonucleotide synthesis, Once the protecting group had been removed then the 1:1 mixture of 1-methylimidazole /tetrahydrofuran and acetic anhydride/pyridine/tetrahydrofuran (capping reagent) would protect the free hydroxyl with an acetate preventing further addition to take place. The step is used in DNA synthesis to acetylate unreacted hydroxyls thereby preventing the growth of side chains and limiting the number of failed sequences formed.

Further experiments, exposing a solution of compound **16** to these capping reagents proved that the Fmoc group was labile in these conditions, especially when the modified nucleoside is added towards the 3' terminus of an oligonucleotide.

3.5.2 UV Thermal Melting

3.5.2.1 Introduction to UV Thermal Melting

The aromatic bases of nucleic acids absorb UV light with a maximum absorption at around 260nm. The absorption is constant for the bases, however the extinction coefficient depends on the environment. Individual nucleotides absorb the greatest quantity of UV light, followed by single stranded DNA and least of all double stranded DNA. The decrease in absorption from single to double stranded DNA is caused by the fixing of the bases in a hydrophobic environment by stacking, the change is known as hypochromicity.

Heating double stranded nucleic acids causes denaturation by disrupting the ordered stacking of the bases through breakdown of the hydrogen bonds. The process can be conveniently monitored by an increase in UV absorbance as the double strands unwind to single strands. The thermal denaturation of dsDNA is co-operative indicating that the ends and the AT-rich internal regions destabilise adjacent regions of the helix. The destabilisation leads to a progressive and concerted melting of the whole structure at a well-defined temperature, corresponding to the mid-point of a smooth transition. The transition temperature is known as the melting temperature (T_m). UV melting experiments can provide both quantitative and qualitative data about the nature, purity and degree of hybridisation of a sample.

3.5.2.2 Results from UV Thermal Melting

A control template, oligonucleotide **46**, was prepared for UV thermal melting. The control has the same sequence as the previous oligonucleotides but does not contain dyes and the modified monomer. Oligonucleotide **43** had a T_M of 47°C, comparing favourably with the control T_M of 48°C. Labelled Oligonucleotide **44** was destabilised by 4°C in comparison to the control having a T_M of 44°C. The destabilisation though evident is not too great and would be minimised if hexaethyleneglycol spacer units were employed to move the bulky dye away from the minor groove of the DNA.

ATTCATACTTTCTTCTTCTTTTCTTT

5' **46** 3'

Fig 3.5.2.2:- Sequence of Oligonucleotide **46**

3.5.3 Fluorescence Results of the Oligonucleotides

Normalised fluorescence traces of the oligonucleotides were obtained. These show good energy transfer from the fluorescein to the ROX. It is evident that the efficiency of energy transfer is less by a factor of 4 when only 2 bases separate the two dyes. It is thought that the reduction in fluorescence is due to a quenching interaction between the dyes.

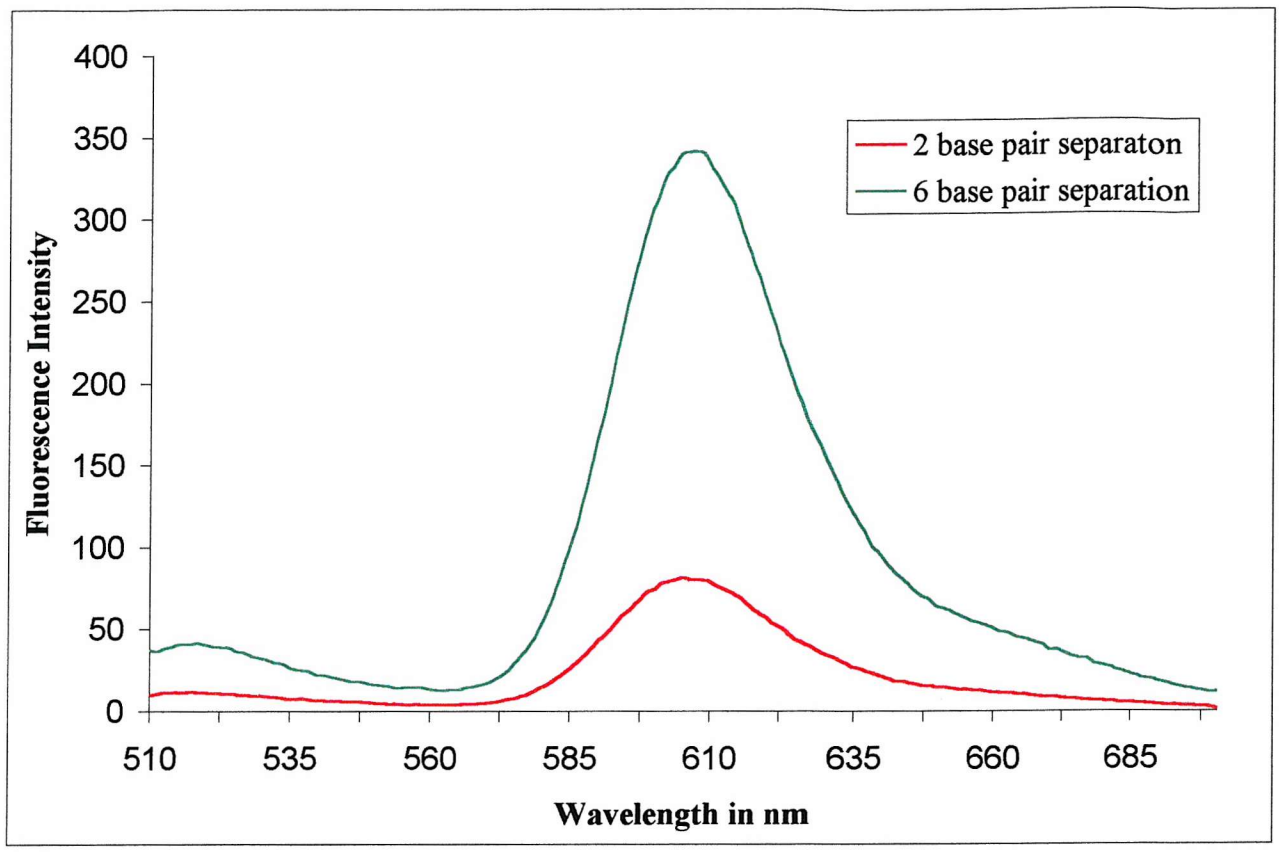


Fig 3.5.3 :- Fluorescence Emission Spectrum Showing Energy Transfer Occurring Between Fluorescein and ROX.

3.5.4 Results From LGC

The Laboratory of the Government Chemist (LGC) has been collaborating with Oswel Research Products on a novel hybridisation probe system called HyBeacons (page 29). Currently LGC use a modified nucleoside where the dye is joined to the C-5 position of uridine by an alkyl chain (fig 3.5.4.1).

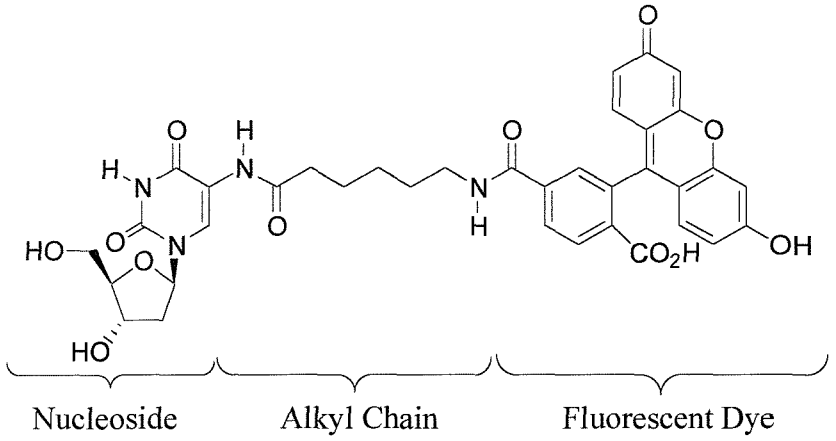
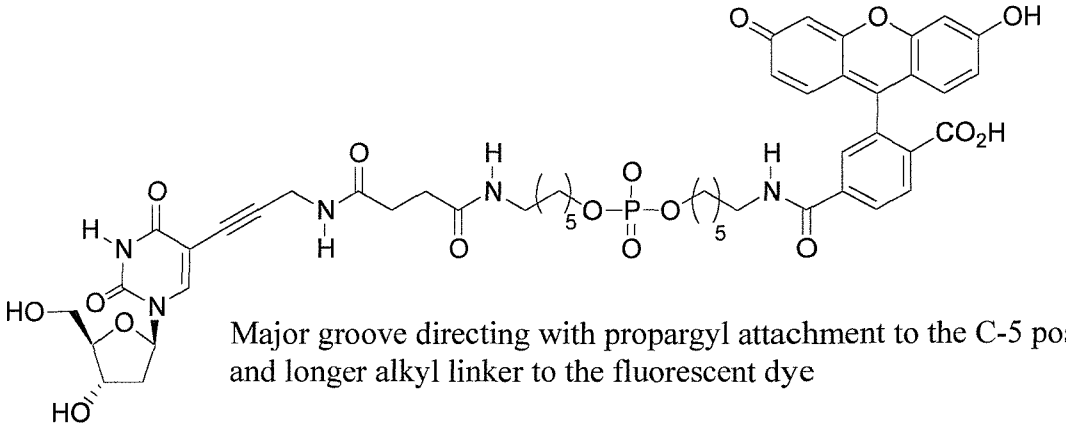


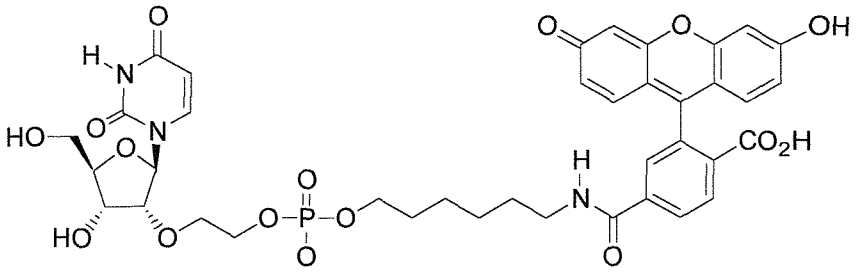
Fig 3.5.4.1 :- Structure of Current LGC Monomer Used (monomer 1)

Attaching a dye to the C-5 position of the base directs the functionality into the major groove of duplex DNA. LGC has been conducting experiments to see whether the position of the dye and the length of the linker affect the performance of the HyBeacon probe. They have been evaluating two monomers in addition to the one in fig 3.5.4.1. The structures of which are shown in fig 3.5.4.2 and fig 3.5.4.3. Monomer 2 was synthesised by Dr Lynda Brown and I am grateful for her permission to use the compound in my research.



Major groove directing with propargyl attachment to the C-5 position and longer alkyl linker to the fluorescent dye

Fig 3.5.4.2 :- Structure of monomer 2 evaluated by LGC



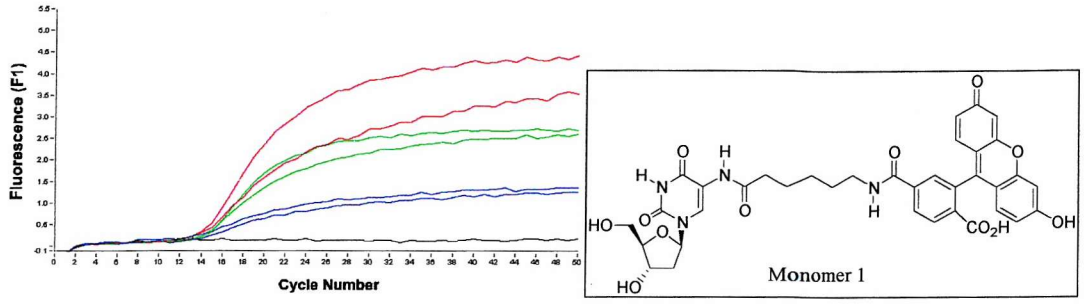
Minor groove directing with short alkyl linker to the fluorescent dye

Fig 3.5.4.3 :- Structure of monomer 3 evaluated by LGC

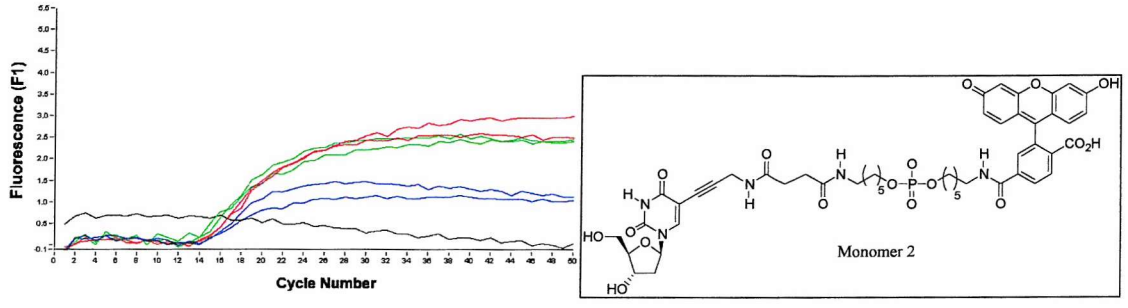
Probes were manufactured with the monomer introduced 6 base pairs in from the 5' terminus of a 21 base sequence. The HyBeacon assay was performed on a LightCycler, a machine produced by Roche. The LightCycler is essentially a thermal cycler with a fluorimeter attached. Samples are introduced in small capillaries and PCR can be performed in real time, with the fluorescence being measured after every PCR cycle. The fluorimeter in the LightCycler can only excite at 495nm but can measure absorbance at 3 frequencies. Channel 1 records absorbances of around 520nm, channel 2 at 640nm and channel 3 at 705nm.

The HyBeacon probes use fluorescein, which is excited at 495nm and emits at 520nm and hence is picked up in channel 1. The locus used for the studies was CYP2D6, a sequence found in all genomic DNA and the probes were set to discriminate between complementary targets and sequences containing 1 nucleotide mismatch with respect to the probe. The results, shown in fig 3.5.4.4 show 4 lines. The red and blue lines represent homozygous samples containing DNA sequences fully complementary and containing a single nucleotide mismatch when hybridised to the probe respectively. The green lines represent heterozygous samples possessing both matched and mismatched alleles and the black lines represent negative controls containing no DNA.

Monomer 1



Monomer 2



Monomer 3

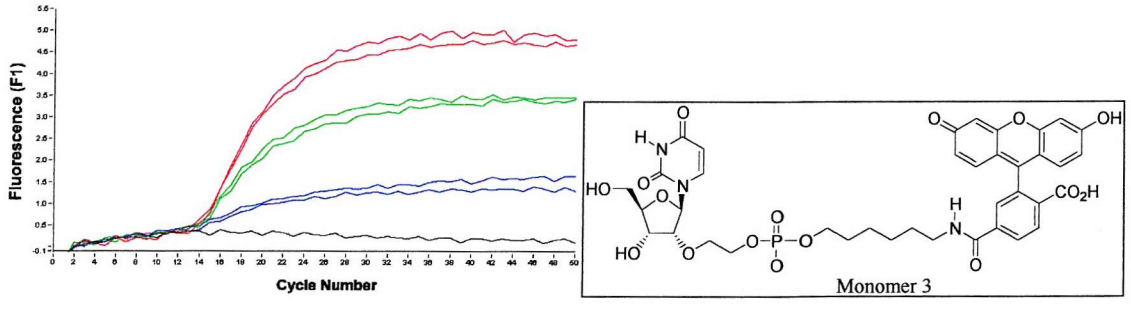


Fig 3.5.4.4 :- Real Time PCR Results of the 3 Monomers Incorporated into HyBeacon Probes

All 3 systems allow the discrimination of the polymorphic alleles however monomer 3 which directs the fluorescein into the minor groove shows markedly better discrimination than the other major groove probes. The discrimination is most notably better between the fully complementary homozygous sample (red line) and the heterozygous sample (green line). It is not known why minor groove direction leads to greater difference between the quenched and fluorescent state of the probe but it is likely to involve the orientation of the dye with respect to the DNA bases.

3.6 Incorporation of the MeNPOC Protected Monomer Into Oligonucleotides

The monomer was incorporated into oligonucleotides with nearly 99% efficiency. The highly efficient incorporation showed that the MeNPOC group did not hinder the addition of the monomer to the oligonucleotide.

3.6.1 Deprotection Conditions for the MeNPOC Group

The MeNPOC group is used in the formation of microarrays, as mentioned in page 75. The deprotection conditions used vary; Pirrung[141] uses a Rayonet photochemical reactor irradiating at 350nm while Beier[143] used a 100W mercury high pressure lamp at 365nm. Due to high cost of mercury arc lamps it was decided to try and utilise a benchtop UV TLC lamp (Model UVGL-58, UVP corp.), irradiating at 365nm. The power of the lamp, measured by using a power meter was found to be 0.6mW/cm^2 at a distance of 10cm from the bulb. Oswel Research product had experience of using benchtop UV lamps to deprotect MeNPOC groups from monomers where the MeNPOC group was held on a long alkyl chain spacer from the 1' position of the ribose. They found that deprotection of photolabile group from the monomer proceeded to ~80% within 1 hour. Deprotection using the mercury arc lamp proceeds at a much greater rate, the group becoming fully deprotected within 5 minutes but, as only 1 deprotection is needed as opposed to the large numbers used in microarray synthesis, the longer time period is not a disadvantage.

To test how long it would take to deprotect the MeNPOC group on the 2' position a polythymidine sequence containing 12 thymidines and the monomer centred 7 base pairs into the sequence was prepared, oligonucleotide **47**.

TTTTTTUTTTTTT U Modified Monomer
5' **47** 3'

Fig 3.6.1.1:- Sequence of Oligonucleotide **47**

The oligonucleotide was cleaved from the solid support using concentrated aqueous ammonia for 10 minutes at room temperature. An HPLC of the product showed just one peak, however the molecular ion, obtained by MALDI mass spectroscopy at 3940gmol^{-1} corresponded to the deprotected free hydroxyl group at the 2' end. Exposing compound **24** to ammonia also resulted in complete removal of the MeNPOC group. The milder deprotection condition of 0.05M potassium carbonate in methanol also resulted in complete cleavage of the MeNPOC group. The fact that the photolabile group is unstable to aqueous ammonia is not disadvantageous as in oligonucleotide synthesis it is never necessary to cleave the oligonucleotide from the solid support with the protecting group intact.

The same oligonucleotide was synthesised again, however after deprotection the oligonucleotide was labelled with fluorescein before cleaving from the solid support with aqueous ammonia. The synthesised oligonucleotides, bound to the solid support were exposed to the UV TLC lamp by opening up the synthesis columns and stirring the resin every half hour. The samples were washed for 10 minutes with the capping solution to prevent dye being attached to the free 5' hydroxyl group, the samples were then exposed to the UV TLC lamp for times ranging from 2 hours to a maximum of 18 hours. The synthesis columns were then resealed and placed back on the ABI DNA synthesiser, where a phosphoramidite dye was joined to the unprotected 2' hydroxyl. The oligonucleotide was then cleaved from the solid support in concentrated aqueous ammonia. The ammonia was removed *in vacuo* and the samples purified by HPLC.

All oligonucleotides gave two main peaks on the HPLC, one labelled with dye, the other colourless and presumably unlabelled. The ratio of the two peaks changed with increasing irradiation time from 4:1 at 2 hours to 1:1 at 18 hours. The first peak on the HPLC is colourless and due to the unlabelled oligonucleotide. A molecular ion of the peak corresponds to the oligonucleotide with a free hydroxyl at the 2' position, any photolabile protecting group present having been deprotected by the ammonia cleavage. The second peak, which increases to a maximum in the 18 hour UV exposure experiment is coloured and has a molecular ion corresponding to the labelled oligonucleotide.

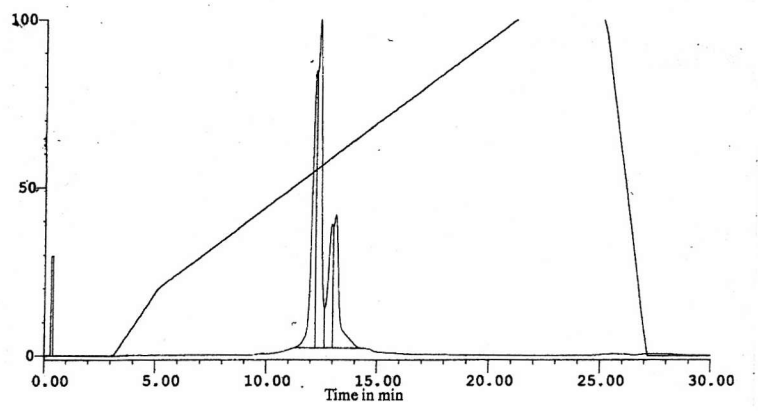


Fig 3.6.1.2 :- HPLC of 2 Hour UV Deprotection of the Oligonucleotide

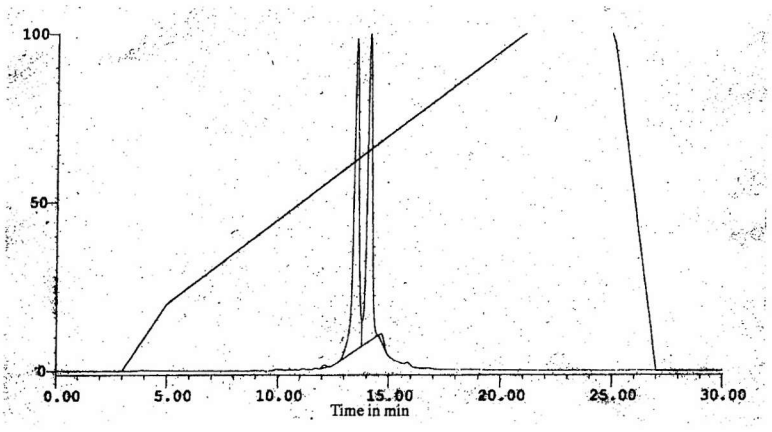


Fig 3.6.1.3 :- HPLC of 18 Hour UV Deprotection of the Oligonucleotide

The bifurcated peaks present most noticeably in fig 3.6.1.2 were due to an old HPLC column being used, upon switching to a new column the peak shape was vastly improved. Only 50% of the oligonucleotide can be deprotected using a UV TLC lamp. It was decided that a mercury arc lamp should be tried to see if using a higher intensity light source would allow further deprotection to occur. Filters were used to block any light with a wavelength below 300nm. Light below this wavelength is absorbed by the DNA bases and would cause photolytic damage to the oligonucleotide.

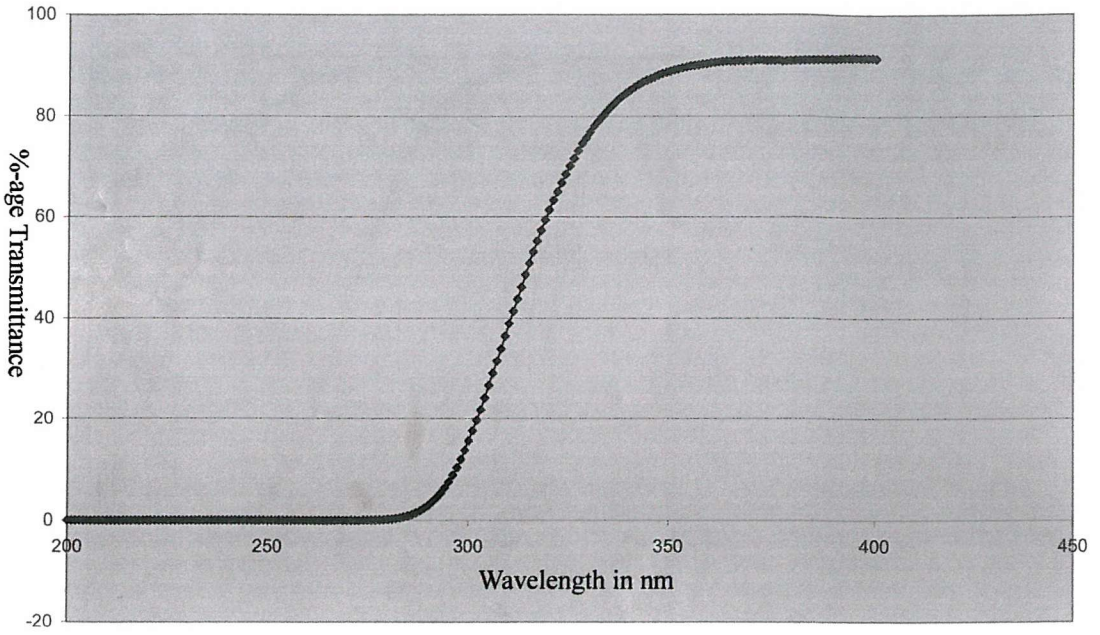


Fig 3.6.1.4 :- UV Transmittance Plot of Filters Used with the High Pressure Mercury Arc Lamp

The mercury arc lamp used for the experiment had a power of 6mW/cm^2 at a distance of 10cm from the lamp. Oligonucleotides were deprotected under the lamp for periods of 2 and 8 hours using the same conditions as for the bench TLC lamp. After deprotection the oligonucleotides were labelled with fluorescein and the amount of deprotection achieved was monitored by HPLC. As can be seen in fig 3.6.1.4 and fig 3.6.1.5 the HPLC traces obtained for the 2 hour and 8 hour mercury arc lamp experiments are nearly identical to that obtained for the 18 hour bench TLC lamp deprotection. The first peak obtained was colourless and unlabelled, the second being labelled and corresponding to the fluorescein attached to the oligonucleotide. The increased power of the mercury arc lamp made no difference to the final amount of deprotection achieved. The time taken to reach the point of maximum deprotection however was decreased from 18 hours to less than 2 hours.

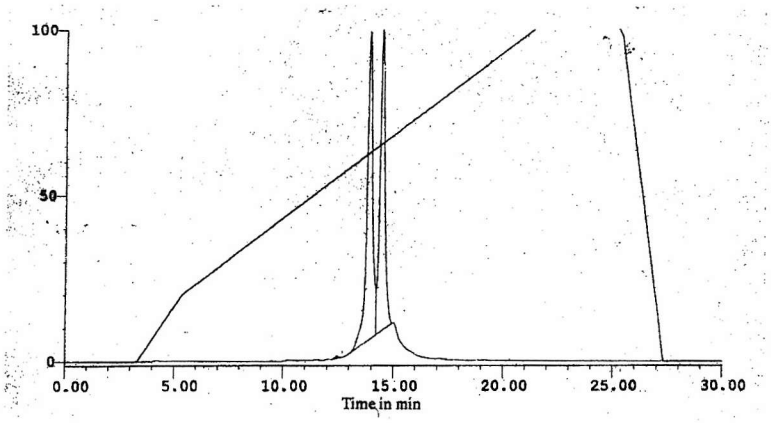


Fig 3.6.1.5 :- HPLC of 2 Hour UV Deprotection of the Oligonucleotide Using a High Pressure Mercury Arc Lamp

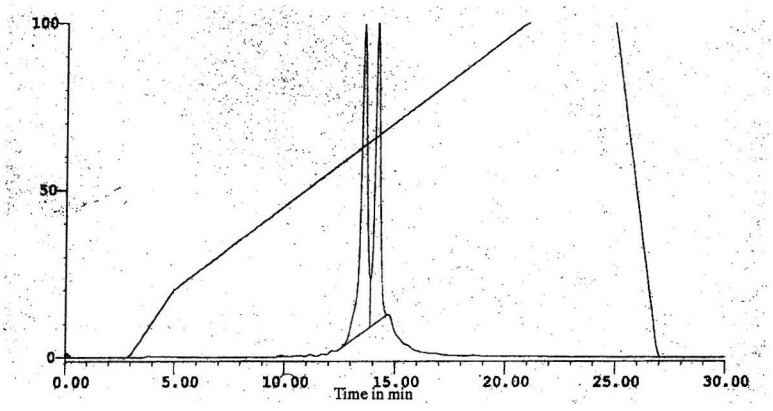


Fig 3.6.1.6 :- HPLC of 8 Hour UV Deprotection of the Oligonucleotide Using a High Pressure Mercury Arc Lamp

One explanation why only 50% of the MeNPOC group could be removed was that the cleaved MeNPOC product was absorbing the UV radiation, preventing it from reaching the oligonucleotide, inhibiting cleavage of further MeNPOC molecules. The resin was washed with acetonitrile after 1 hour to remove the deprotected MeNPOC groups, placed back underneath the light source and irradiated for a further hour. One final wash was carried out and the sample was irradiated for a third hour. The oligonucleotide was then labelled with fluorescein and purified by HPLC. HPLC traces (figs 3.6.1.5 and 3.6.1.6) identical to that shown in fig 3.6.1.4 were observed disproving the theory.

Another hypothesis was that the MeNPOC was not completely stable to the reagents employed to cap the 5' hydroxyl group before UV deprotection of the photolabile

group. To test whether MeNPOC was stable to the capping conditions, compound **22** was taken and exposed to the capping solution. After 10 minutes, a TLC showed that some deprotection (approximately 30%) had taken place. The growing oligonucleotide is exposed to approximately 30 seconds of the capping reagents per base added so for a 30mer sequence with the modified nucleotide in the centre of the sequence the MeNPOC group will be exposed to the capping reagent for 7.5 minutes, leading to 20% of the expected protected oligonucleotide. In the sequences prepared above, the oligonucleotide was in addition exposed to a 10 minute manual capping step, a total exposure time of 13 minutes. The slight lability of the MeNPOC group in the capping reagent is therefore not a major problem in any oligonucleotide prepared except if a manual capping step is performed. Subsequent oligonucleotides prepared without the manual capping step showed deprotections in the order of 80%.

3.6.2 Distance Dependent Fluorescence Measurements of Energy Transfer Probes

Two or more molecules of the same dye can be introduced into a probe giving the potential advantage of a much brighter signal than that obtained from just one dye. Placing two identical dyes within an oligonucleotide should give a doubling of the fluorescent signal obtained, provided the dyes are far enough away from each other to prevent quenching from occurring. However if the dyes are placed within the Förster distance of each other then it is possible that the amount of fluorescence produced by the system can be greater than a factor of two. The fluorescence increase is enhanced as energy transfer can occur between the two dyes, non-radiative energy transfer being more efficient than irradiation by light.

Three oligonucleotides were synthesised, with a fluorescein attached to the 5' end of the sequence. The second fluorescein dye was attached to the modified monomer that was held a varying distance from the first fluorescein. These separations were:

1. Both dyes attached to the monomer at the 5' end of the oligonucleotide with no hexaethyleneglycol spacers, oligonucleotide **48**
2. 3 base pairs between the 2 fluoresceins, oligonucleotide **49**
3. 6 base pairs between the dyes, oligonucleotide **50**

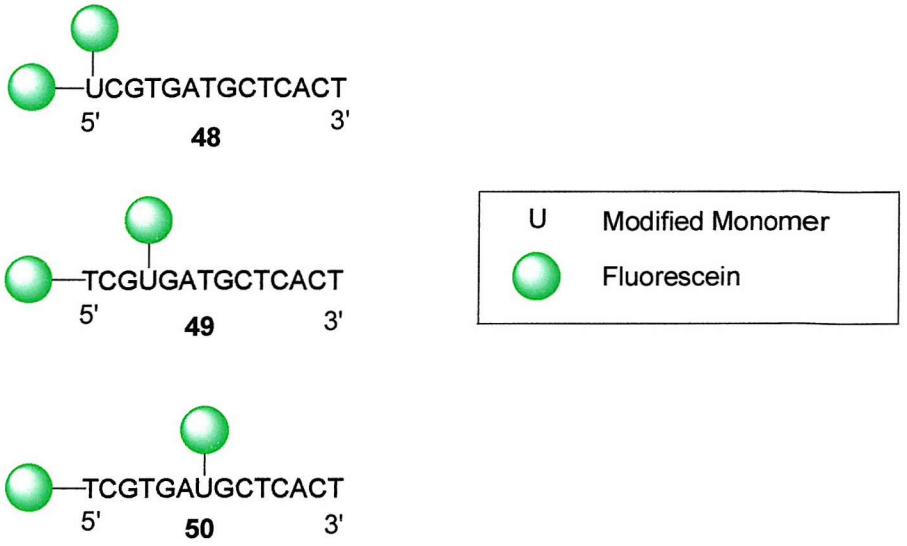


Fig 3.6.2.1:- Sequences of Oligonucleotides **48**, **49** and **50**

A control oligonucleotide labelled with one fluorescein at the 5' position was also synthesised. The oligonucleotides, at normalised concentrations were excited at 495nm, the emission from the dyes was recorded from 510nm to 600nm using a fluorescence spectrometer (fig 3.6.2.1). The control oligonucleotide had an intensity of approximately 400 units. The fluorescence produced by oligonucleotides **48** and **49** was markedly reduced giving intensities of 60 units and 170 units respectively. Oligonucleotide **50** however gave an intensity of 560 units, considerably higher than that of the control. A further fluorescence trace containing the control at twice the concentration was run. If the intensity obtained from oligonucleotide **50** was higher than that of the double control then energy transfer enhancement of the fluorescence was being observed. The control at double concentration gave an intensity of 780 units, slightly more than oligonucleotide **50**. It is probable that a small amount of collisional quenching is taking place with oligonucleotide **50**. As the oligonucleotide has no complementary strand to hybridise to, the dyes could cause the oligonucleotide to bend allowing the fluoresceins to become closer to each other and thereby quench each other. The introduction of a complementary sequence should cause an increase in fluorescence to occur as the duplex will be rigid.

A complementary oligonucleotide, **51** was prepared and introduced to the cell containing oligonucleotide **50** at triple the concentration of oligonucleotide **50**.

AGTGAGCATCACGA
5' **51** 3'

Fig 3.6.2.2:- Sequence of Oligonucleotide **51**

The cell was heated to 60°C and allowed to cool to room temperature. The heating was carried out to dissociate any secondary structure that might have been present in the oligonucleotides. A fluorescent emission spectrum recorded at room temperature showed that the fluorescence obtained from oligonucleotide **50** in the presence of the complement was still not at the same level as that obtained from the "double concentration" control. As the fluorescence intensity of oligonucleotide **50** is less than that of the control some quenching must still be occurring.

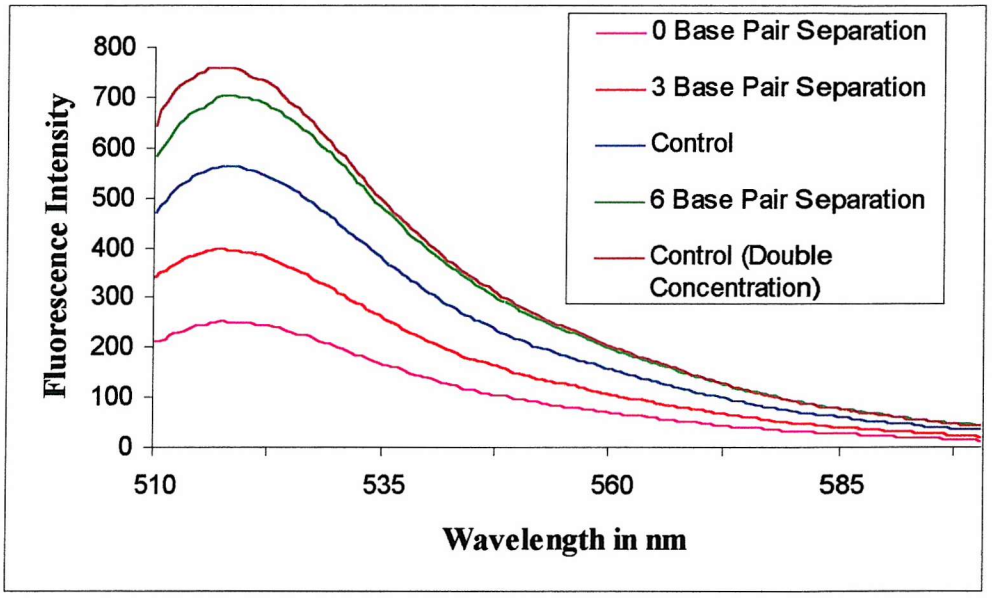


Fig 3.6.2.3 :- Fluorescence Emission Spectrum of Double Fluorescein Labelled Oligonucleotides with Varying Separations

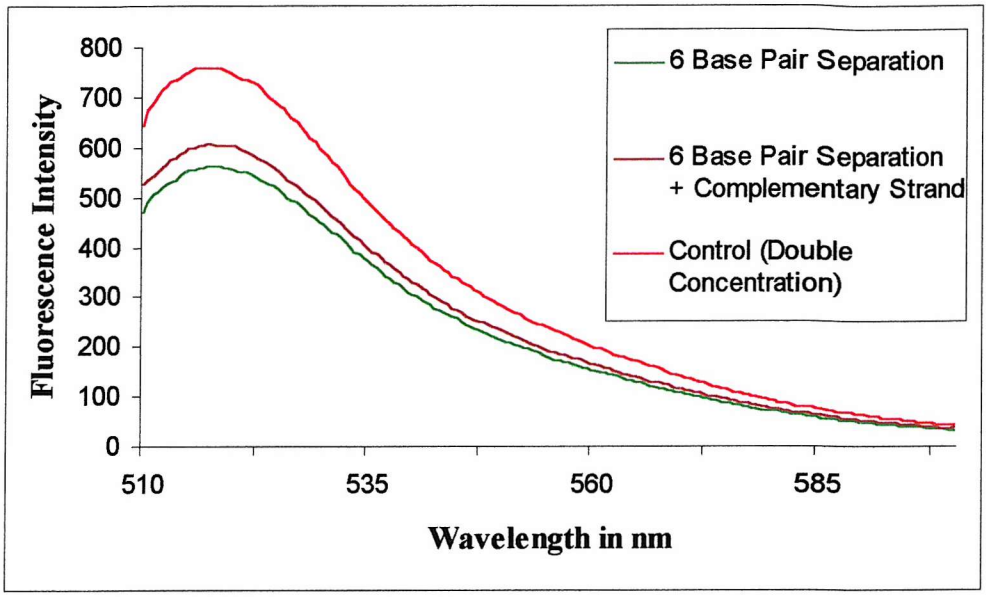


Fig 3.6.2.4 :- Fluorescence Emission Spectrum of Double Fluoresein Labelled Oligonucleotides with the Complement Sequence Introduced

In summary, oligonucleotides containing two fluorescein dyes separated by varying distances were prepared. Out of the distances synthesised a 6 base pair separation was shown to give the best fluorescence. The amount of fluorescence produced by the system is still not as great as that from a control oligonucleotide containing one fluorescein at double the concentration, showing that some quenching must occur in the energy transfer system.

3.6.3 Synthesis and Testing of Energy Transfer Taqman Probes

Taqman, a technique discussed on page 33 is a hybridisation probe system that uses fluorescence to detect hybridisation. In a standard Taqman assay, a quencher and fluorophore, held a set distance from each other are cleaved from the oligonucleotide by *Taq* polymerase. Upon being cleaved the fluorophore is no longer quenched and an increase in fluorescence is observed.

Taqman assays are performed using the LightCycler. For multiplexing to take place an energy transfer version of Taqman needs to be developed. Convential methods of designing energy transfer systems, produced by attaching each dye to a modification of the DNA base (fig 3.6.3.1) or from a modified ribose (fig 3.6.3.2) are of no use in

Taqman. The two dyes used in a FRET system are both attached to separate modified nucleotides and when these are cleaved by the *Taq* polymerase, no energy transfer can occur as both dyes are now free in solution.

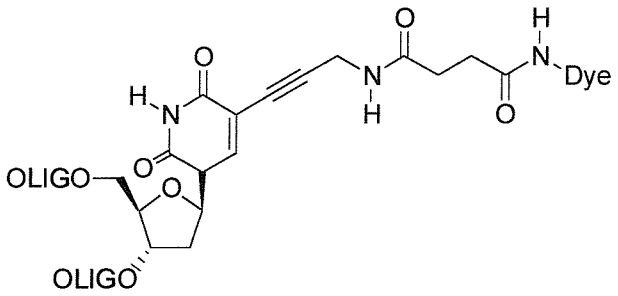


Fig 3.6.3.1 :- Modification Allowing the Introduction of a Dye into an Oligonucleotide from the C-5 Position of Thymidine

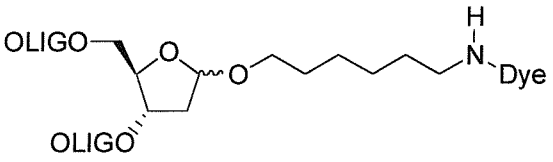


Fig 3.6.3.2 :- Modification Allowing the Introduction of a Dye into an Oligonucleotide using a modified deoxyribose

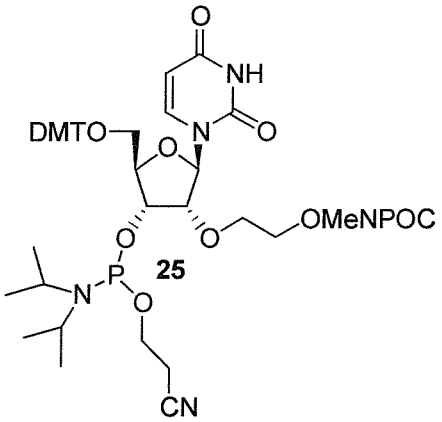


Fig 3.6.3.3 :- Structure of Compound **25**

It is possible to design an energy transfer system that it is compatible with the Taqman assay by using compound **25** as the monomer. Compound **25** allows the functionalisation of 2 dyes to one monomer provided the monomer is used at the 5'-end of an oligonucleotide chain. A quencher moiety introduced to the oligonucleotide provides a way of generating an increased fluorescent signal upon cleavage by the *Taq*

polymerase (fig 3.6.3.4). An advantage of compound **25** over other modified nucleotide systems is the fact that compound **25** has a uracil functionality. For Taqman to be successful the polymerase has to be capable of reading through the modified nucleotide, allowing cleavage of the energy transfer system from the oligonucleotide. Having an unmodified base should mean that compound **25** more closely resembles a natural nucleotide and the chance of the polymerase recognising the modified nucleotide are increased.

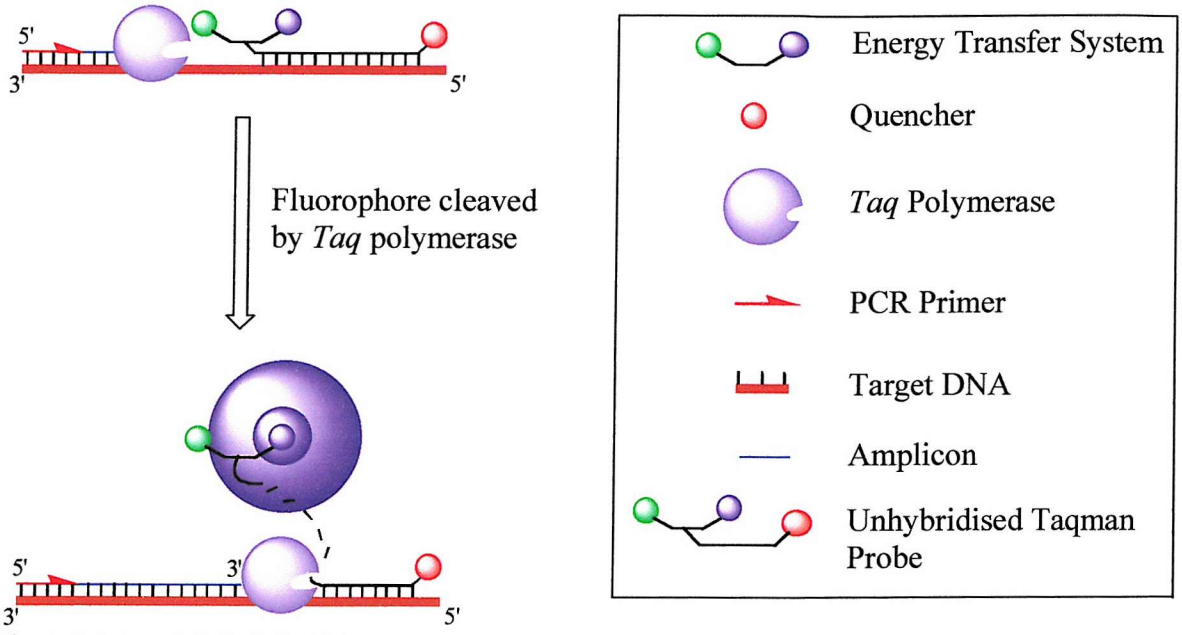


Fig 3.6.3.4 :- A Modified Energy Transfer Taqman Assay

The oligonucleotide, **52**, has been synthesised (complementary to the GAPDH exon). GAPDH is a DNA sequence found in human genomic DNA. The use of an exon sequence means that both genomic and copy DNA (cDNA) can be identified. A 22 base pair sequence was synthesised containing the modified monomer at the 5'-end and a methyl red dye at the 3' position. The modified monomer comprised a fluorescein attached to the 5' hydroxyl and a 6-carboxy-6-rhodamine (ROX) dye joined to the 2' hydroxyl by 4 hexaethyleneglycol spacers and an aminolink molecule (fig 3.6.3.5).

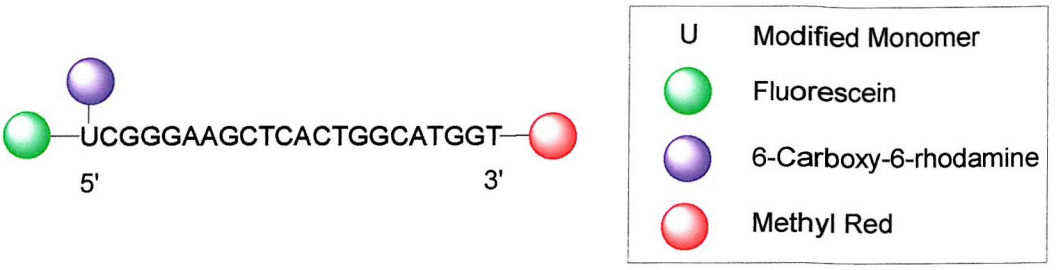


Fig 3.6.3.5 :- Sequence of GAPDH Energy Transfer Taqman Probe, Oligonucleotide **52**

A fluorescence emission spectrum (fig 3.6.3.6) of the oligonucleotide showed that very little emission was given out by the ROX whether it was excited at 495nm, the excitation wavelength of fluorescein or at 585nm, the excitation wavelength of ROX. The conclusion drawn from these experiments was that the methyl red was successfully quenching the fluorescence given out by the ROX.

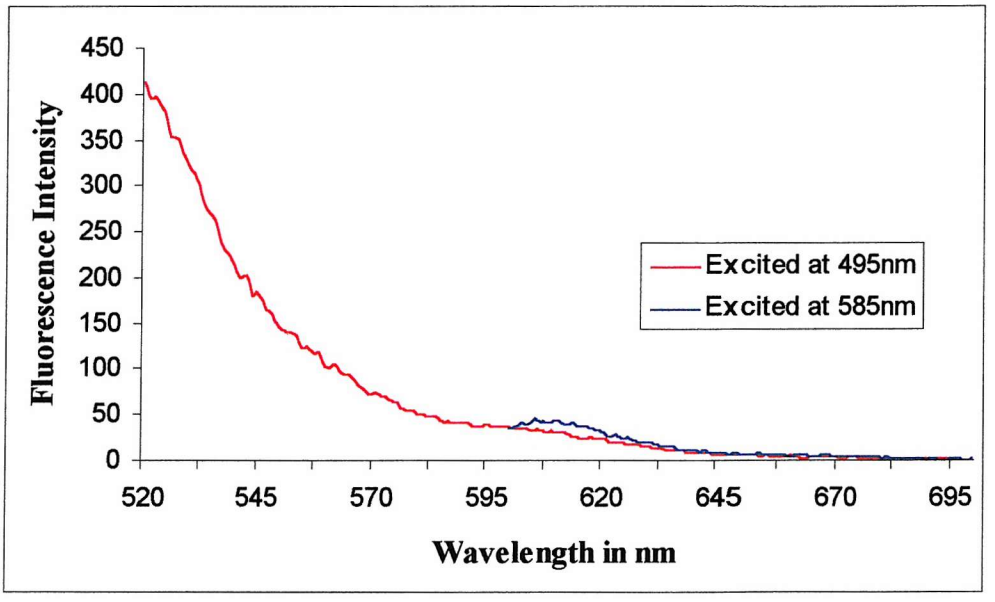


Fig 3.6.3.6 :- Fluorescence Emission Spectrum of GAPDH Energy Transfer Probe

The PCR results obtained to date are negative, giving no amplification of a fluorescence signal even after 40 cycles. The background fluorescence of the assay is low which suggests that good quenching is occurring. The most probable explanation for the failure of the experiment was that the monomer inhibits the binding of the *Taq* polymerase to the probe-target hybrid preventing the cleavage of the methyl red dye.

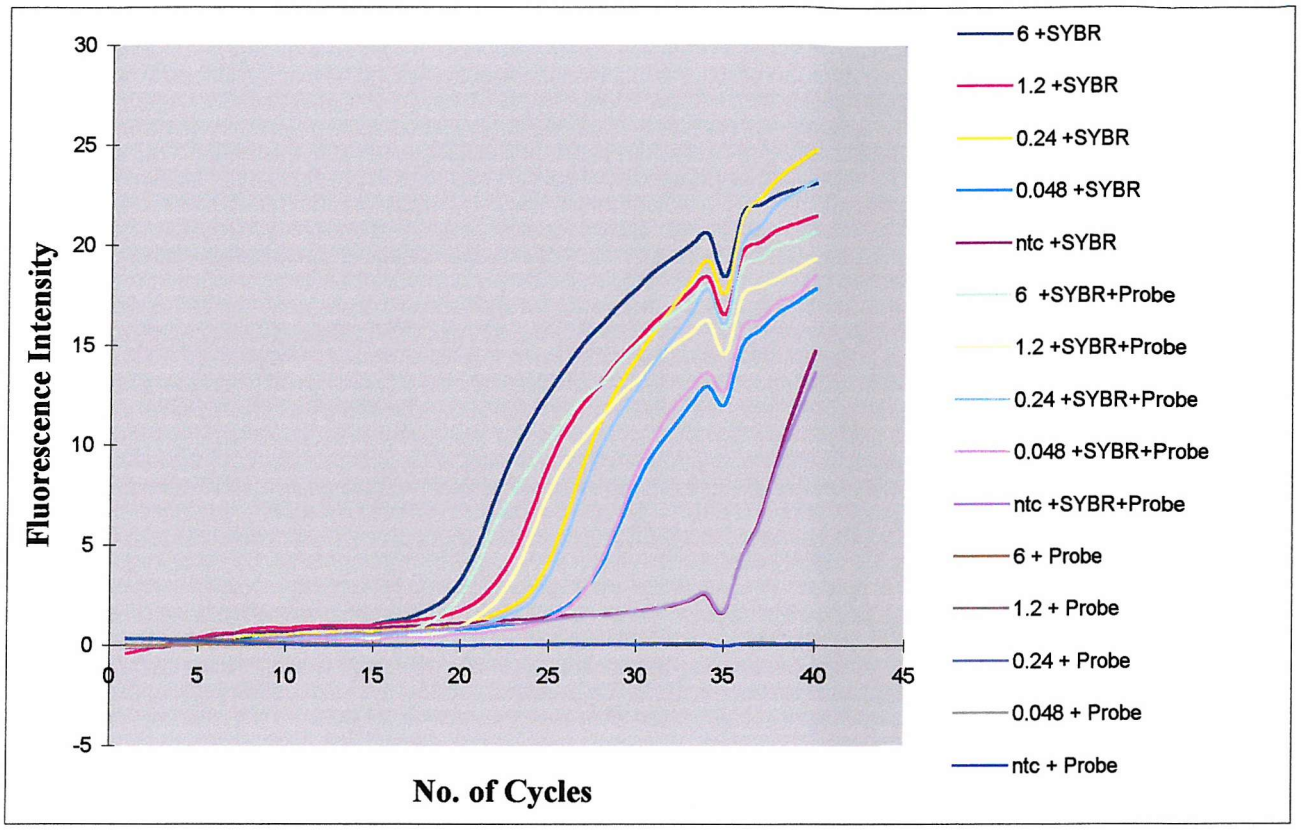


Fig 3.6.3.7 :- PCR Results of the Synthesised FRET Taqman Probes

If the problem is due to the *Taq* polymerase not recognising the modified monomer then it is possible to construct the probe in reverse, with the modified monomer at the 3'-end of the probe and the methyl red at the 5'-end. The polymerase can then bind and cleave the methyl red leaving the energy transfer system attached to the double stranded DNA, a fluorescence increase can then be detected. These experiments have not yet been carried out due to time constraints but will be performed in the near future by Oswel Research Products Ltd.

Another way of constructing an efficient energy transfer Taqman probe is to use 3 dyes as opposed to 2 dyes and a quencher. The LightCycler measures emissions at 3 distinct bands, called channels. Channel 1 records emissions at 520nm, channel 2 at around 605nm and channel 3 at approximately 700nm. If the 3 dyes used in the probe are chosen carefully it is possible to utilise a system where the second, emitting at channel 2 is dark when the 3 dyes are present and emits when the third dye has been cleaved by the polymerase (fig 3.6.3.8). The 3 dyes chosen were fluorescein, ROX and Cy 5.

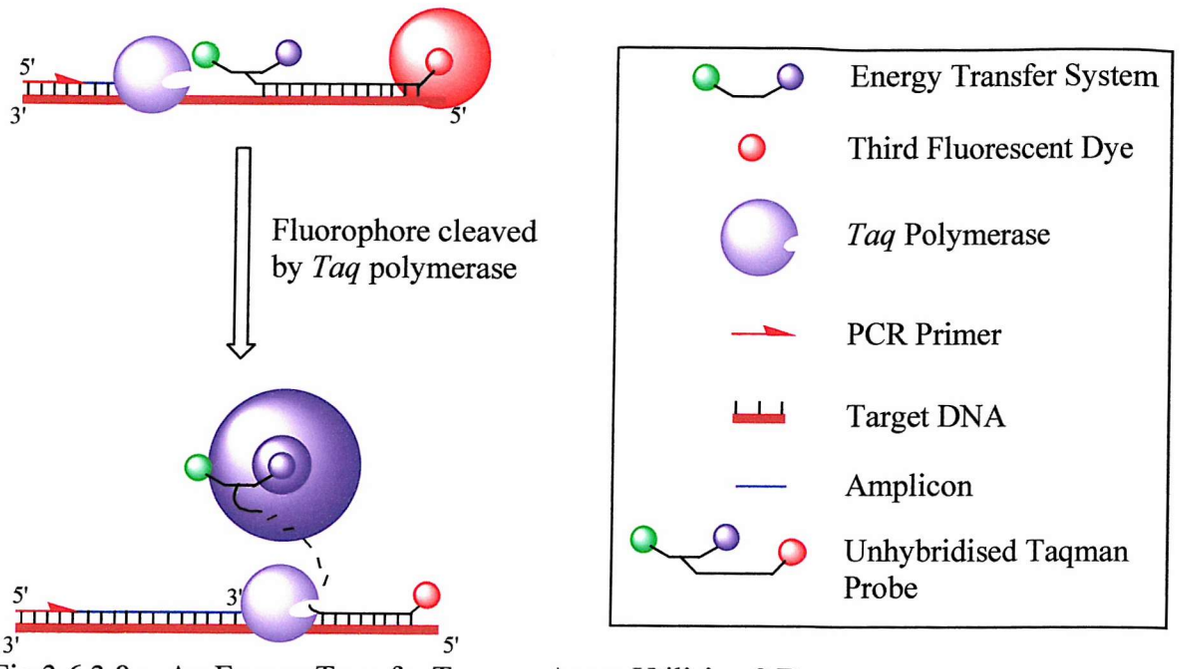


Fig 3.6.3.8 :- An Energy Transfer Taqman Assay Utilising 3 Dyes

The same GAPDH sequence (fig 3.6.3.5) was synthesised with the methyl red being replaced with a Cy 5 dye. The synthesis carried out on a 0.2μM scale was successful but yielded only 0.5μM of DNA, not enough for PCR to be conducted. A fluorescence plot of the oligonucleotide, exciting at 495nm, 585nm and 650nm showed that efficient energy transfer between fluorescein, ROX and Cy 5 was obtained.

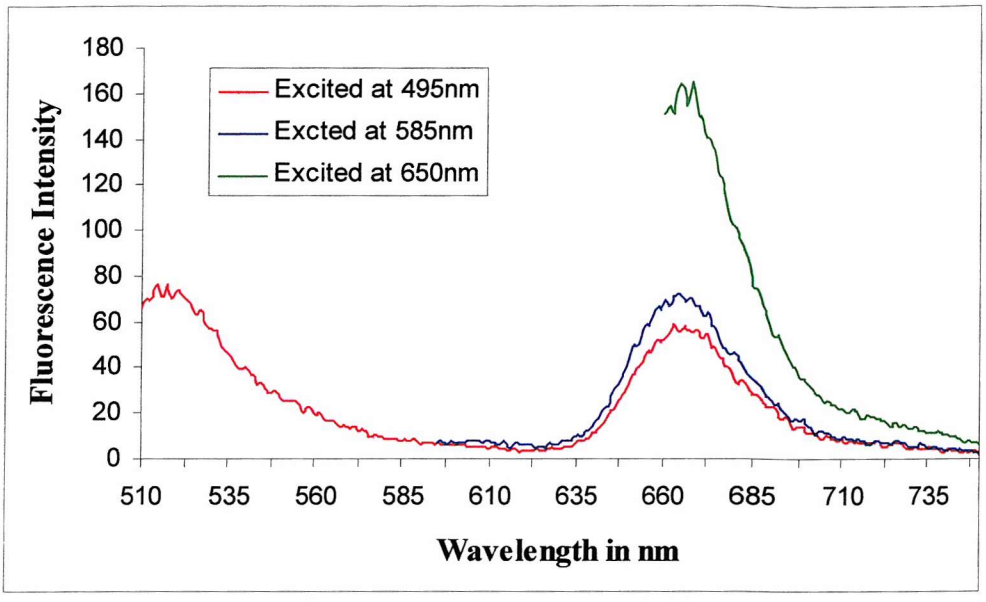


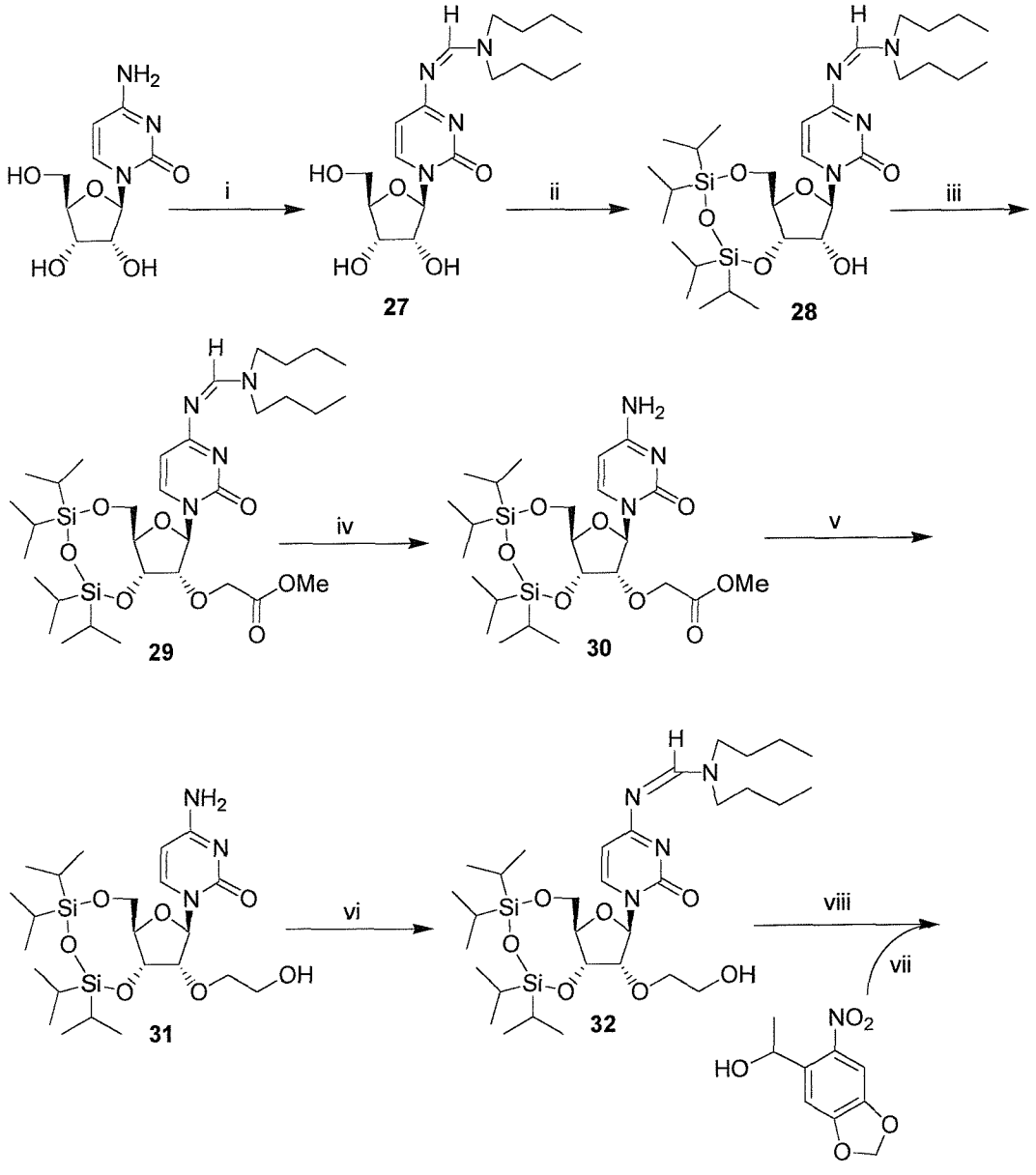
Fig 3.6.3.9 :- Fluorescence Emission Spectrum of 3 Dye GAPDH Energy Transfer Probe

Due to the PCR results obtained from the methyl red energy transfer probe the 3 dye probe will also be synthesised by Oswel in the near future in reverse with the Cy 5 being attached to the 5'-end of the oligonucleotide and the modified monomer joined to the 3'-end.

In summary, the advantages of a FRET Taqman system have been discussed and various ideas for a design of such a system have been put forward. Oligonucleotides have been synthesised and tested. Although the background of the probes is very low no fluorescence signal amplification took place after PCR. The most probable cause for the lack of amplification is that the DNA polymerase could not read through the modified nucleoside.

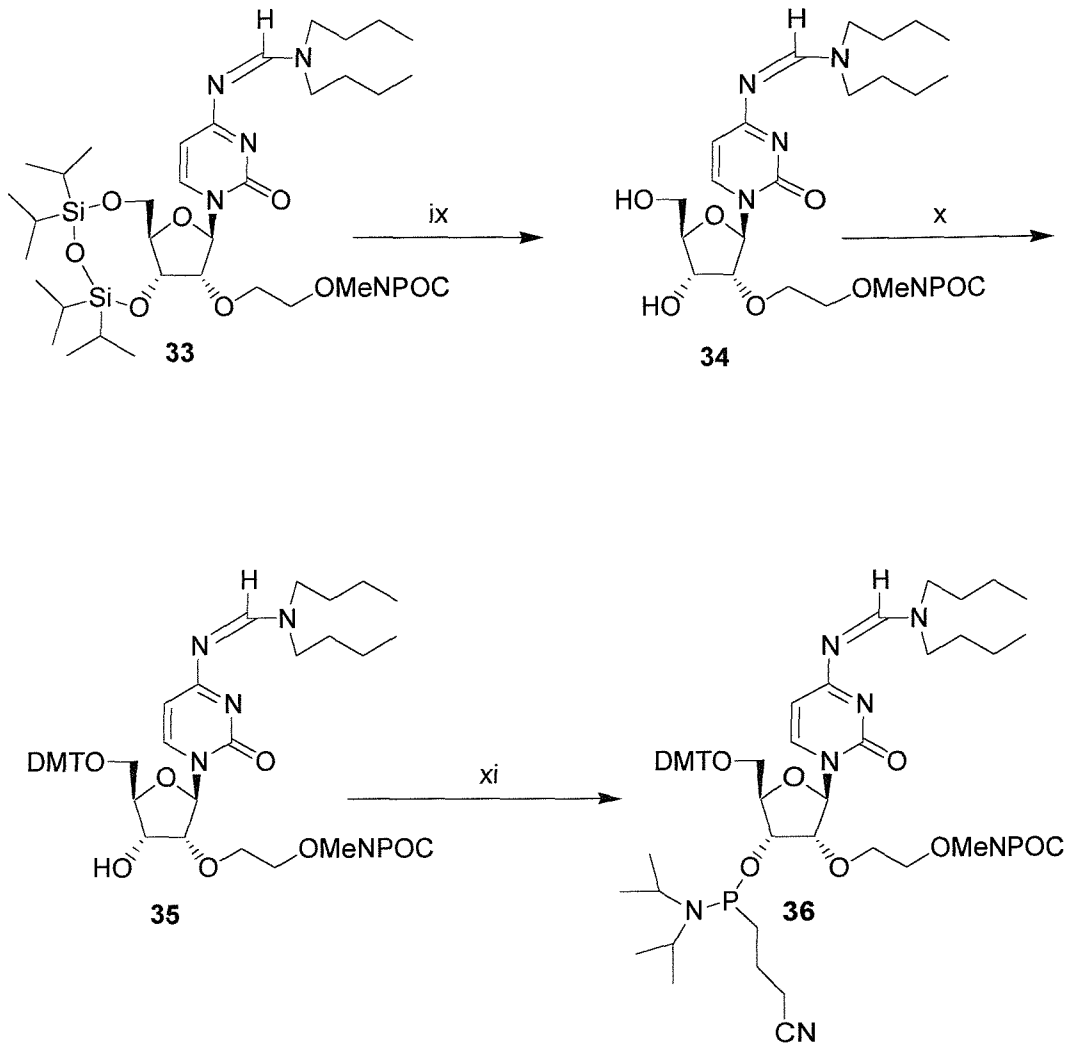
3.7 Synthesis of Cytidine Monomer[144]

It was mentioned at the beginning of the chapter that a cytidine nucleoside containing the 2'-*O*-modification will be synthesised. The reaction scheme used was very similar to that of the modified uridine nucleoside.



Reagents and conditions: i, *N,N*-dibutylformamide dimethylacetal, DMF, *88%*; ii, 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane, imidazole, pyridine, *63%*; iii, methyl bromoacetate, NaH, DMF, *69%*; iv, MeOH:ammonia (1:1), *54%*; v, $LiBH_4$, MeOH:THF (1:4), *91%*; vi, *N,N*-dibutylformamide dimethylacetal, DMF, *80%*; vii carbonyldiimidazol, nitromethane; viii 1eq **32**, pyridine, *54%*

Fig 3.7.1 :- Reaction Scheme for Cytidine Monomer



Reagents and conditions: ix, TBAF, THF, *80%*; x, 4,4'-dimethoxytrityl chloride, pyridine, *81%*; xi, 2-cyanoethyldiisopropylchlorophosphoramidite, DIPEA, THF, *36%*

Fig 3.7.1 :- Reaction Scheme for Cytidine Monomer

The synthetic plan for preparing compound **36** was nearly identical to that of compound **25**. The same conditions were used to perform the 2' alkylation and the MeNPOC protecting group were used at the 2' position. The N-4 amino group needs to be protected throughout the synthesis and also during DNA synthesis as the primary amine can react with the phosphoramidite nucleosides causing branched sequences to grow. The di-*n*-butylmethylidene group was chosen for its ease of addition and ease of deprotection in basic conditions, allowing it to be removed when the oligonucleotide is cleaved from the resin.

The first three steps proceeded smoothly giving consistent yields above 60%. When the reduction of compound **29** was attempted using 4 equivalents of lithium borohydride in a mixture of methanol:tetrahydrofuran (1:4) the correct product, compound **32** was not obtained. The spot obtained on TLC was still strongly UV active suggesting that unlike compound **11**, the base had not been reduced. Cytidine is known to be less activated than uridine so the lack of reduction of the cytidine ring is not surprising. The only other novel part of the molecule was the di-*n*-butylmethylidene group. It was suspected that the protecting group was being reduced in some way but it was difficult to tell by NMR as the butyl protons all come in the same region as the tetraisopropyl protons of the tetraisopropyldisiloxane group. To try and identify the structure of the over reduced product, compound **28** was reduced with the lithium borohydride conditions used to convert compound **29** into compound **30**. The reaction however gave a complex mixture of products that could not be separated.

It was decided to remove the N-4 protecting group, carry out the reduction and then reintroduce the di-*n*-butylmethylidene. The deprotection was carried out in a 1:1 solution of methanol and ammonia and gave a yield of 54%. The reduction proved very successful using lithium borohydride in tetrahydrofuran:methanol (4:1) giving a yield of 91%. The N-4 protecting group was reintroduced using the same conditions used to synthesise compound **27**.

The rest of the synthetic pathway was straightforward, giving yields for all steps above 50%. The only exception was for the phosphitylation of compound **35**. The reaction proceeded quite slowly giving a yield of 36%. The reaction has not been optimised and adjusting the reaction conditions will probably increase the yield of this final step.

In summary, the modified cytidine nucleoside has been prepared allowing the modified monomer to couple with both a guanine and adenine base on the opposing complementary strand. The synthesis proceeded smoothly with only the reduction of the 2'-*O*-methyl ester causing a problem. Removal of the N-4 protecting group before carrying out the reduction allowed the final phosphoramidite to be synthesised.

3.8 Conclusions

Two modified nucleosides have been prepared, based on uridine and cytidine.

When compound **11** was reduced using the conditions reported on page 48 the correct product, compound **12** was only formed in 28%. Two over-reduction products, **20a** and **20b** were the major constituents. The reaction conditions were altered to give compound **12** in a 50 to 60% yield.

An Fmoc group was used to protect the 2'-*O*-hydroxyethyl group initially. However when oligonucleotide **45** was prepared the Fmoc group was found to have been removed in the course of automated DNA synthesis. Further experiments showed that the Fmoc group was labile to the capping conditions employed in DNA synthesis.

A photolabile group, MeNPOC was used instead of Fmoc as the literature suggested that it was more stable to the capping reagents. The group was found to be more stable but was still deprotected slightly by the capping reagents. Deprotection of the MeNPOC was carried out by exposing the oligonucleotide to a benchtop UV lamp for 10 hours.

The modified nucleoside when incorporated into the centre of an oligonucleotide (oligonucleotide **44**) had a T_M of 44°C destabilised by 4°C from that of a control. The destabilisation is modest and would be minimised if hexaethyleneglycol spacers were used to move the bulky fluorescent dyes from the Oligonucleotide.

The modified nucleoside was used in the Hybeacon assay developed by LGC (page 84). In the discrimination of mutant and wild type samples of the CYP2D6 locus the modified monomer was shown to function better than existing monomers used.

Two fluorescein dyes were attached various distances between each other using DNA bases as spacers. It was found that a distance of 6 base pairs between the dyes, oligonucleotide **50** gave the highest fluorescence intensity, however the intensity was still not as great as a control (containing one fluorescein labelled at the 5'-end) at twice

the concentration of oligonucleotide **50**. Adding an oligonucleotide complementary to oligonucleotide **50**, did lead to small increase in fluorescence. The fluorescence recorded was still not at the level seen in the control so a small amount of quenching was hypothesised to be taking place.

On page 96 the design and synthesis of FRET Taqman probes is discussed. Oligonucleotide **52** was synthesised and although seemed to function well as an energy transfer probe with the emission of light from ROX being successfully quenched, no increase in fluorescence signal was observed upon using the probe in a Taqman assay.

Chapter 4

Conclusions

4.0 Conclusions

There is a need for fluorescence resonance energy transfer versions of many of the assays used for genetic analysis, for example Taqman. If a reliable energy transfer probe is developed then more than one DNA mismatch can be identified in one test, a procedure known as multiplexing.

In chapter 2 the development of various energy transfer linkers was discussed. On page 41 the attempted synthesis of a biphenyl linker is reported.

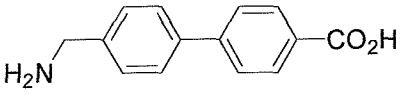


Fig 4.0.1 :- Structure of the Biphenyl Energy Transfer Linker

The reaction was found to be unsuccessful and even though all the reaction variables were altered the correct product was never obtained.

It had been reported in the literature (Page 45) that nucleosides could be used as scaffolds to attach two fluorescent dyes in such a position that energy transfer could occur between them. In the literature, one dye was attached to the 5' hydroxyl with the second being attached to:

- The base by a linkage attached to the 5 position
- The 2' hydroxyl by an alkoxy chain

Having a dye attached to the base of the nucleoside can cause the hybridisation of the probe with the target DNA to be destabilised so a decision was made to attach the second dye to the 2' hydroxyl.

On page 48 the synthesis of a modified 1'-*O*-methylribose phosphoramidite is outlined.

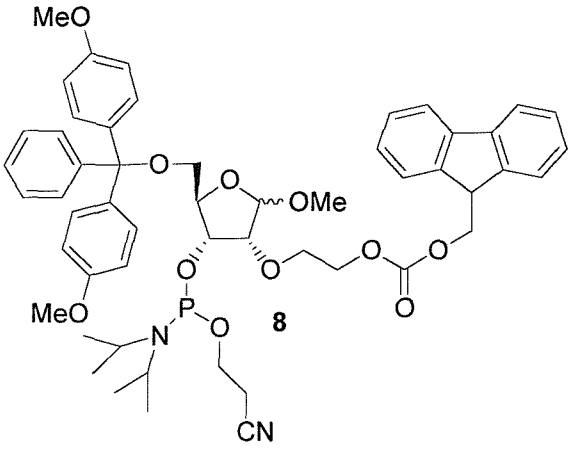


Fig 4.0.2 :- Structure of the 1'-*O*-methylribose phosphoramidite

The sugar was prepared as the synthesis of the 1'-*O*-methylribose was much simpler than that of a nucleoside. The ability of the molecule, (with a dye attached to the 5' position and to the alkylated 2' hydroxyl), to act as efficient energy transfer linker could be assessed. If the design proved to be effective then a nucleoside containing the same 2' modification would be synthesised.

On page 53 a fluorescence emission spectrum showing the fluorescence emission spectrum obtained from the linker containing both fluorescein (FAM) and hexachlorofluorescein (HEX) is reported. The plot showed that energy transfer was occurring from FAM to HEX. An experiment detailed on page 55 illustrated that some quenching of fluorescence must be occurring as exciting the linker at 535nm (the absorption frequency of HEX) gave a higher fluorescence emission intensity than exciting the energy transfer linker at 495nm (the absorption frequency of FAM). If all of the energy absorbed by the FAM was being transferred to the HEX then the fluorescence intensity of the plots (exciting at 495nm and 535nm) should have been the same.

On page 56 the method chosen to reduce quenching is documented. A series of hexaethyleneglycol (HEG) spacers were attached between the dye and the 2' hydroxyl.

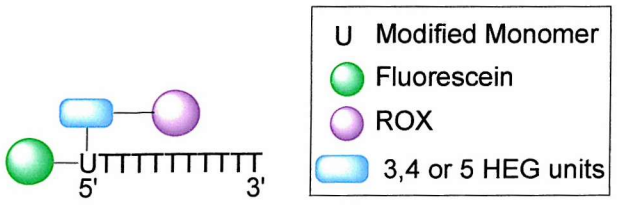


Fig 4.0.3 :- Sequence of an Oligonucleotide with HEG Spacers Introduced.

The fluorescence emission spectrum on page 57 illustrates the effect that the addition of the HEG spacers had. The fluorescence intensity increased by a twofold amount with the addition of 4 to 5 HEG's. The addition of a spacer unit between the 2 dyes was successful.

With efficient energy transfer between the 5' and 2' positions of the ribose demonstrated and optimised a decision was taken to synthesise two nucleosides containing the 2'-*O*-modification, using both uridine and cytidine. The synthesis route to the uridine monomer is documented on pages 64, 65 and 76. The synthetic scheme for the cytidine monomer is reported on pages 103 and 104.

Problems with the initial alkylation of the N-3 position and of the alkylation of the 2' position and their respective solutions are discussed on pages 66 and 67. When the reduction of the 2' methyl ethanoate (compound **11**) was attempted, the correct product (compound **12**) was formed in only a 20% yield. The major products of the reaction were two much more polar compounds, **20a** and **20b**. A discussion of the structures of products **20a** and **20b** can be found on pages 70 to 73. The reaction conditions were altered and the reaction taken only 50% to completion. If the recovered compound **11** were to be taken through the reaction again, compound **12** might be obtained in an average yield of between 50 to 60%.

A fluorenylmethyloxycarbonyl group (Fmoc) was attached to the 2'-*O*-ethoxy group and the synthesis of oligonucleotides **43**, **44**, and **45** was attempted.

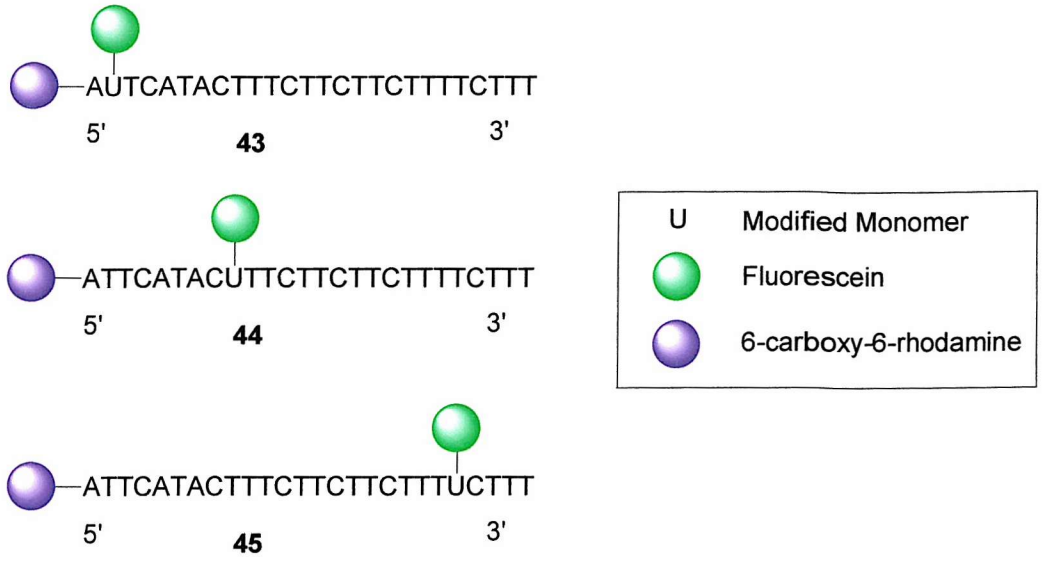


Fig 4.0.4 :- Sequences of Oligonucleotides **43**, **44** and **45**

It was found with oligonucleotide **45** that the fluorescein dye could not be attached. The Fmoc group was found to be slightly labile to the capping reagents employed in automated DNA synthesis and when the monomer was exposed to large amounts of the capping reagents, as happens when the modification is attached near the 3'-end of the oligonucleotide the Fmoc group is removed (page 81).

UV melting experiments were carried out with oligonucleotides **43** and **44** (page 80). Oligonucleotide **43** had a T_M of 47°C and oligonucleotide **44**, a T_M of 44°C. These compared to the control T_M of 48°C. Only a slight destabilisation was evident with oligonucleotide **44**.

LGC carried out experiments using the modified nucleoside in their Hybeacon probes (page 29). The experiments used three monomers, shown below.

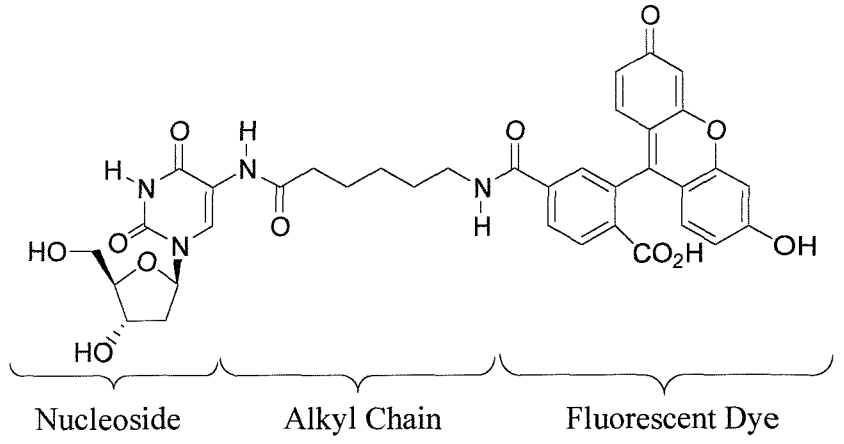
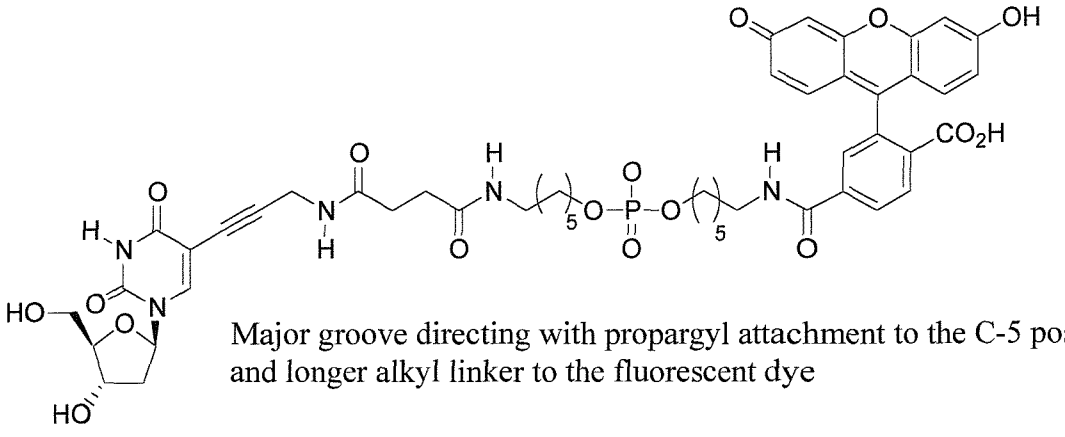
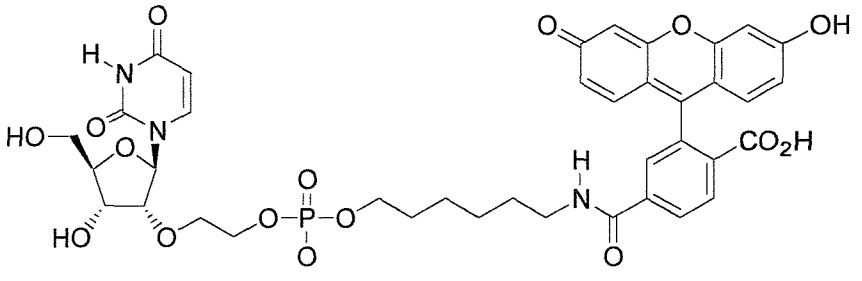


Fig 4.0.5 :- Structure of Monomer 1



Major groove directing with propargyl attachment to the C-5 position and longer alkyl linker to the fluorescent dye

Fig 4.0.6 :- Structure of Monomer 2



Minor groove directing with short alkyl linker to the fluorescent dye

Fig 4.0.7 :- Structure of Monomer 3

The monomers were evaluated for their ability to discriminate between a homozygous wild type, homozygous mutant and heterozygous sequence. The results, shown on page 84 illustrate that the 2'-*O*-ethoxy monomer (monomer 3) shows better discrimination between the three samples than either monomer 1 or 2.

Because of the inability to label an oligonucleotide close to the 3'-end with the Fmoc group attached it was decided to use a different protecting group for the 2'-*O*-ethoxy position. A photolabile group, α-methyl-2-nitropiperonyloxycarbonyl (MeNPOC) was chosen (page 73). The MeNPOC group had been utilised as a protecting group in oligonucleotide synthesis on microarrays where it is used in conjunction with a mask to grow a large number of oligonucleotides quickly and selectively.

Page 76 documents the reaction scheme used in the addition of the MeNPOC group to the nucleoside. A new method of reducing compound **12** is also reported in this scheme using sodium borohydride in methanol and *t*-butanol instead of lithium borohydride in THF and methanol. A discussion of the novel reduction conditions is reported on pages 76 to 77.

On page **88** the conditions used to deprotect the MeNPOC group are discussed. The group was eventually cleaved by irradiating the sample under a benchtop UV lamp for 10 hours.

On page 93 experiments to measure the fluorescence intensity increase obtained when separating two fluorescein dyes by different numbers of base pairs are reported. The graph on page 95 shows that the fluorescence intensity increases as the distance between the FAM dyes is increased but only surpasses a control oligonucleotide (labelled at the 5'-end with FAM) when the separation reaches 6 base pairs. On page 96 a complementary sequence was added to see if the DNA duplex formed separated the dyes any more, but only a slight increase in fluorescence was observed.

Pages 96 to 102 detail the design and synthesis of FRET Taqman probes. In Taqman (page 34) a single fluorophore held in close proximity to a quencher is cleaved from the oligonucleotide by DNA polymerase. In FRET Taqman, a FRET system replaces the single fluorophore.

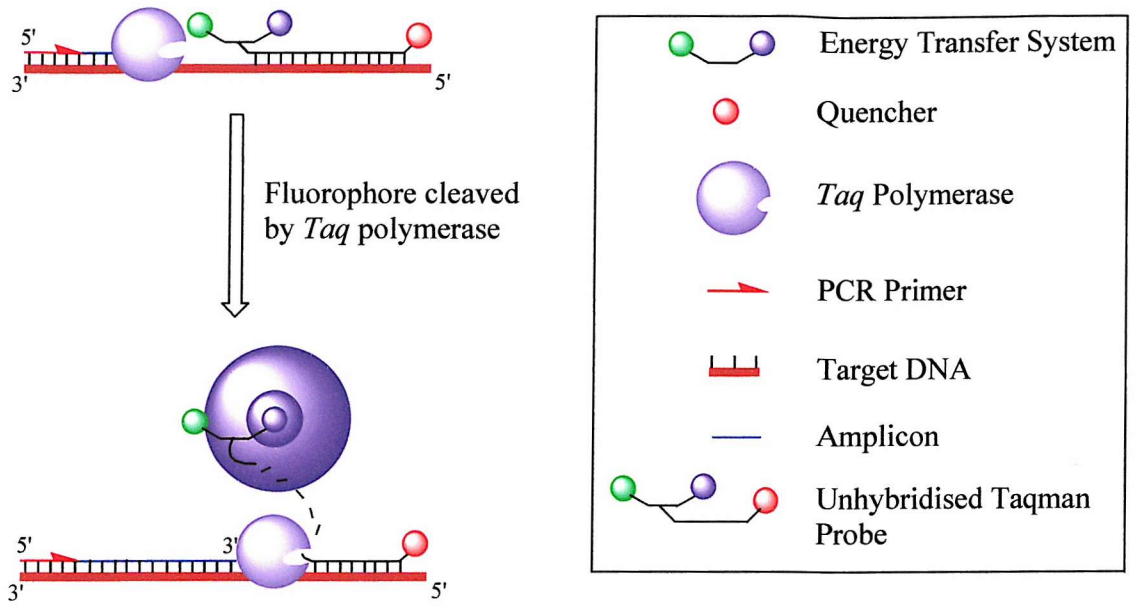


Fig 4.0.8 :- A FRET Taqman Assay

Oligonucleotide **52** was synthesised containing a FAM and ROX energy transfer system at the 5'-end and a methyl red quencher at the 3'-end. Although the probe seemed to function well, in subsequent PCR (page 100) no increase in fluorescence was observed suggesting that cleavage of the FRET system from the oligonucleotide was not taking place.

4.1 Further Work

Work will be carried on by Oswel to refine the FRET Taqman probes. An oligonucleotide, similar in structure to oligonucleotide **52** will be synthesised but with the quencher at the 5'-end and the FRET system at the 3'-end. If the FRET system is inhibiting DNA polymerase binding then this oligonucleotide should prove effective. DNA polymerase cleaves from the 5'-end to the 3'-end so the quencher should be cleaved from the oligonucleotide leaving the FRET system attached, leading to an increased fluorescent signal. An oligonucleotide similar in structure to oligonucleotide **53** will also be prepared with the Cy5 being labelled on the 5'-end and the FRET system on the 3'-end.

LGC has requested more of the modified uridine monomer and is intending to commercialise the Hybeacon probe using the 2'-*O*-ethoxy modified uridine as part of the assay.

Chapter 5

Experimental

5.0 EXPERIMENTAL

5.1 Preparation of Compounds

5.11 General Methods

All reactions requiring anhydrous conditions were performed in dry glassware under an atmosphere of nitrogen. Pyridine and DCM were distilled from calcium hydride. Tetrahydrofuran was freshly distilled from sodium wire and benzophenone. Methanol was distilled from magnesium and iodine. Anhydrous dimethylformamide was purchased from Aldrich. Other reagents were purchased from Aldrich/Sigma, Cruachem, Avocado or Lancaster.

Column chromatography was carried out under pressure on Merck 60-mesh silica. TLC was carried out using Merck Kieselgel 60 F_{24} (0.22mm thickness, aluminium backed). Compounds were visualised by irradiation at 254nm or staining with ρ-anisaldehyde: glacial acetic acid: concentrated sulphuric acid: ethanol (5:1:1:100) or phosphomolybdic acid: ethanol (1:10) or ammonium molybdate: cerium sulfate: concentrated sulphuric acid: water (5:0.2:5:100).

Infrared spectra were measured on a Nicolet 400 FT-IR instrument using a Golden Gate adapter and OMNIC software, or on a BIORAD FT-IR instrument using a Golden Gate adapter and BIORAD WIN-IR software. Absorptions are described as strong (s), medium (m), weak, or broad (br).

Melting points were measured on a Gallenkamp electrothermal melting point apparatus.

Ultraviolet spectra were measured on a Perkin Elmer UV/Vis Lambda 2 spectrometer.

Proton NMR was recorded at 400MHz or 300MHz and carbon NMR at 75.5MHz or 100MHz on a Bruker DPX 400 or AM 300 spectrometer in either deuterated chloroform, D_5-pyridine or D_6-DMSO. The multiplicities of carbon-13 signals were

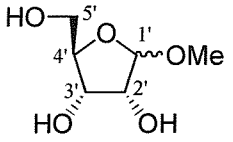
determined using distortionless enhancement by phase transfer (DEPT) spectral editing technique. Gradient heteronuclear multiple quantum correlation (HMQC) and gradient cosy experiments were recorded on a Bruker DPX 400. Where anomeric mixtures were prepared, NMR for only the major anomer was reported.

Mass Spectra were recorded on a Fisons VG platform instrument, Finnagan Trace GC/MS with combined EI/CI source fitted with a macherey-nagel optima-delta 3-0.25μm GC column or a micromass TOFSpec2E recorded in either linear or reflectron mode using a matrix of a saturated solution of either 2,5-dihydroxybenzoic acid (DHB) or alpha-cyan-ρ-hydroxycinnamic acid (α).

Microanalytical data were obtained from University College, London or MEDAC Ltd.

5.1.2 List of Compounds

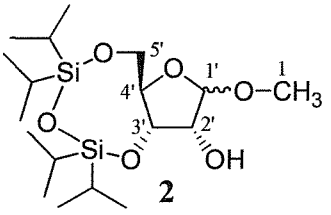
5.1.3 Experimental



1

$C_6H_{12}O_5$

Methyl-*β*-D-ribofuranoside (1). Under a nitrogen atmosphere, to a solution of ribose (3.02g, 20.00mmol) in methanol (10.0ml) was added a 1% HCl solution (5.4ml), prepared by the addition of acetyl chloride (0.17ml) to methanol (10.0ml). After stirring for 3 hours, the reaction was quenched with solid sodium bicarbonate (1.00g), filtered and concentrated *in vacuo*. The residue, which formed as an oil was used crude in the next reaction. [R_F =0.4, 0.3 (20% methanol/DCM), anisaldehyde (α and β anomers)].



2

$C_{18}H_{38}O_6Si_2$

3',5'-*O*-1,1,3,3-Tetraisopropyldisiloxan-1,3-diyl-methyl-*β*-D-ribofuranoside (2). Under a nitrogen atmosphere, to a solution of the furanoside **1** (3.34g, 20.00mmol) in pyridine (30.0ml) was added 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (7.7ml, 24.00mmol). After stirring for 8 hours the reaction was extracted with ethyl acetate (3x50ml), washed with 2M $HCl_{(aq)}$ (3x100ml), dried over sodium sulfate and concentrated *in vacuo*. The residue was purified by column chromatography (hexane/ethyl acetate 4:1) to give the title compound **2** as an oil as an anomeric mixture (2:8, α:β), (4.42g, *47% over 2 steps*). [R_F =0.5 (hexane/ethyl acetate 4:1), anisaldehyde].

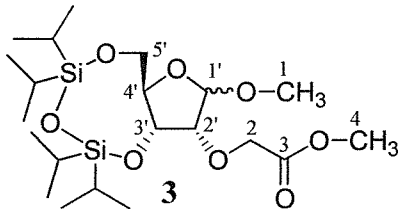
^{1}H NMR (400MHz, $CDCl_3$): δ = 4.80 (s, 1H, **H**$^{1'}$), 4.43 (dd, J=5.7, 5.1 Hz, 1H, **H**$^{3'}$), 3.92-4.01 (m, 3H, **H**$^{2'}$,**H**$^{4'}$,**H**$^{5'}$), 3.68 (dd, J=11.6, 9.1 Hz, 1H, **H**$^{5'}$), 3.25 (s, 3H, **H**^{1}), 0.80-1.05 (m, 28H, **^{i}Pr-CH, ^{i}Pr-CH$_3$**)

^{13}C NMR (100MHz, $CDCl_3$): δ = 106.29 (**C**H$^{1'}$), 81.66 (**C**H$^{4'}$), 74.78 (**C**H$^{2'}$), 74.01 (**C**H$^{3'}$), 65.16 (**C**H$_2$$^{5'}$), 53.89 (**C**H$_3$^{1}), 16.50, 16.41, 16.38, 16.35, 16.17, 16.06, 16.00, 15.97 (**^{i}Pr-CH$_3$**), 12.29, 12.08, 11.84, 11.60 (**^{i}Pr-CH**)

IR (film, cm^{-1}): υ_{max} = 2941 (br), 2860 (m), 2345 (w), 1560 (w), 1460 (m)

LRMS (Scan CI): m/z = 393 ((M-OMe+NH_4)$^+$, 100%), 425 ((M+NH_4)$^+$, 25%)

HRMS (Scan EI): m/z = Found: 424.2572 ((M+NH_4)$^+$, 100%); Expected: 424.2551



3

$C_{21}H_{42}O_8Si_2$

1'-Methyl-3',5'-*O*-1,1,3,3-tetraisopropyldisiloxan-1,3-diyl-2'-methylethanoate-methyl-*β*-D-ribofuranoside (3). Under a nitrogen atmosphere, to a solution of the ribofuranoside **2** (3.97g, 9.90mmol) in DMF (40.0ml) was added methyl bromoacetate (4.7ml, 49.50mmol) at -5°C (methanol/ice). After stirring for 15 minutes, sodium hydride (0.80g, 19.80mmol) was added portionwise over a period of 2 hours. Saturated potassium chloride (5.0ml) was added and the product was extracted with diethyl ether (150ml). The organic layer was washed with brine (2x50ml), dried over sodium sulfate and concentrated *in vacuo*. The residue was purified by column chromatography (hexane/ethyl acetate 5:1) to give the title compound **3** in the form of an oil (1.61g, *35%*). [R_F =0.4 (hexane/ethyl acetate 4:1), anisaldehyde].

^{1}H NMR (400MHz, $CDCl_3$): δ = 4.80 (s, 1H, **H**$^{1'}$), 4.42 (dd, J = 7.8, 4.1 Hz, 1H, **H**$^{3'}$), 4.39 (d, J = 16.6 Hz, 1H, **H**^{2}), 4.26 (d, J = 16.6 Hz, 1H, **H**^{2}), 3.98 (ddd, J = 7.9, 5.9, 3.1

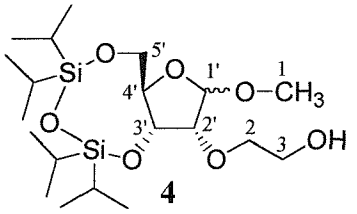
Hz, 1H, $\mathbf{H^{4'}}$), 3.92 (dd, J = 12.1, 3.1 Hz, 1H, $\mathbf{H^{5'}}$), 3.80 (dd, J = 12.2, 5.9 Hz, 1H, $\mathbf{H^{5'}}$), 3.77 (d, J = 4.1 Hz, 1H, $\mathbf{H^{2'}}$), 3.68 (s, 3H, $\mathbf{H^{4}}$), 3.27 (s, 3H, $\mathbf{H^{1}}$), 1.00 (m, 28H, **^{i}Pr-CH**, **iPr-CH$_3$**)

^{13}C NMR (100MHz, $CDCl_3$): δ = 104.90 ($\mathbf{CH^{1'}}$), 82.16 ($\mathbf{CH^{4'}}$), 79.84 ($\mathbf{CH^{2'}}$), 74.77 ($\mathbf{CH^{3'}}$), 67.26 ($\mathbf{CH_2^{2}}$), 62.73 ($\mathbf{CH_2^{5'}}$), 53.72 ($\mathbf{CH_3^{1}}$), 50.75 ($\mathbf{CH_3^{4}}$), 16.45, 16.33, 16.21, 16.12, 15.96 (**^{i}Pr-CH$_3$**), 12.36, 12.22, 11.74, 11.70 (**^{i}Pr-CH**)

IR (film, cm^{-1}): υ_{max} = 2931 (br), 2860 (m), 1758 (m), 1460 (m)

LRMS (Scan TofSpec-E): m/z = 501 ($(M+Na)^+$, 100%), 517 ($(M+K)^+$, 70%)

HRMS (Scan EI): m/z = Found: 496.2762 ($(M+NH_4)^+$, 60%); Expected: 496.2762



4

$C_{20}H_{42}O_7Si_2$

3',5'-*O*-1,1,3,3-Tetraisopropyldisiloxan-1,3-diyl-2'-ethoxy-methyl-*β*-D-ribofuranoside (4). Under a nitrogen atmosphere, to a solution of the ribofuranoside **3** (2.41g, 5.00mmol) in THF (35.0ml) was added lithium borohydride (0.20g, 9.90mmol). A solution of methanol (1.0ml) in THF (5.0ml) was added. After stirring for 15 minutes, methanol (10.0ml) was added. The product was extracted with diethyl ether (200ml), washed with water (2x50ml), dried over sodium sulfate and the solvent removed *in vacuo*. The residue was purified by column chromatography (hexane/ethyl acetate 5:1 to hexane/ethyl acetate 2:1) to give the title compound **4** in the form of an oil (1.69g, *76%*). [R_F =0.1 (hexane/ethyl acetate 4:1), anisaldehyde].

^{1}H NMR (400MHz, $CDCl_3$): δ = 4.78 (s, 1H, $H^{1'}$), 4.51 (dd, J = 8.1, 4.4 Hz, 1H, $H^{3'}$), 4.01 (m, 1H, $H^{4'}$), 3.99 (dd, J = 11.8, 2.9 Hz, 1H, $H^{5'}$), 3.88 (m, 2H, H^{3}), 3.82 (dd, J =

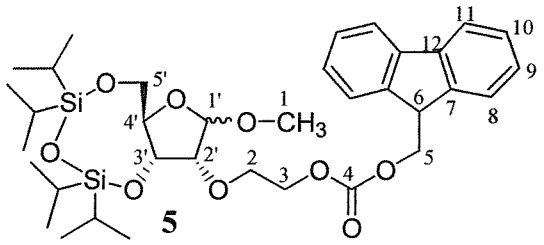
11.8, 4.4 Hz, 1H, $H^{5'}$), 3.75 (d, J = 4.4 Hz, 1H, $H^{2'}$), 3.71 (m, 2H, H^{2}), 3.35 (s, 3H, H^{1}), 2.87 (t, J = 5.9 Hz, 1H, OH), 1.00 (m, 28H, **^{i}Pr-CH**, **iPr-CH_3**)

^{13}C NMR (100MHz, $CDCl_3$): δ = 106.05 (**$CH^{1'}$**), 83.71 (**$CH^{4'}$**), 81.19 (**$CH^{2'}$**), 73.64 (**$CH^{3'}$**), 73.24 (**CH_2^{2}**), 63.77 (**$CH_2^{5'}$**), 61.81 (**CH_2^{3}**), 54.79 (**CH_3^{1}**), 17.44, 17.33, 17.30, 17.24, 17.15, 16.94, 16.88 (**^{i}Pr-CH_3**), 13.33, 13.21, 12.65 (**^{i}Pr-CH**)

IR (film, cm^{-1}): υ_{max} = 2943 (br), 2867 (m), 1748 (m), 1463 (s), 1387 (s)

LRMS (Scan TofSpec-E): m/z = 489 ($(M+K)^+$, 100%)

HRMS (Scan EI): m/z = Found: 460.2806 ($(M+NH_4)^+$, 30%); Expected: 460.2813



$C_{35}H_{52}O_9Si_2$

3',5'-*O*-1,1,3,3-Tetraisopropyldisiloxan-1,3-diyl-2'-ethoxy-(9*H*-9-fluorenylmethyl) oxycarbonyl-methyl-*β*-D-ribofuranoside (5). Under a nitrogen atmosphere, to a solution of the ribofuranoside **4** (0.44g, 1.00mmol) in pyridine (10.0ml) was added 9-fluorenylmethyl chloroformate (0.30g, 1.10mmol). After stirring for 2 hours the product was extracted with ethyl acetate (200ml), washed with 2M $HCl_{(aq)}$ (3x100ml), dried over sodium sulfate and the solvent removed *in vacuo*. The residue was purified by column chromatography (hexane/ethyl acetate 6:1) to give the title compound **5** in the form of an oil (0.57g, *92%*). [R_F =0.4 (hexane/ethyl acetate 4:1), U.V., anisaldehyde].

^{1}H NMR (400MHz, $CDCl_3$): δ = 7.68 (d, J = 7.5 Hz, 2H, **H^{11}**), 7.55 (d, J = 7.5 Hz, 2H, **H^{8}**), 7.32 (dd, J = 7.5, 7.5 Hz, 2H, **H^{10}**), 7.23 (dd, J = 7.5, 7.5 Hz, 2H, **H^{9}**), 4.70 (s, 1H, **$H^{1'}$**), 4.42 (dd, J = 7.5, 4.0 Hz, 1H, **$H^{3'}$**), 4.32 (d, J = 7.5 Hz, 2H, **H^{5}**), 4.26 (m, 2H, **H^{2}**),

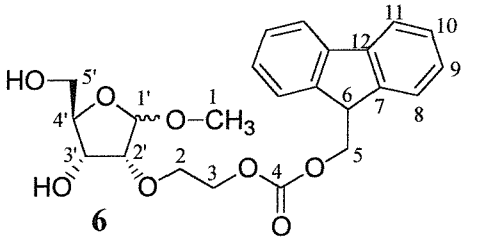
4.19 (t, J = 7.5 Hz, 1H, **H**^{6}), 4.10 (m, 2H, **H**^{3}), 3.97 (m, 1H, **H**$^{4'}$), 3.92 (dd, J = 12.1, 3.0 Hz, 1H, **H**$^{5'}$), 3.78 (m, 1H, **H**$^{5'}$), 3.71 (d, J = 4.0 Hz, 1H, **H**$^{2'}$), 3.27 (s, 3H, **H**^{1}), 1.00 (m, 28H, **^{i}Pr-CH, iPr-CH$_3$**)

^{13}C NMR (100MHz, $CDCl_3$): δ = 154.20 (**C**^{4}), 142.42 (**C**^{7}), 140.32 (**C**^{12}), 129.19 (**CH**^{10}), 126.89 (**CH**^{9}), 124.19 (**CH**^{8}), 119.07 (**CH**^{11}), 105.07 (**CH**$^{1'}$), 82.47 (**CH**$^{2'}$), 79.94 (**CH**$^{4'}$), 73.07 (**CH**$^{3'}$), 68.99 (**CH$_2$**^{5}), 68.14 (**CH$_2$**^{3}), 66.47 (**CH$_2$**^{2}), 62.79 (**CH$_2$**$^{5'}$), 53.66 (**CH$_3$**^{1}), 45.75 (**CH$_3$**^{6}), 16.21, 16.46, 16.38, 16.33, 16.22, 16.05, 16.00 (**^{i}Pr-CH$_3$**), 12.50, 12.35, 12.21, 11.80 (**^{i}Pr-CH**)

IR (film, cm^{-1}): υ_{max} = 2944 (br), 2866 (m), 1746 (s), 1450 (m), 1391 (m)

LRMS (Scan TofSpec-E): m/z = 695 ($(M+Na)^+$, 70%), 711 ($(M+K)^+$, 100%)

HRMS (Scan EI): m/z = Found: 695.3047 ($(M+Na)^+$, 50%); Expected: 695.3043



$C_{23}H_{26}O_8$

2'-Ethoxy-(9*H*-9-fluorenylmethyl) oxycarbonyl-methyl-*β*-D-ribofuranoside (6). Under a nitrogen atmosphere, to a solution of the ribofuranoside **5** (1.45g, 2.20mmol) in THF (20.0ml) was added HF-pyridine (1.70ml, 11.20mmol) in a plastic syringe. After stirring for 6 hours, saturated sodium bicarbonate solution (50.0ml) was added. The product was extracted with diethyl ether (200ml), washed with water (3x50ml), dried over sodium sulfate and the solvent removed *in vacuo*. The residue was purified by recrystallisation from ethyl acetate and hexane to give the title compound **6** in the form of a solid (0.73g, *69%*). [R_F =0.4 (10% methanol/DCM), U.V., anisaldehyde].

Melting Point = 94-96°C

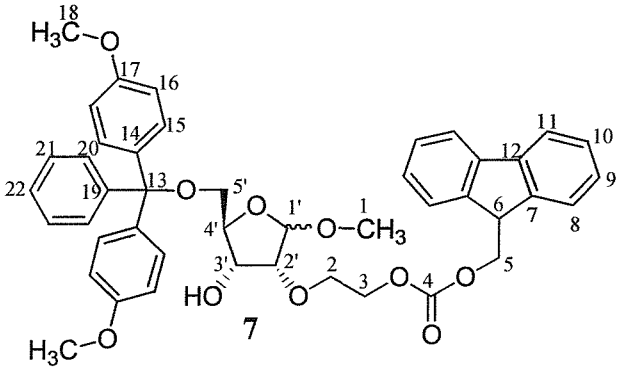
^{1}H NMR (400MHz, $CDCl_3$): δ = 7.79 (d, J = 7.4 Hz, 2H, **H**^{11}), 7.63 (d, J = 7.4 Hz, 2H, **H**^{8}), 7.43 (dd, J = 6.6, 7.4 Hz, 2H, **H**^{10}), 7.34 (dd, J = 6.6, 7.4 Hz, 2H, **H**^{9}), 4.94 (d, J = 1.5 Hz, 1H, **H**$^{1'}$), 4.44 (d, J = 6.6 Hz, 2H, **H**^{5}), 4.28 (m, 2H, **H**^{2}), 4.15 - 4.24 (m, 2H, **H**$^{3'}$, **H**^{6}), 3.98 (m, 1H, **H**$^{2'}$), 3.85 (m, 2H, **H**^{3}), 3.69 - 3.77 (m, 2H, **H**$^{4'}$, **H**$^{5'}$), 3.57 (m, 1H, **H**$^{5'}$), 3.32 (s, 3H, **H**^{1})

^{13}C NMR (100MHz, $CDCl_3$): δ = 154.12 (**C**^{4}), 142.24 (**C**^{7}), 140.28 (**C**^{12}), 126.90 (**C**H^{10}), 126.17 (**C**H^{9}), 124.12 (**C**H^{8}), 119.06 (**C**H^{11}), 105.56 (**C**H$^{1'}$), 84.52 (**C**H$^{2'}$), 82.82 (**C**H$^{4'}$), 69.96 (**C**H$^{3'}$), 68.88 (**C**H_2^{5}), 67.99 (**C**H_2^{3}), 65.72 (**C**H_2^{2}), 62.09 (**C**$H_2^{5'}$), 54.72 (**C**H_3^{1}), 45.68 (**C**H_3^{6})

IR (film, cm^{-1}): υ_{max} = 2986 (br), 1734 (s), 1449 (m), 1389 (m)

LRMS (Scan EI): m/z = 453 ($(M+Na)^+$, 100%)

HRMS (Scan EI): m/z = Found: 453.1541 ($(M+Na)^+$, 30%); Expected: 453.1525



7

$C_{44}H_{44}O_{10}$

2'-Ethoxy-(9*H*-9-fluorenylmethyl) oxycarbonyl-5'-dimethoxytrityl-methyl-β-D-ribofuranoside (7). All materials were weighed and dried in a desiccator overnight before commencing the reaction. Under a nitrogen atmosphere, to a solution of the ribofuranoside **6** (0.52g, 1.20mmol) in pyridine (2.5ml) was added 4,4'-dimethoxytrityl chloride (0.40g, 1.30mmol) in pyridine (2.5ml) dropwise at 0°C. After stirring for 3 hours at room temperature the product was extracted with ethyl acetate (2x100ml), washed with water (3x50ml), dried over sodium sulfate and the solvent removed *in vacuo*. The residue was purified by column chromatography (hexane/ethyl acetate 2:1) to give the title compoud **7** in the form of a white foam (0.58g, *72%*). [R_F =0.2 (hexane/ethyl acetate 1:1), U.V., anisaldehyde, yellow on heating].

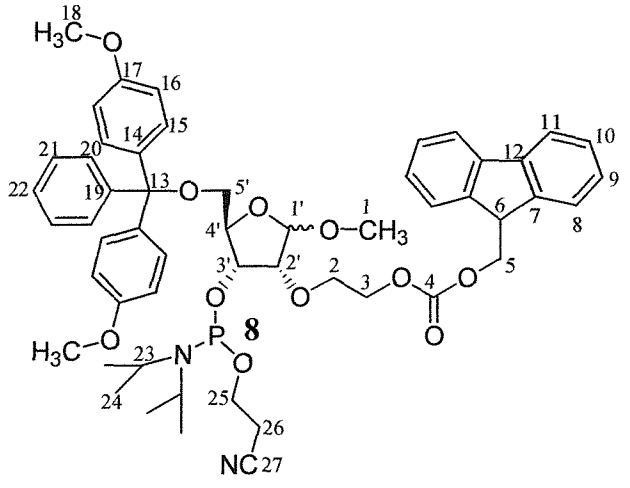
^{1}H NMR (400MHz, $CDCl_3$): δ = 7.68 (d, J = 7.5 Hz, 2H, **H**^{11}), 7.53 (d, J = 7.5 Hz, 2H, **H**^{8}), 7.41 (d, J = 7.5 Hz, 4H, **H**^{16}), 7.29 (d, J = 9.0 Hz, 4H, **H**^{15}), 7.16-7.26 (m, 5H, **H**^{9}, **H**^{20}, **H**^{22}), 7.07-7.13 (m, 3H, **H**^{10}, **H**^{21}), 4.87 (s, 1H, **H**$^{1'}$), 4.33 (d, J = 7.5 Hz, 2H, **H**^{5}), 4.27 (m, 2H, **H**^{2}), 4.14-4.20 (m, 1H, **H**$^{3'}$), 4.04 (t, J = 7.5 Hz, 1H, **H**^{6}), 3.97-4.02 (m, 1H, **H**$^{2'}$), 3.83-3.90 (m, 2H, **H**^{3}), 3.71-3.77 (m, 1H, **H**$^{4'}$), 3.68 (s, 6H, **H**^{18}), 3.30 (s, 3H, **H**^{1}), 3.22 (dd, J = 4.0, 10.0 Hz, 1H, **H**$^{5'}$), 3.10 (dd, J = 5.5, 10.0 Hz, 1H, **H**$^{5'}$)

^{13}C NMR (100MHz, $CDCl_3$): δ = 157.62 (s, **C**^{17}), 154.14 (s, **C**^{4}), 146.37 (s, **C**^{19}), 143.96 (s, **C**^{7}), 142.29 (s, **C**^{12}), 135.19 (s, **C**^{14}), 129.10 (d, **CH**^{15}), 128.12 (d, **CH**^{20}), 127.25 (d, **CH**^{21}), 126.88 (d, **CH**^{9}), 126.72 (d, **CH**^{22}), 126.19 (d, **CH**^{10}), 124.15 (d, **CH**^{8}), 119.03 (d, **CH**^{11}), 112.14 (d, **CH**^{16}), 104.73 (d, **CH**$^{1'}$), 84.91 (s, **C**^{13}), 84.61 (d, **CH**$^{2'}$), 82.34 (d, **CH**$^{4'}$), 70.65 (d, **CH**$^{3'}$), 69.05 (t, **CH$_2$**^{5}), 68.01 (t, **CH$_2$**^{3}), 65.76 (t, **CH$_2$**^{2}), 63.56 (t, **CH$_2$**$^{5'}$), 54.76 (q, **CH$_3$**^{1}), 54.21 (q, **CH$_3$**^{18}), 45.68 (d, **CH**^{6})

IR (film, cm^{-1}): υ_{max} = 2950 (br), 2172 (w), 2049 (w), 1744 (s), 1606 (m)

LRMS (Scan EI): m/z = 755 ($(M+Na)^+$, 100%), 771 ($(M+K)^+$, 55%)

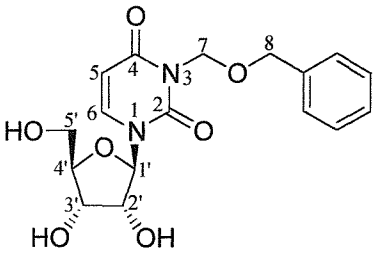
HRMS (Scan EI): m/z = Found: 733.3078 ($(M+H)^+$, 40%); Expected: 733.3013



$C_{53}H_{61}N_2O_{11}P$

2'-Ethoxy-(9*H*-9-fluorenylmethyl) oxycarbonyl-3'-(2-cyanoethyldiisopropyl phosphoramidite)-5'-dimethoxytrityl-methyl-*β*-D-ribofuranoside (8). Under a nitrogen atmosphere, to a solution of the ribofuranoside **7** (0.29g, 0.40mmol) in THF (5.0ml) was added 2-cyanoethyldiisopropylchlorophosphoramidite (0.2ml, 0.80mmol) and DIPEA (0.2ml, 1.00mmol). After stirring for 4 hours, degassed ethyl acetate (20.0ml) was added, the mixture was transferred into a flask containing saturated potassium chloride (15.0ml). After agitation the organic layer was transferred to a separate flask. The above procedure was repeated two more times. The combined organic layers were taken, dried in a nitrogen atmosphere over sodium sulfate and the solvent removed *in vacuo*. The residue was purified by column chromatography (hexane/ethyl acetate 2:1) and then precipitated from cold hexane (-78°C) to give the title compound **8** in the form of a white foam (0.33g, *68%*). [R_F =0.4 (hexane/ethyl acetate 1:1), U.V., anisaldehyde, yellow on heating].

LRMS (Scan EI): m/z = 933 ($(M+H)^+$, 100%), 955 ($(M+Na)^+$, 100%), 971 ($(M+K)^+$, 30%)



9

$C_{17}H_{20}N_2O_7$

3-Benzyloxymethyluridine (9). Under a nitrogen atmosphere, to a solution of uridine (10.04g, 41.00mmol) in DMF (100.0ml) was added DBU (12.3ml, 82.00mmol) and benzyl chloromethyl ether (8.5ml, 61.50mmol) at 0°C. The reaction was allowed to return to room temperature and after stirring for 16 hours methanol (50.0ml) was added. After stirring for a further 30 minutes the solvent was removed *in vacuo*. The residue was purified by column chromatography (5% methanol/DCM), then recrystallised from methanol to give the title compound **9** as a white crystalline solid (12.21g, *82%*). [R_F =0.3 (10% methanol/DCM), U.V., ammonium molybdate].

Melting point = 168-170°C

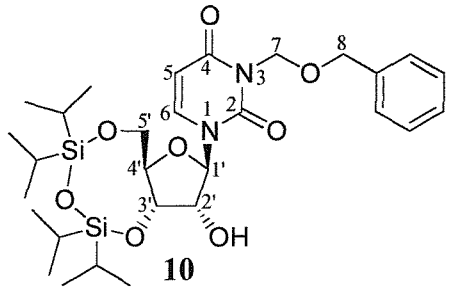
^{1}H NMR (400MHz, DMSO-d_6): δ = 8.22 (d, J = 8.5 Hz, 1H, **H^6**), 7.35-7.50 (m, 5H, **Ar-H**), 5.94 (d, J = 5.0 Hz, 1H, **H$^{1'}$**), 5.89 (d, J = 8.5 Hz, 1H, **H^5**), 5.55 (d, J = 5.5 Hz, 1H, **OH$^{2'}$**), 5.46 (d, J = 10.0 Hz, 1H, **H^8**), 5.42 (d, J = 10.0 Hz, 1H, **H^8**), 5.27 (t, J = 5.0 Hz, 1H, **OH$^{5'}$**), 5.25 (d, J = 5.5 Hz, 1H, **OH$^{3'}$**), 4.70 (s, 2H, **H^7**), 4.16 (dd, J = 10.5, 5.0 Hz, 1H, **H$^{2'}$**), 4.11 (dd, J = 10.5, 5.0 Hz, 1H, **H$^{3'}$**), 4.00 (dd, J = 5.0, 3.0 Hz, 1H, **H$^{4'}$**), 3.78 (ddd, J = 12.5, 5.0, 3.0 Hz, 1H, **H$^{5'}$**), 3.68 (ddd, J = 12.5, 5.0, 3.0 Hz, 1H, **H$^{5'}$**)

^{13}C NMR (100MHz, DMSO-d_6): δ = 162.46 (**C^4**), 151.43 (**C^2**), 140.50 (**CH^6**), 138.53 (**C**-Ar), 128.66, 128.33, 128.05 (**CH**-Ar), 101.41 (**CH^5**), 89.41 (**CH$^{1'}$**), 85.29 (**CH$^{4'}$**), 74.24 (**CH$^{2'}$**), 71.49 (**CH$_2^7$**), 70.41 (**CH$_2^8$**), 70.06 (**CH$^{3'}$**), 61.01 (**CH$_2^{5'}$**)

IR (film, cm^{-1}): υ_{max} = 3500 (br), 2016 (w), 1703 (s), 1645 (s)

LRMS (Scan EI): m/z = 365 ((M+H)$^+$, 20%), 387 ((M+Na)$^+$, 20%), 751 ((2M+Na)$^+$, 100%)

Microanalysis = Calc. (C, 56.04; H, 5.53; N, 7.69); Found (C, 55.85; H, 5.55; N, 7.64)



10

$C_{29}H_{46}N_2O_8Si_2$

3',5'-*O*-1,1,3,3-Tetraisopropyldisiloxan-1,3-diyl-3-benzyloxymethyluridine (10). Under a nitrogen atmosphere, to a solution of the nucleoside **9** (10.04g, 27.50mmol) and imidazole (2.00g, 30.00mmol) in pyridine (100.0ml) was added 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (10.5ml, 33.00mmol) dropwise. After stirring for 7 hours the product was extracted with ethyl acetate (3x100ml), washed with 2M $HCl_{(aq)}$ (3x200ml), dried over sodium sulfate and the solvent removed *in vacuo*. The residue was purified by column chromatography (hexane/ethyl acetate 4:1) to give the title compound **10** as a colourless oil (15.19g, *91%*). [R_F =0.1 (hexane/ethyl acetate 4:1), U.V., ammonium molybdate].

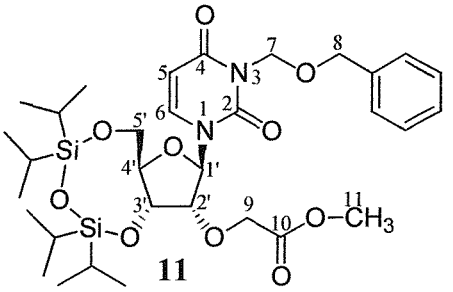
^{1}H NMR (400MHz, DMSO-d_6): δ = 7.84 (d, J = 8.0 Hz, 1H, **H^6**), 7.34-7.48 (m, 5H, **Ar-H**), 5.78 (d, J = 8.0 Hz, 1H, **H^5**), 5.69 (s, 1H, **H$^{1'}$**), 5.45 (d, J = 9.5 Hz, 1H, **H^8**), 5.41 (d, J = 9.5 Hz, 1H, **H^8**), 4.70 (s, 2H, **H^7**), 4.21-4.29 (m, 3H, **H$^{2'}$**, **H$^{3'}$**, **H$^{5'}$**), 4.11-4.17 (m, 1H, **H$^{4'}$**), 4.04 (dd, J = 13.1, 2.5 Hz, 1H, **H$^{5'}$**), 1.04-1.20 (m, 28H, **^{i}Pr-CH**, **iPr-CH$_3$**)

^{13}C NMR (100MHz, DMSO-d_6): δ = 162.49 (**C^4**), 150.80 (**C^2**), 139.28 (**CH^6**), 138.59 (**C**-Ar), 128.58, 127.87, 127.72 (**CH**-Ar), 100.71 (**CH^5**), 91.65 (**CH$^{1'}$**), 81.44 (**CH$^{4'}$**), 74.08 (**CH$^{2'}$**), 71.57 (**CH$_2^7$**), 70.37 (**CH$_2^8$**), 69.01 (**CH$^{3'}$**), 60.46 (**CH$_2^{5'}$**), 17.79, 17.68, 17.61, 17.55, 17.40, 17.32, 17.28, 17.23 (**^{i}Pr-CH$_3$**), 13.26, 12.82, 12.78, 12.41 (**^{i}Pr-CH**)

IR (film, cm^{-1}): υ_{max} = 2944 (br), 2866 (m), 1714 (s), 1656 (s), 1452 (s)

LRMS (Scan EI): m/z = 608 ($(M+H+1)^+$, 100%)

HRMS (Scan EI): m/z = Found (629.272 $(M+Na)^+$, 100%); Expected 629.2726



11

$C_{32}H_{50}N_2O_{10}Si_2$

3',5'-*O*-1,1,3,3-Tetraisopropyldisiloxan-1,3-diyl-3-benzyloxymethyl-2'-methylethanoateuridine (11). Under a nitrogen atmosphere, to a solution of the nucleoside **10** (13.04g, 21.50mmol) in DMF (130.0ml) was added methyl bromoacetate (12.0ml, 129.00mmol) at -5°C (methanol/ice). After stirring for 15 minutes, sodium hydride (2.60g, 107.00mmol) was added portionwise over a period of 2 hours. Saturated potassium chloride (50.0ml) was added and the product was extracted with ethyl acetate (2x100ml). The organic layer was washed with brine (3x50ml), dried over sodium sulfate and concentrated *in vacuo* on a high vacuum pump at room temperature for 3 hours. The residue was purified by recrystallisation from boiling ethanol to give the title compound **11** as a white crystalline solid (10.45g, *72%*). [R_F =0.3 (hexane/ethyl acetate 3:1, double eluted), U.V., ammonium molybdate].

Melting point = 97 - 99°C

^{1}H NMR (400MHz, $CDCl_3$): δ = 7.75 (d, J = 8.5 Hz, 1H, **H^6**), 7.10-7.30 (m, 5H, **Ar-H**), 5.70 (s, 1H, **H$^{1'}$**), 5.62 (d, J = 8.5 Hz, 1H, **H^5**), 5.42 (d, J = 10.0 Hz, 1H, **H^8**), 5.38 (d, J = 10.0 Hz, 1H, **H^8**), 4.63 (s, 2H, **H^7**), 4.53 (d, J = 16.6 Hz, 1H, **H^9**), 4.37 (d, J = 16.6 Hz, 1H, **H^9**), 4.10 - 4.20 (m, 3H, **H$^{2'}$**, **H$^{3'}$**, **H$^{5'}$**), 3.92 (m, 2H, **H$^{4'}$**, **H$^{5'}$**), 3.67 (s, 3H, **H^{11}**), 1.00 (m, 28H, **^{i}Pr-CH**, **iPr-CH$_3$**)

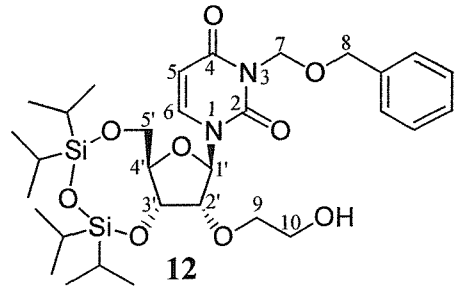
^{13}C NMR (100MHz, $CDCl_3$): δ = 169.38 (**C^{10}**), 161.67 (**C^4**), 149.67 (**C^2**), 137.10 (**CH^6**), 128.31 (**C-Ar**), 127.28, 126.69, 126.64 (**CH-Ar**), 100.26 (**CH^5**), 88.06 (**CH$^{1'}$**),

81.49 (**C**H$^{4'}$), 80.56 (**C**H$^{2'}$), 71.36 (**C**H$_2$^{7}), 69.24 (**C**H$_2$^{8}), 67.28 (**C**H$^{3'}$), 66.43 (**C**H$_2$^{9}), 58.33 (**C**H$_2$$^{5'}$), 50.81 (**C**H$_3$^{11}), 16.47, 16.40, 16.28, 16.24, 16.02, 15.99, 15.98, 15.76 (**^{i}Pr-CH$_3$**), 12.04, 11.88, 11.50, 11.30 (**^{i}Pr-CH**)

IR (film, cm^{-1}): υ_{max} = 2940 (br), 2865 (m), 1735 (s), 1712 (s), 1665 (s), 1653 (s)

LRMS (Scan EI): m/z = 679 ((M+H)$^+$, 80%), 696 ((M+NH$_4^+$)$^+$, 100%)

Microanalysis: Calc. (C, 56.61; H, 7.42; N, 4.13); Found (C, 56.30; H, 7.46; N, 4.07)



12

$C_{31}H_{50}N_2O_9Si_2$

3',5'-*O*-1,1,3,3-Tetraisopropyldisiloxan-1,3-diyl-3-benzyloxymethyl-2'-ethoxyuridine (12). Under a nitrogen atmosphere, to a solution of the nucleoside **11** (1.97g, 3.00mmol) in THF (16.0ml) and methanol (4.0ml) was added lithium borohydride (0.10g, 5.90mmol) at 5°C (ice bath). After stirring for 30 minutes a further portion of lithium borohydride (0.10g, 5.90mmol) was added. After stirring for 30 minutes the reaction was slowly warmed to 15°C and stirred for a further 6 hours. Ammonium chloride (5.0ml) was added and the product was extracted with DCM (2x100ml), washed with saturated brine (3x50ml), dried over sodium sulfate and the solvent removed *in vacuo*. The residue was purified by column chromatography (hexane/ethyl acetate 2:1 (loaded in diethyl ether)) to give the title compound **12** as a colourless oil (0.49g, *28%*) and recovered starting material **11** (0.92g, *45%*). [R_F =0.3 (hexane/ethyl acetate 1:1), U.V., ammonium molybdate].

^{1}H NMR (400MHz, $CDCl_3$): δ = 7.70 (d, J = 8.0 Hz, 1H, **H^6**), 7.15-7.35 (m, 5H, **Ar-H**), 5.62 (m, 2H, **H$^{1'}$**, **H^5**), 5.42 (d, J = 9.5 Hz, 1H, **H^8**), 5.38 (d, J = 9.5 Hz, 1H, **H^8**),

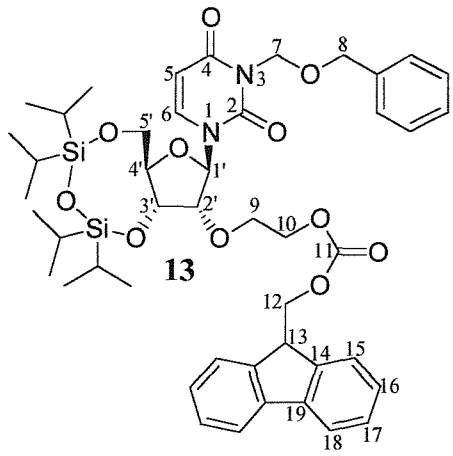
4.65 (s, 2H, **H**^{7}), 4.17 (d, J = 14.0 Hz, 1H, **H**^{10}), 4.02-4.11 (m, 2H, **H**$^{2'}$, **H**$^{3'}$), 3.78-3.97 (m, 4H, **H**$^{4'}$, **H**^{9}, **H**^{10}), 3.60-3.72 (m, 2H, **H**$^{5'}$), 1.00 (m, 28H, ^{i}**Pr-CH**, ^{i}**Pr-CH$_3$**)

^{13}C NMR (100MHz, $CDCl_3$): δ = 161.62 (**C**^{4}), 149.81 (**C**^{2}), 136.89 (**CH**^{6}), 136.75 (**C-Ar**) 127.29, 126.70, 126.61 (**CH-Ar**), 100.3 (**CH**^{5}), 88.85 (**CH**$^{1'}$), 81.91 (**CH**$^{4'}$), 80.78 (**CH**$^{2'}$), 72.11 (**CH$_2$**^{7}), 71.39 (**CH$_2$**^{8}), 69.27 (**CH$_2$**^{9}), 67.25 (**CH**$^{3'}$), 60.64 (**CH$_2$**$^{5'}$), 58.27 (**CH$_2$**^{10}), 16.45, 16.38, 16.27, 16.21, 16.05, 15.99, 15.96, 15.19 (^{i}**Pr-CH$_3$**), 12.51, 12.00, 11.87, 11.61 (^{i}**Pr-CH**)

IR (film, cm^{-1}): υ_{max} = 2943 (br), 2886 (m), 2150 (w), 1735 (m), 1714 (m), 1660 (s), 1451 (s)

LRMS (Scan EI): m/z = 651 ($(M+H)^+$, 50%), 1323 ($(2M+Na)^+$, 100%)

HRMS (Scan EI): m/z = Found: 673.2947 ($(M+Na)^+$, 100%), Expected: 673.2953



13

$C_{46}H_{60}N_2O_{11}Si_2$

3-Benzyloxymethyl-3',5'-*O*-1,1,3,3-tetraisopropyldisiloxan-1,3-diyl-2'-ethoxy-(9*H*-9-fluorenylmethyl) oxycarbonyluridine (13). Under a nitrogen atmosphere, to a solution of the nucleoside **12** (3.21g, 4.92mmol) in pyridine (20.0ml) was added a solution of 9-fluorenylmethyl chloroformate (2.50g, 9.80mmol) in pyridine (10.0ml). After stirring for 24 hours the product was extracted with ethyl acetate (3x100ml), washed with aqueous 2M $HCl_{(aq)}$ (3x200ml), dried over anhydrous sodium sulfate, and concentrated *in vacuo*. The residue was purified by column chromatography (hexane/ethyl acetate 4:1) to give the title compound **13** as a white waxy solid (3.70g, *86%*). [R_F =0.20 (hexane/ethyl acetate 4:1), U.V., ammonium molybdate].

^{1}H NMR (400MHz, $CDCl_3$): δ = 7.76 (d, J = 8.5 Hz, 1H, **H^6**), 7.68 (d, J = 8.2 Hz, 2H, **H^{18}**), 7.54 (d, J = 8.2 Hz, 2H, **H^{15}**), 7.14 - 7.34 (m, 9H, **Ar-H**, **H^{16}**, **H^{17}**), 5.65 (s, 1H, **H$^{1'}$**), 5.61 (d, J = 8.5 Hz, 1H, **H^5**), 5.35 (s, 2H, **H^7**), 4.28 - 4.37 (m, 9H, **H$^{3'}$**, **H^8**, **H^9**, **H^{10}**, **H^{12}**), 4.18 (t, J = 8.0 Hz, 1H, **H^{13}**), 4.16 (d, J = 13.6 Hz, 1H, **H$^{5'}$**), 4.04 (m, 1H, **H$^{4'}$**), 3.88 (d, J = 13.6 Hz, 1H, **H$^{5'}$**), 3.79 (m, 1H, **H$^{2'}$**), 1.00 (m, 28H, **^{i}Pr-CH**, **iPr-CH$_3$**)

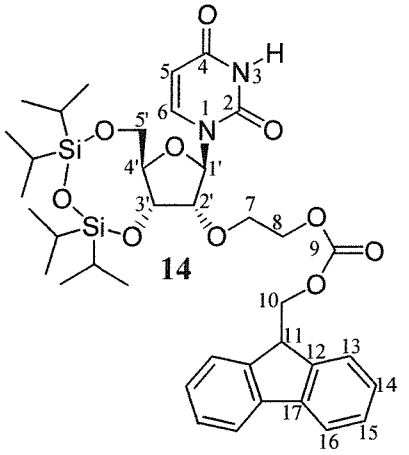
^{13}C NMR (100MHz, $CDCl_3$): δ = 161.69 (**C^4**), 154.17 (**CH^{11}**), 149.65 (**C^2**), 142.44 (**CH^{14}**), 140.26 (**CH^{19}**), 137.04 (**CH^6**), 136.90 (**C-Ar**), 127.26, 126.84, 126.66 (**CH-Ar**), 126.61 (**CH^{17}**), 126.18 (**CH^{16}**), 124.20 (**CH^{15}**), 119.01 (**CH^{18}**), 100.18 (**CH^5**), 88.45 (**CH$^{1'}$**), 81.70 (**CH$^{2'}$**), 80.61 (**CH$^{4'}$**), 71.34 (**CH$_2$^{7}**), 69.22 (**CH$_2$^{8}**), 68.92 (**CH$_2$^{9}**), 67.93 (**CH$_2$^{12}**), 67.30 (**CH$^{3'}$**), 66.15 (**CH$_2$^{10}**), 58.36 (**CH$_2$$^{5'}$**), 45.73 (**CH^{13}**), 16.47, 16.41,

16.29, 16.24, 16.16, 16.02, 15.99, 15.85 (**^{i}Pr-CH_3**), 12.50, 12.03, 11.87, 11.56 (**^{i}Pr-CH**)

IR (film, cm^{-1}): υ_{max} = 2943 (br), 2868 (m), 1741 (s), 1713 (s), 1662 (s)

LRMS (Scan Cl): m/z = 896 ($(M+Na)^+$, 40%)

HRMS (Scan El): m/z = Found: 895.3638 ($(M+Na)^+$, 100%), Expected: 895.3633



14

$C_{38}H_{52}N_2O_{10}Si_2$

3',5'-*O*-1,1,3,3-Tetraisopropyldisiloxan-1,3-diyl-2'-ethoxy-(9*H*-9-fluorenylmethyl) oxycarbonyluridine (14). Under a nitrogen atmosphere, Pd/C 20wt% (0.10g) was placed into a RB flask containing 2M HCl (0.2ml, 4.00mmol) and a solution of the nucleoside **13** (0.70g, 0.83mmol) in THF (2.5ml) and methanol (2.5ml). A balloon filled with hydrogen gas was used to introduce hydrogen to the reaction and a positive pressure of hydrogen was kept in the reaction at all times. After stirring for 6 hours the palladium was removed from the reaction by filtration through celite, washed with ethyl acetate (400.0ml) and the solvent removed *in vacuo*. The residue was purified by column chromatography (hexane/ethyl acetate 2:1 to hexane/ ethyl acetate 1:1) to give the title compound **14** as a white foam (0.37g, *62%*). [R_F =0.40 hexane/ethyl acetate 1:1, U.V., ammoniumm molybdate].

^{1}H NMR (400MHz, $CDCl_3$): δ = 9.30 (s, 1H, **NH^3**), 7.89 (d, J = 8.0 Hz, 1H, **H^6**), 7.76 (d, J = 7.5 Hz, 2H, **H^{16}**), 7.63 (d, J = 7.5 Hz, 2H, **H^{13}**), 7.41 (t, J = 7.5 Hz, 2H, **H^{15}**),

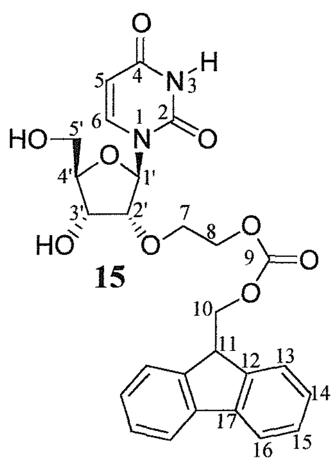
7.30 (t, J = 7.5 Hz, 2H, **H14**), 5.73 (s, 1H, **H1'**), 5.68 (d, J = 8.0 Hz, 1H, **H5**), 4.34-4.48 (m, 5H, **H3'**, **H7**, **H8**) 4.04-4.30 (m, 5H, **H4'**, **H5'**, **H10**, **H11**), 3.97 (dd, J = 13.6, 1.5 Hz, 1H, **H5'**), 3.91 (d, J = 3.5 Hz, 1H, **H2'**), 0.85-1.04 (m, 28H, **iPr-CH, iPr-CH3**)

^{13}C NMR (100MHz, $CDCl_3$): δ = 165.38 (C^4), 156.90 (C^9), 151.70 (C^2), 145.20 (C^{12}), 143.12 (C^{17}), 141.22 (CH^6), 129.57 (CH^{15}), 128.86 (CH^{14}), 126.95 (CH^{13}), 121.75 (CH^{16}), 103.28 (CH^5), 90.79 ($CH^{1'}$), 84.43 ($CH^{2'}$), 83.37 ($CH^{4'}$), 71.64 ($CH_2{}^7$), 70.64 ($CH_2{}^{10}$), 70.01 ($CH^{3'}$), 68.95 ($CH_2{}^8$), 61.13 ($CH_2{}^{5'}$), 48.48 (CH^{11})

IR (film, cm^{-1}): υ_{max} = 2959 (br), 2847 (m), 2830 (m), 1740 (w), 1728 (s), 1667 (s)

LRMS (Scan CI): m/z = 776 ($(M+Na)^+$, 60%)

HRMS (Scan EI): m/z = Found: 775.3046 ($(M+Na)^+$, 100%), Expected: 775.3059



15

$C_{26}H_{26}N_2O_9$

2'-Ethoxy-(9*H*-9-fluorenylmethyl) oxycarbonyluridine (15). Under a nitrogen atmosphere, to a solution of the nucleoside **14** (1.04g, 1.31mmol) in THF (10.0ml) was added HF-pyridine (1.7ml, 11.00mmol) via a plastic syringe. After stirring for 6 hours the reaction was quenched with saturated aqueous sodium bicarbonate (30.0ml). The product was extracted with ethyl acetate (2x50ml), washed with aqueous 2M $HCl_{(aq)}$ (3x25ml), dried over anhydrous sodium sulfate, and concentrated *in vacuo*. The residue was purified by column chromatography (ethyl acetate/hexane 4:1 to methanol/

DCM 1:19) to give the title compound **15** as a white foam (0.4g, *54%*). [R_F =0.2 ethyl acetate, U.V., ammonium molybdate].

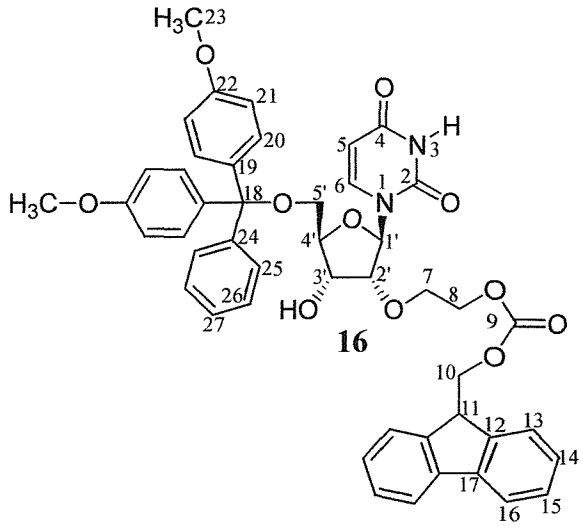
^{1}H NMR (400MHz, $CDCl_3$): δ = 9.95 (s, 1H, N**H**^{3}), 7.77 (d, J = 7.0 Hz, 1H, **H**^{6}), 7.64 (d, J = 7.0 Hz, 2H, **H**^{16}), 7.48 (d, J = 7.0 Hz, 2H, **H**^{13}), 7.28 (t, J = 7.0 Hz, 2H, **H**^{14}), 7.20 (t, J = 7.0 Hz, **H**^{15}), 5.66 (d, J = 2.5 Hz, 1H, **H**$^{1'}$), 5.60 (d, J = 8.0 Hz, 1H, **H**^{5}), 4.30 (d, J = 7.5 Hz, 2H, **H**^{10}), 4.16-4.27 (m, 3H, **H**$^{3'}$, **H**^{8}), 4.13 (t, J = 7.5 Hz, 1H, **H**^{11}), 3.97 (m, 1H, **H**$^{5'}$), 3.89-3.93 (m, 2H, **H**$^{2'}$, **H**$^{4'}$), 3.69-3.80 (m, 3H, **H**$^{5'}$, **H**^{7})

^{13}C NMR (100MHz, $CDCl_3$): δ = 170.25 (**C**^{4}), 163.09 (**C**^{2}), 154.18 (**C**^{9}), 149.48 (**C**^{12}), 142.26 (**C**^{17}), 140.23 (**CH**^{6}), 126.89 (**CH**^{14}), 126.17 (**CH**^{15}), 124.10 (**CH**^{13}), 119.06 (**CH**^{16}), 101.06 (**CH**^{5}), 88.16 (**CH**$^{1'}$), 83.51 (**CH**$^{2'}$), 81.18 (**CH**$^{4'}$), 69.03 (**CH**$_2^{10}$), 67.89 (**CH**$_2^{7}$), 67.21 (**CH**$^{3'}$), 65.78 (**CH**$_2^{8}$), 59.50 (**CH**$_2^{5'}$), 45.63 (**CH**^{11})

IR (film, cm^{-1}): υ_{max} = 2971 (br), 2868 (m), 1742 (m), 1667 (s)

LRMS (Scan CI): m/z = 533 ($(M+Na)^+$, 50%)

HRMS (Scan EI): m/z = Found: 533.1532 ($(M+Na)^+$, 100%), Expected: 533.1536



16

$C_{47}H_{44}N_2O_{11}$

5'-Dimethoxytrityl-2'-ethoxy-(9*H*-9-fluorenylmethyl) oxycarbonyluridine (16). Under a nitrogen atmosphere 4, 4'-dimethoxytrityl chloride (0.40g, 1.10mmol) and the nucleoside **15** (0.37g, 0.70mmol) were dissolved in pyridine (3.0ml). After stirring for 8 hours the product was extracted with ethyl acetate (2x50ml), washed with brine (3x25ml), dried over anhydrous sodium sulfate, and concentrated *in vacuo*. The residue was purified by column chromatography (hexane/ethyl acetate 1:1 to ethyl acetate) to give the title compound **16** as a white foam (0.52g, *80%*). [R_F =0.4 ethyl acetate, U.V., ammonium molybdate, yellow upon heating].

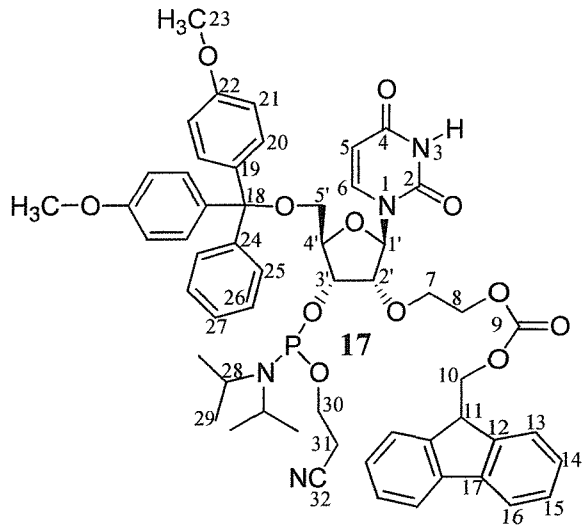
^{1}H NMR (400MHz, $CDCl_3$): δ = 7.68 (m, 3H, **H**^{6}, **H**^{16}), 7.51 (d, J = 8.0 Hz, 2H, **H**^{13}), 7.14-7.31 (m, 11H, **H**^{14}, **H**^{15}, **H**^{26}, **H**^{27}, **H**^{20}), 7.09 (D, J = 9.0 Hz, 2H, **H**^{25}), 6.75 (m, 4H, **H**^{21}), 5.60 (d, J = 9.0 Hz, 1H, **H**^{5}), 4.12-4.42 (m, 8H, **H**$^{1'}$, **H**^{7}, **H**^{8}, **H**^{10}, **H**^{11}), 3.75-4.06 (m, 4H, **H**$^{2'}$, **H**$^{3'}$, **H**$^{4'}$, **H**$^{5'}$), 3.62 (s, 6H, **H**^{23}), 3.45 (m, 1H, **H**$^{5'}$)

^{13}C NMR (100MHz, $CDCl_3$): δ = 162.32 (**C**^{4}), 157.73 (**C**^{22}), 15416 (**C**^{9}), 149.17 (**C**^{2}), 146.34 (**C**^{24}), 142.26 (**C**^{12}), 140.09 (**CH**^{6}), 138.48 (**C**^{17}), 134.10 (**C**^{19}), 129.19 (**CH**^{20}), 128.13 (**CH**^{26}), 127.16, 126.92, 126.89, 126.82, 126.76, 126.17 (**CH**^{13}, **CH**^{14}, **CH**^{15}, **CH**^{16}, **CH**^{25}), 126.05 (**CH**^{27}), 112.16 (**CH**^{21}), 101.27 (**CH**^{5}), 88.67 (**C**^{18}), 83.64 (**CH**$^{1'}$), 82.11 (**CH**$^{2'}$), 81.03 (**CH**$^{4'}$), 69.07 (**CH**$^{3'}$), 68.00 (**CH_2**^{7}), 67.34 (**CH_2**^{10}), 65.67 (**CH_2**^{8}), 59.90 (**CH_2**$^{5'}$), 54.23 (**CH_3**^{23}), 45.66 (**CH**^{11})

IR (film, cm^{-1}): υ_{max} = 2940 (br), 1747 (m), 1711 (m), 1661 (s)

LRMS (Scan CI): m/z = 835 ($(M+Na)^+$, 40%)

HRMS (FTMS): m/z = Found (835.2829 $(M+Na)^+$, 100%), Expected 835.2843



$C_{56}H_{61}N_4O_{12}P$

5'-Dimethoxytrityl-2'-ethoxy-(9*H*-9-fluorenylmethyl) oxycarbonyl-3'-2-cyanoethyl diisopropylphosphoramiditeuridine (17). Under a nitrogen atmosphere 2-cyanoethyl diisopropylchlorophosphoramidite (0.1ml, 0.60mmol) and DIPEA (0.1ml, 0.80mmol) were added to a solution of the nucleoside **16** (0.41g, 0.54mmol) in THF (4.0ml). After stirring for 6 hours the reaction was quenched by addition of degassed ethyl acetate (20.0ml). The mixture was washed with aqueous saturated potassium chloride (20ml). The organic layer was dried over anhydrous sodium sulfate flask the solvent was removed *in vacuo*. The residue (0.80g) was purified by column chromatography (degassed ethyl acetate) to give the title compound **17** as a white foam (0.37g, *72%*). [R_F =0.4 (ethyl acetate/hexane 3:1), U.V., ammonium molybdate, yellow upon heating].

^{1}H NMR (400MHz, $CDCl_3$): δ = 8.02 (d, J = 8.5 Hz, 0.5H, $\mathbf{H^6}$), 7.94 (d, J = 8.5 Hz, 0.5H, $\mathbf{H^6}$), 7.66-7.71 (m, 2H, $\mathbf{H^{16}}$), 7.49-7.55 (m, 1H, $\mathbf{H^{27}}$), 7.13-7.38 (m, 14H, $\mathbf{H^{13}}$, $\mathbf{H^{14}}$, $\mathbf{H^{15}}$, $\mathbf{H^{20}}$, $\mathbf{H^{25}}$, $\mathbf{H^{26}}$), 6.72-6.81 (m, 4H, $\mathbf{H^{21}}$), 5.88 (d, J = 2.0 Hz, 0.5H, $\mathbf{H^{1'}}$), 5.84

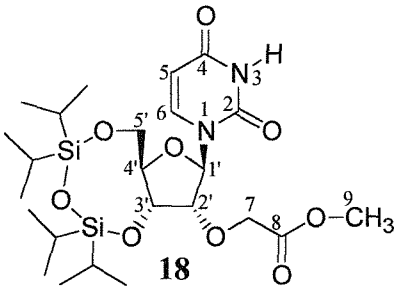
(d, J = 2.0 Hz, 0.5H, $\mathbf{H}^{1'}$), 5.15 (d, J = 8.0 Hz, 0.5H, $\mathbf{H}^{5}$), 5.10 (d, J = 8.0 Hz, 0.5H, $\mathbf{H}^{5}$), 4.12-4.42 (m, 6H, $\mathbf{H}^{3'}$, $\mathbf{H}^{4'}$, $\mathbf{H}^{7}$, $\mathbf{H}^{10}$), 3.84-4.06 (m, 3H, $\mathbf{H}^{2'}$, $\mathbf{H}^{8}$), 3.72 (m, 6H, $\mathbf{H}^{23}$), 3.43-3.67 (m, 4H, $\mathbf{H}^{5'}$, $\mathbf{H}^{30}$), 3.34-3.41 (m, 2H, $\mathbf{H}^{28}$), 2.45 (t, J = 6.0 Hz, 1H, $\mathbf{H}^{31}$), 2.32 (t, J = 6.0 Hz, 1H, $\mathbf{H}^{31}$), 0.91-1.15 (m, 12H, $\mathbf{H}^{29}$)

^{13}C NMR (100MHz, $CDCl_3$): δ = 162.01 (s, $\mathbf{C}^{4}$), 157.77 (s, $\mathbf{C}^{22}$), 154.14 (s, $\mathbf{C}^{9}$), 148.99 (s, $\mathbf{C}^{2}$), 143.35 (s, $\mathbf{C}^{24}$), 142.31 (s, $\mathbf{C}^{12}$), 140.27 (d, $\mathbf{C}H^{6}$), 139.00 (s, $\mathbf{C}^{17}$), 134.36 (s, $\mathbf{C}^{19}$), 129.29 (d, $\mathbf{C}H^{20}$), 127.33 (d, $\mathbf{C}H^{26}$), 127.31, 126.96, 126.88, 126.84, 126.18, 126.14 (d, $\mathbf{C}H^{13}$, $\mathbf{C}H^{14}$, $\mathbf{C}H^{15}$, $\mathbf{C}H^{25}$), 124.15 (d, $\mathbf{C}H^{27}$), 119 .15 (d, $\mathbf{C}H^{16}$) 119.06 (s, $\mathbf{C}^{32}$), 112.24 (d, $\mathbf{C}H^{21}$), 101.00 (d, $\mathbf{C}H^{5}$), 87.46 (s, $\mathbf{C}^{18}$), 86.12 (d, $\mathbf{C}H^{1'}$), 81.56 (d, $\mathbf{C}H^{2'}$), 80.78 (d, $\mathbf{C}H^{4'}$), 68.84 (d, $\mathbf{C}H^{3'}$), 67.48 (t, $\mathbf{C}H_2{}^{7}$), 67.31 (t, $\mathbf{C}H_2{}^{10}$), 66.07 (t, $\mathbf{C}H_2{}^{8}$), 59.96 (t, $\mathbf{C}H_2{}^{5'}$), 57.30 (t, $\mathbf{C}H_2{}^{30}$), 54.26 (q, $\mathbf{C}H_3{}^{23}$), 45.77 (d, $\mathbf{C}H^{11}$), 28.68 (d, $\mathbf{C}H^{28}$), 23.59 (q, $\mathbf{C}H_3{}^{29}$), 19.32 (t, $\mathbf{C}H_2{}^{31}$)

^{31}P NMR ($CDCl_3$): δ = 150.50 (P III)

IR (film, cm^{-1}): υ_{max} = 2940 (br), 2870 (m), 1733 (w), 1714 (m), 1662 (s)

LRMS (Scan Cl): m/z = 1013 ($(M+H)^+$, 40%)



18

$C_{24}H_{42}N_2O_9Si_2$

3',5'-*O*-1,1,3,3-Tetraisopropyldisiloxan-1,3-diyl-2'-methylethanoateuridine (18). A flask containing a solution of the nucleoside **11** (7.00g, 10.31mmol) and 20 wt% Pd/C (10% wt/wt, 0.70g) in THF (40.0ml) and methanol (40.0ml) was flushed with nitrogen gas. Hydrogen gas (balloon) was bubbled through the reaction and the solution was left stirring under a positive pressure of hydrogen gas. After stirring for 12 hours the Pd/C was removed via filtration through celite washing with ethyl acetate (500.0ml). The residue was purified by column chromatography (hexane/ethyl acetate 2:1) to give the title compound **18** as a colourless oil (5.03g, *86%*). [R_F =0.3 (hexane/ethyl acetate 1:1), U.V., ammonium molybdate].

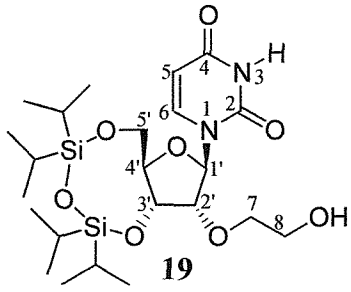
^{1}H NMR (400MHz, $CDCl_3$): δ = 9.30 (s, 1H, N**H**^{3}), 8.85 (d, J = 8.0 Hz, 1H, **H**^{6}), 5.73 (s, 1H, **H**$^{1'}$), 5.56 (d, J = 8.0 Hz, 1H, **H**^{5}), 4.51 (dd, J = 4.0, 10.0 Hz, 1H, H^7)4.43 (dd, J = 4.0, 10.0 Hz, 1H, **H**^{7}), 4.16-4.37 (m, 3H, **H**$^{3'}$, **H**$^{4'}$, **H**$^{5'}$), 3.94-4.12 (m, 2H, **H**$^{2'}$, **H**$^{5'}$), 3.60 (s, 3H, **H**^{9}), 1.00 (m, 28H, **^{i}Pr-CH, iPr-CH$_3$**)

^{13}C NMR (100MHz, $CDCl_3$): δ = 170.77 (**C**^{4}), 163.97 (**C**^{7}), 150.82 (**C**^{8}), 140.01 (**CH**^{6}), 102.01 (**CH**^{5}), 89.09 (**CH**$^{1'}$), 82.95 (**CH**$^{2'}$), 81.98 (**CH**$^{4'}$), 68.70 (**CH**$^{3'}$), 67.87 (**CH$_2$**^{7}), 59.76 (**CH$_2$**$^{5'}$), 52.25 (**CH$_3$**^{9}), 17.88, 17.80, 17.69, 17.63, 17.45, 17.40, 17.36, 17.13 (**^{i}Pr-CH$_3$**), 13.85, 13.50, 13.44, 13.28 (**^{i}Pr-CH**)

IR (film, cm^{-1}): υ_{max} = 2936 (br), 2860 (m), 2363 (w), 2321 (w), 1720 (m), 1663 (s), 1451 (s)

LRMS (Scan CI): m/z = 581 ($(M+Na)^+$, 20%), 1140 ($(2M+Na)^+$, 20%)

HRMS (FTMS): m/z = Found: 559.2499 ($(M+H)^+$, 4.0e+06), Expected: 559.2502



19

$C_{23}H_{42}N_2O_8Si_2$

3',5'-*O*-1,1,3,3-Tetraisopropyldisiloxan-1,3-diyl-2'-ethoxyuridine (19). Under a nitrogen atmosphere, to a solution of the nucleoside **18** (10.00g, 18.00mmol) and sodium borohydride (1.70g, 45.00mmol) in *t*-butanol (100.0ml) was added methanol (11.0ml, 269.00mmol). The reaction was refluxed (80°C) for 1.25 hours. Ammonium chloride (25.0ml) was added and the product was extracted with DCM (2x100ml), washed with saturated brine (3x50ml), dried over sodium sulfate and the solvent removed *in vacuo*. The residue was purified by column chromatography (hexane/ethyl acetate 2:1) to give the title compound **19** as a white foam (7.24g, *76%*). [R_F =0.2 (hexane/ethyl acetate 1:1), U.V., ammonium molybdate].

Melting Point: 94-96°C

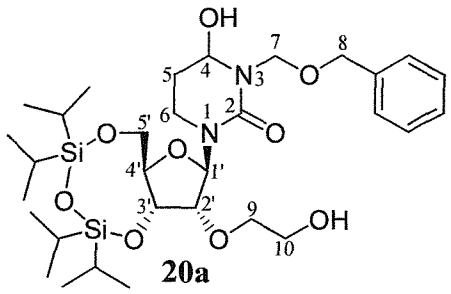
^{1}H NMR (400MHz, $CDCl_3$): δ = 7.97 (d, J = 8.0 Hz, 1H, **H^6**), 5.70 (s, 1H, **H$^{1'}$**), 5.65 (d, J = 8.0 Hz, 1H, **H^5**), 4.21 (d, J = 10.0 Hz, 1H, **H$^{5'}$**), 3.95-4.08 (m, 3H, **H$^{4'}$**, **H^7**), 3.63-3.84 (m, 3H, **H$^{2'}$**, **H$^{3'}$**, **H$^{5'}$**), 3.54 (m, 2H, **H^8**), 1.00 (m, 28H, **^{i}Pr-CH**, **iPr-CH$_3$**)

^{13}C NMR (100MHz, $CDCl_3$): δ = 170.13 (**C^4**), 162.69 (**C^2**), 149.44 (**CH^6**), 100.81 (**CH^5**), 88.45 (**CH$^{1'}$**), 81.86 (**CH$^{2'}$**), 80.81 (**CH$^{4'}$**), 72.02 (**CH$^{3'}$**), 67.21 (**CH$_2^7$**), 60.61 (**CH$_2^8$**), 58.29 (**CH$_2^{5'}$**), 16.46, 16.37, 16.28, 16.20, 16.04, 15.96, 15.93, 15.78 (**^{i}Pr-CH$_3$**), 12.44, 12.05, 11.87, 11.56 (**^{i}Pr-CH**)

IR (film, cm^{-1}): υ_{max} = 2950 (br), 2926 (m), 2870 (m), 2359 (w), 2335 (w), 1697 (s), 1682 (s)

LRMS (Scan CI): m/z = 553 ((M+Na)$^+$, 30%), 1083 (2M+Na)$^+$, 50%)

HRMS (FTMS): m/z =Found: 559.2381 ($(M+Na)^+$, 2.0e+06), Expected: 553.2378



20a

$C_{31}H_{54}N_2O_9Si_2$

3',5'-O-1,1,3,3-tetraisopropyldisiloxan-1,3-diyl--3-benzyloxymethyl-2'-ethoxy-tetrahydrouridine (20a). Under a nitrogen atmosphere, to a solution of the nucleoside **11** (2.00g, 3.00mmol) in THF (16.0ml) and methanol (4.0ml) at 5°C (ice bath) was added lithium borohydride (0.30g, 13.60mmol). After stirring for 3 hours at 5°C another portion of lithium borohydride (0.30g, 13.60mmol) was added. After stirring for a further 3 hours the product was extracted with DCM (3x50ml), washed with saturated brine (3x50ml), dried over soidum sulfate and the solvent removed *in vacuo*. The residue was purified by column chromatography (5% methanol/ DCM) to give the title compond **20a** as a colourless oil (1.42g, *74%*). [R_F =0.5 (5% methanol/ DCM), ammonium molybdate].

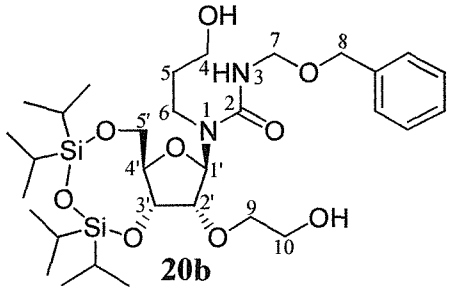
^{1}H NMR (400MHz, $CDCl_3$): δ = 7.45 (m, 5H, ArH), 5.91 (dd, J = 1.5, 14.0 Hz, 1H, **H**^{4}), 5.31 (d, J = 7.0 Hz, 1H, **H**^{8}), 5.28 (d, J = 7.0 Hz, 1H, **H**^{8}), 4.72 (d, J = 10.5 Hz, 1H, **H**$^{2'}$), 4.64 (m, 1H, **H**$^{4'}$), 4.67 (s, 2H, **H**^{7}), 4.30 (dd, J = 5.5, 8.5 Hz, 1H, **H**$^{3'}$), 4.05-4.13 (m, 1H, **H**$^{5'}$), 3.95-4.05 (m, 2H, **H**$^{4'}$, **H**$^{5'}$), 3.84-3.95 (m, 1H, **H**^{9}), 3.77-3.84 (m, 1H, **H**$^{1'}$), 3.67-3.74 (m, 1H, **H**^{9}), 3.60-3.67 (m, 2H, **H**^{10}), 3.10 (m, 2H, **H**^{6}), 1.87 (m, 2H, **H**^{5}), 1.00 (m, 28H, **^{i}Pr-CH, iPr-CH$_3$**)

^{13}C NMR (100MHz, $CDCl_3$): δ = 154.38 (**C**^{2}), 139.17 (**C-Ar**), 128.59, 127.93, 128.87, 127.70 (**CH-Ar**), 89.82 (**CH**^{4}), 81.04 (**CH**$^{4'}$), 79.65 (**CH**$^{1'}$), 79.58 (**CH**$^{2'}$), 74.82 (**CH$_2$**^{8}), 72.90 (**CH$_2$**^{9}), 71.04 (**CH**$^{3'}$), 69.68 (**CH$_2$**^{7}), 60.90 (**CH$_2$**^{10}), 60.52 (**CH$_2$**$^{5'}$), 35.67 (**CH$_2$**^{6}), 29.19 (**CH$_2$**^{5}), 17.70, 17.57, 17.54, 17.47,17.39, 17.35, 17.31, 17.25, (**^{i}Pr-CH$_3$**), 12.89, 12.68, 12.59, 12.56 (**^{i}Pr-CH**)

IR (film, cm^{-1}): υ_{max} = 2941 (br), 2860 (m), 2335 (w), 1630 (s), 1498 (s)

LRMS (Scan CI): m/z = 655 ($(M+H)^+$, 10%), 677 ($(M+Na)^+$, 40%)

HRMS (FTMS): m/z = Expected: 677.3260; Found: 677.3262 ($(M+Na)^+$, 2.0 e^{06})



20b

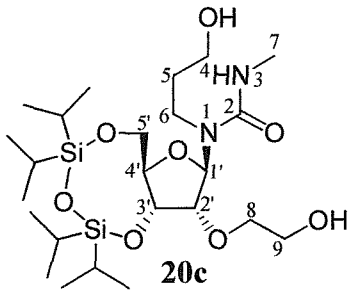
$C_{31}H_{56}N_2O_9Si_2$

3',5'-*O*-1,1,3,3-Tetraisopropyldisiloxan-1,3-diyl-3-benzyloxymethyl-2'-ethoxy-4-hydroxy-1-(2,3,4-tetrahydroxypentyl)-hexahydro-2-pyrimidinone (20b). Under a nitrogen atmosphere, to a solution of the nucleoside **11** (2.00g, 3.00mmol) in THF (16.0ml) and methanol (4.0ml) at room temperature was added lithium borohydride (0.60g, 27.20mmol). After stirring for 6 hours the product was extracted with DCM (3x50ml), washed with saturated brine (3x50ml), dried over soidum sulfate and the solvent removed *in vacuo*. The residue was purified by column chromatography (5% methanol/ DCM) to give the reduced nucleoside **20a** as a colourless oil (1.17g, *61%*) and the title compound **20b** as a colourless oil (0.44g, *23%*). [R_F =0.5 (5% methanol/ DCM), ammonium molybdate].

^{1}H NMR (400MHz, $CDCl_3$): δ = 7.20-7.34 (m, 5H, Ar-**H**), 6.47 (m, 1H, **OH**$^{4'}$), 5.32 (s, 1H, **H**$^{1'}$), 4.69 (m, 2H, **H**^{7}), 4.43 (s, 2H, **H**^{8}), 3.10-4.18 (m, 13H, **H**$^{2'}$, **H**$^{3'}$, **H**$^{4'}$, **H**$^{5'}$, **H**^{4}, **H**^{6}, **H**^{9}, **H**^{10}), 1.84 (m, 2H, **H**^{5}), 1.00 (m, 28H, **^{i}Pr-CH**, **iPr-CH$_3$**)

^{13}C NMR (100MHz, $CDCl_3$): δ = 157.11 (**C**^{2}),137.35 (**C-Ar**), 127.51, 127.34, 126.84, 126.65, 126.53 (**CH-Ar**), 91.84 (**CH**$^{1'}$), 81.46 (**CH**$^{4'}$), 79.21 (**CH**$^{2'}$), 72.36 (**CH$_2$**^{7}), 70.58 (**CH$_2$**^{8}), 69.39 (**CH$_2$**^{9}), 69.30 (**CH**$^{3'}$), 60.99 (**CH$_2$**^{4}), 58.83 (**CH$_2$**^{10}), 57.51 (**CH$_2$**$^{5'}$), 39.79 (**CH$_2$**^{6}), 33.08 (**CH$_2$**^{5}), 16.50, 16.34, 16.22, 16.12, 16.07, 15.96, 15.81, 15.75 (**^{i}Pr-CH$_3$**), 12.44, 12.05, 11.96, 11.63 (**^{i}Pr-CH**)

LRMS (Scan CI): m/z = 658 ($(M+H)^+$, 20%)



20c

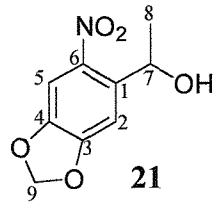
$C_{24}H_{50}N_2O_8Si_2$

3',5'-*O*-1,1,3,3-Tetraisopropyldisiloxan-1,3-diyl-3-methyl-2'-ethoxy-4-hydroxy-1-(2,3,4-tetrahydroxypentyl)-hexahydro-2-pyrimidinone (20c). Under a nitrogen atmosphere, to a solution of the nucleoside **11** (1.00g, 1.80mmol) and sodium borohydride (0.17g, 4.50mmol) in *t*-butanol (10.0ml) was added methanol (1.1ml, 26.90mmol). The reaction was refluxed (80°C) for 1.25 hours. Ammonium chloride (2.5ml) was added and the product was extracted with DCM (3x50ml), washed with saturated brine (3x50ml), dried over sodium sulfate and the solvent removed *in vacuo*. The residue was purified by column chromatography (hexane/ethyl acetate 2:1) to give the title compound **19** as a white foam (0.51g, *67%*). [R_F =0.2 (hexane/ethyl acetate 1:1), U.V., ammonium molybdate].

^{1}H NMR (400MHz, $CDCl_3$): δ = 5.22 (s,1H, **H**$^{1'}$), 4.20 (dd, J = 5.9, 9.1 Hz, 1H, **H**$^{3'}$), 4.05 (dd, J = 1.3, 13.1 Hz, 1H, **H**^{9}), 3.88 (dd, J = 2.7, 13.1 Hz, 1H, **H**^{9}), 3.85 (m, 1H, **H**^{8}), 3.77 (dd, J = 2.0, 5.9, 1H, **H**$^{4'}$), 3.40-3.72 (m, 7H, **H**$^{2'}$, **H**$^{5'}$, **H**^{4}, **H**^{6}, **H**^{8}), 3.2 (dt, J = 5.8, 15.2 Hz, 1H, **H**^{6}), 2.76 (s, 3H, **H**^{7}), 1.40 (m, 2H, **H**^{5}), 1.00 (m, 28H, **^{i}Pr-CH**, **iPr-CH$_3$**)

^{13}C NMR (100MHz, $CDCl_3$): δ = 161.85 (**C**^{2}), 92.34 (**CH**$^{1'}$), 82.72 (**CH**$^{4'}$), 78.98 (**CH**$^{2'}$), 71.23 (**CH$_2$**^{8}), 67.63 (**CH**$^{3'}$), 59.49 (**CH$_2$**$^{5'}$), 58.61 (**CH$_2$**^{9}), 57.52 (**CH$_2$**^{4}), 40.51 (**CH$_2$**^{6}), 31.69 (**CH$_2$**^{5}), 26.31 (**CH$_3$**^{7}), 16.50, 16.34, 16.25, 16.11, 16.07, 15.95, 15.80 (**^{i}Pr-CH$_3$**), 12.14, 11.97, 11.64, 11.47 (**^{i}Pr-CH**)

LRMS (Scan CI): m/z = 573 ($(M+Na)^+$, 100%)



21

$C_9H_9NO_5$

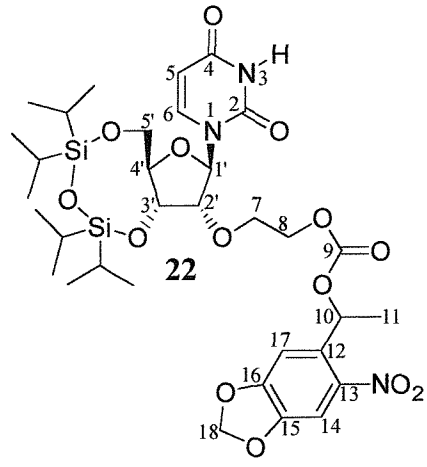
1-(6-Nitro-1,3-benzodioxol-5-yl)-1-ethanol (21). Under a nitrogen atmosphere, to a solution of 6-nitropiperonal (10.00g, 51.30mmol) in DCM (500.00ml) was added 2M trimethylammonium in hexane (51.3ml, 102.50mmol) dropwise over 15 minutes. After stirring for 2 hour at room temperature the reaction was quenched by the addition of 2M $HCl_{(aq)}$ (300.0ml) in ice. The product was extracted with DCM (3x300ml), washed with water (3x50ml), dried over sodium sulfate and the solvent removed *in vacuo*. The residue was purified by column chromatraphy (hexane/ethyl acetate 1:1) and recrystallisation in boiling ethyl acetate/hexane to give the title compound **21** as an orange solid (5.52g, *51%*). [R_F =0.3 (hexane/ethyl acetate 1:1), U.V., phosphomolybdic acid].

Melting Point: 110-112°C

^{1}H NMR (400MHz, DMSO-d_6): δ = 7.45 (s, 1H, **H**^{5}), 7.21 (s, 1H, **H**^{2}), 5.95 (s, 2H, **H**^{9}), 5.35 (q, J = 6.5 Hz, 1H, **H**^{7}), 2.50 (s, 1H, O**H**), 1.43 (d, J = 6.5 Hz, 3H, **H**^{8})

^{13}C NMR (100MHz, DMSO-d_6): δ = 151.40 (**C**^{3}), 145.88 (**C**^{4}), 140.43 (**C**^{6}), 131.10 (**C**^{1}), 105.32 (**CH**^{2}), 104.06 (**CH**^{5}), 101.92 (**CH**$_2$^{9}), 64.64 (**CH**^{7}), 23.23 (**CH**$_3$^{8})

LRMS (Scan CI): m/z = 194 ((M-H_2O+H)$^+$, 30%), 211 ((M-H_2O+NH_4)$^+$, 50%), 229 ((M+NH_4)$^+$, 30%)



22

$C_{33}H_{49}N_3O_{14}Si_2$

3',5'-*O*-1,1,3,3-Tetraisopropyldisiloxan-1,3-diyl-2'-ethoxy-1-(6-nitro-1,3-benzodioxol-5-yl)-uridine (22). Under a nitrogen atmosphere, to a solution of carbonyldiimidazole (4.00g, 24.00mmol) in nitromethane (40.0ml) was added methyl triflate (5.0ml, 48.00mmol) dropwise at 5°C (ice bath). The reaction was warmed to room temperature and after stirring for 30 minutes the solution was transferred to a flask containing the nucleoside **21** (5.00g, 24.00mmol) and stirred for 2 hours. Under a nitrogen atmosphere to a solution of the alcohol **19** (4.00g, 7.50mmol) in pyridine (40.0ml) was added the above solution (22.5ml). After stirring for 8 hours the solvent was removed *in vacuo*. The residue was purified by column chromatography (hexane/ethyl acetate 1:1) to give the title compound **22** as a yellow foam (4.53g, *78%*). [R_F =0.3 (hexane/ethyl acetate 1:1), U.V., brown upon heating, ammonium molybdate].

Melting Point: 84-86°C

^{1}H NMR (400MHz, $CDCl_3$): δ = 7.80 (d, J = 8.0 Hz, 1H, **H**^{6}), 7.40 (s, 1H, **H**^{17}), 7.05 (s, 1H, **H**^{14}), 6.20 (App dq, J = 6.5, 3.5 Hz, 1H, **H**^{10}), 6.05 (m, 2H, **H**^{18}), 5.59-5.64 (m, 2H, **H**$^{1'}$, **H**^{5}), 4.13-4.29 (m, 4H, **H**^{7}, **H**^{8}), 4.07-4.13 (m, 2H, **H**$^{4'}$, **H**$^{5'}$), 3.85-4.06 (m, 3H, **H**$^{2'}$, **H**$^{3'}$, **H**$^{5'}$), 3.79 (dd, J = 12.6, 4.0 Hz, 1H, **H**$^{5'}$), 1.55 (d, J = 6.5 Hz, 3H, **H**^{11}), 1.00 (m, 28H, **^{i}Pr-CH**, **iPr-CH$_3$**)

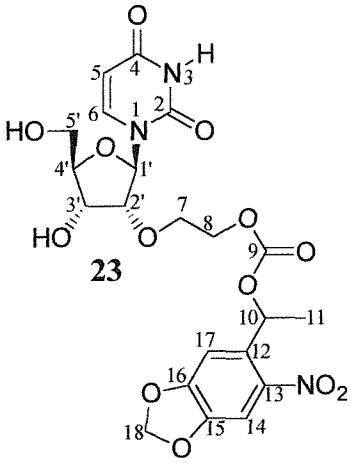
^{13}C NMR (100MHz, $CDCl_3$): δ = 162.61 (**C**^{4}), 153.08 (**C**^{13}), 151.44 (**C**^{16}), 148.91 (**C**^{2}), 146.27 (**C**^{9}), 140.51 (**C**^{15}), 138.51 (**CH**^{6}), 134.12 (**C**^{12}), 104.89 (**CH**^{14}), 104.19 (**CH**^{17}),

102.08 ($\mathbf{CH_2}^{18}$), 100.50 ($\mathbf{CH}^{5}$), 88.07 ($\mathbf{CH}^{1'}$), 81.73 ($\mathbf{CH}^{2'}$), 80.60 ($\mathbf{CH}^{4'}$), 71.14 ($\mathbf{CH}^{10}$), 67.93 ($\mathbf{CH_2}^{8}$), 67.34 ($\mathbf{CH}^{3'}$), 66.26 ($\mathbf{CH_2}^{7}$), 58.34 ($\mathbf{CH_2}^{5'}$), 21.09 ($\mathbf{CH_3}^{11}$), 16.48, 16.38, 16.30, 16.21, 16.11, 15.97, 15.81 (^{i}**Pr-CH$_3$**), 12.40, 12.06, 11.85, 11.52 (^{i}**Pr-CH**)

IR (film, cm^{-1}): υ_{max} = 2926 (br), 2364 (w), 1749 (s), 1682 (s), 1640 (s)

LRMS (Scan CI): m/z = 769 ((M+H+1)$^{+}$, 100%)

Microanalysis: Expected (C, 51.61; H, 6.43; N, 5.47); Found (C, 51.41; H, 6.13; N, 5.51)



23

$C_{21}H_{23}N_3O_{13}$

2'-Ethoxy-1-(6-nitro-1,3-benzodioxol-5-yl)-uridine (23). A solution of the nucleoside **22** (5.00g, 6.50mmol) in 1M tetrabutylammonium fluoride in THF (50.0ml) was stirred at room temperature for 2 hours. The product was extracted with DCM (3x50ml), washed with saturated brine (3x20ml), dried over sodium sulfate and the solvent removed *in vacuo*. The residue was purified by column chromatography (5% methanol/ DCM) to give the title compound **23** as a yellow foam (2.39g, *67%*). [R_F =0.3 (10% methanol/ DCM), U.V., brown upon heating, ammonium molybdate].

Melting Point: 86-88°C

^{1}H NMR (400MHz, $CDCl_3$): δ = 8.04 (d, J = 8.0 Hz, 1H, $\mathbf{H}^{6}$), 7.68 (s, 1H, $\mathbf{H}^{17}$), 7.28 (d, J = 5.0 Hz, 0.5H, $\mathbf{H}^{14}$), 7.26 (d, J = 5.0 Hz, 0.5H, $\mathbf{H}^{14}$), 6.35 (d, J = 4.0 Hz, 2H,

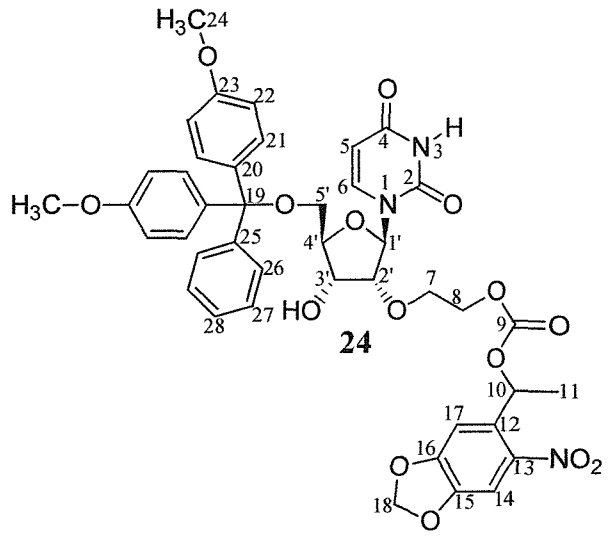
H^{18}), 6.10 (q, J = 6.5, Hz, 1H, **H**^{10}), 5.92 (d, J = 4.5 Hz, 0.5H, **H**$^{1'}$), 5.90 (d, J = 4.5 Hz, 0.5H, **H**$^{1'}$), 5.64 (d, J = 8.0 Hz, 1H, **H**^{5}), 4.30 (m, 1H, **H**^{7}), 4.26 (m, 1H, **H**^{7}), 4.22 (m, 1H, **H**$^{3'}$), 4.05 (t, J = 4.5 Hz, 1H, **H**$^{2'}$), 3.94 (m, 1H, **H**$^{4'}$), 3.85 (m, 2H, **H**^{8}), 3.77 (m, 1H, **H**$^{5'}$), 3.67 (m, 1H, **H**$^{5'}$), 1.68 (d, J = 6.5 Hz, 3H, **H**^{11})

^{13}C NMR (100MHz, $CDCl_3$): δ = 161.39 (**C**^{4}), 151.90 (**C**^{2}), 150.53 (**C**^{9}), 148.78 (**C**^{16}), 145.61 (**C**^{15}), 139.85 (**C**^{12}), 138.64 (**C**H^{6}), 131.68 (**C**^{13}), 104.01 (**C**H^{14}), 102.84 (**C**H^{17}), 101.88 (**C**H_2^{18}), 99.96 (**C**H^{5}), 84.72 (**C**H$^{1'}$), 82.99 (**C**H$^{4'}$), 79.76 (**C**H$^{2'}$), 69.47 (**C**H^{10}), 66.42 (**C**H$^{3'}$), 65.81 (**C**H_2^{8}), 65.18 (**C**H_2^{7}), 58.48 (**C**$H_2$$^{5'}$), 19.52 (**C**$H_3$^{11})

IR (film, cm^{-1}): υ_{max} = 2957 (br), 2161 (w), 1745 (m), 1683 (s), 1518 (s)

LRMS (Scan CI): m/z = 548 ($(M+Na)^+$, 30%)

Microanalysis: Expected (C, 48.01; H, 4.41; N, 7.99); Found (C, 47.88; H, 4.99; N, 7.48)



24

$C_{42}H_{41}N_3O_{15}$

5'-Dimethoxytrityl-2'-ethoxy-1-(6-nitro-1,3-benzodioxol-5-yl)-uridine (24). Under a nitrogen atmosphere 4, 4'-dimethoxytrityl chloride (2.80g, 8.40mmol) and the nucleoside **23** (4.00g, 7.60mmol) were dissolved in pyridine (40.0ml). After stirring for 8 hours the product was extracted with ethyl acetate (2x100ml), washed with brine (3x50ml), dried over sodium sulfate, and the solvent removed *in vacuo*. The residue was purified by column chromatography (hexane/ethyl acetate 1:1 to ethyl acetate) to give the title compound **24** as a yellow foam (5.16g, *83%*). [R_F =0.5 ethyl acetate, U.V., brown upon heating, ammonium molybdate].

Melting Point: 108-112°C

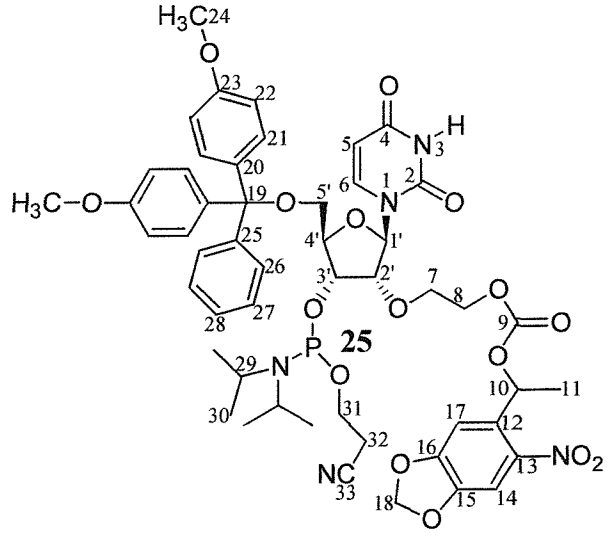
^{1}H NMR (400MHz, $CDCl_3$): δ = 9.45 (s, 0.5H, **NH**), 9.40 (s, 0.5H, **NH**), 7.95 (d, J = 8.0 Hz, 0.5H, $\mathbf{H^6}$), 7.75 (d, J = 8.0 Hz, 0.5H, $\mathbf{H^6}$), 7.38 (s, 0.5H, $\mathbf{H^{14}}$), 7.35 (s, 0.5H, $\mathbf{H^{14}}$), 7.31 (d, J = 7.5 Hz, 1H, $\mathbf{H^{28}}$), 7.05-7.28 (m, 8H, $\mathbf{H^{21}}$, $\mathbf{H^{26}}$, $\mathbf{H^{27}}$), 6.98 (s, 0.5H, $\mathbf{H^{17}}$), 6.97 (s, 0.5H, $\mathbf{H^{17}}$), 6.77 (d, J = 9.0 Hz, 2H, $\mathbf{H^{22}}$), 6.74 (d, J = 9.0 Hz, 2H, $\mathbf{H^{22}}$), 6.18 (q, J = 6.5 Hz, 1H, $\mathbf{H^{10}}$), 5.89-6.04 (m, 2H, $\mathbf{H^{18}}$), 5.78 (d, J = 3.5 Hz, 0.5H, $\mathbf{H^{1'}}$), 5.66 (d, J = 3.5 Hz, 0.5H, $\mathbf{H^{1'}}$), 5.62 (d, J = 8.0 Hz, 0.5H, $\mathbf{H^5}$), 5.21 (d J = 8.0 Hz, 0.5H, $\mathbf{H^5}$), 4.33-4.42 (m, 2H, H^8), 3.74-4.32 (m, 6H, $\mathbf{H^{2'}}$, $\mathbf{H^{3'}}$, $\mathbf{H^{4'}}$, $\mathbf{H^{5'}}$, $\mathbf{H^7}$), 3.70 (s, 6H, $\mathbf{H^{24}}$), 3.45 (m, 1H, $\mathbf{H^{5'}}$), 1.55 (m, 3H, $\mathbf{H^{11}}$)

^{13}C NMR (100MHz, $CDCl_3$): δ = 170.16 ($\mathbf{C}^{4}$), 162.42 ($\mathbf{C}^{23}$), 157.70 ($\mathbf{C}^{13}$), 151.76 ($\mathbf{C}^{2}$), 151.51 ($\mathbf{C}^{9}$), 149.25 ($\mathbf{C}^{16}$), 146.36 ($\mathbf{C}^{15}$), 143.39 ($\mathbf{C}^{25}$), 140.61, 140.00 ($\mathbf{CH}^{6}$), 138.88, 138.50 ($\mathbf{C}^{12}$), 134.32 ($\mathbf{C}^{20}$), 129.20, 129.09, 128.13, 127.15, 127.01, 126.81, 126.77, 126.15, 126.04 ($\mathbf{CH}^{21}$, $\mathbf{CH}^{26}$, $\mathbf{CH}^{27}$, $\mathbf{CH}^{28}$), 112.31, 112.15 ($\mathbf{CH}^{22}$), 104.73 ($\mathbf{CH}^{17}$), 104.18 ($\mathbf{CH}^{14}$), 102.19 ($\mathbf{CH_2}^{18}$), 101.26, 101.06 ($\mathbf{CH}^{5}$), 88.48, 86.67 ($\mathbf{CH}^{1'}$), 85.82 ($\mathbf{C}^{19}$), 83.57, 82.08 ($\mathbf{CH}^{4'}$), 81.18, 81.10 ($\mathbf{CH}^{2'}$), 71.39 ($\mathbf{CH}^{10}$), 67.97, 67.84 ($\mathbf{CH_2}^{8}$), 67.84, 67.28 ($\mathbf{CH}^{3'}$), 65.73, 65.64 ($\mathbf{CH_2}^{7}$), 59.92, 59.39 ($\mathbf{CH_2}^{5'}$), 54.25 ($\mathbf{CH_3}^{24}$), 20.94 ($\mathbf{CH_3}^{11}$)

IR (film, cm^{-1}): υ_{max} = 2161 (w), 1745 (m), 1683 (s), 1518 (s)

LRMS (Scan ES+): m/z = 851 ($(M+Na+1)^+$, 30%), 867 ($(M+K+1)^+$, 20%)

Microanalysis: Expected (C, 60.94; H, 4.99; N, 5.07); found (C, 60.61; H, 5.20; N, 4.79)



$C_{51}H_{58}N_5O_{16}P$

5'-Dimethoxytrityl-2'-ethoxy-1-(6-nitro-1,3-benzodioxol-5-yl)-3'-2-cyanoethyl diisopropylphosphoramidite-uridine (25). Under a nitrogen atmosphere 2-cyanoethyl diisopropylchlorophosphoramidite (0.6ml, 2.70mmol) and DIPEA (0.6ml, 3.60mmol) were added to a solution of the nucleoside **24** (2.00g, 2.40mmol) in THF (20.0ml). After stirring for 6 hours the reaction was quenched by addition of degassed ethyl acetate (50.0ml). The mixture was washed with aqueous saturated potassium

chloride (3x25ml), dried over sodium sulfate and the solvent removed *in vacuo*. The residue (2.50g) was purified by column chromatography (degassed ethyl acetate) to give the title compound **17** as a white foam (2.18g, *84%*). [R_F = 0.2 (ethyl acetate/hexane 3:1), U.V., ammonium molybdate, brown upon heating].

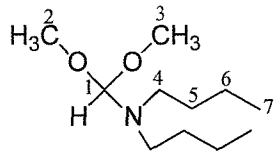
^{1}H NMR (400MHz, $CDCl_3$): δ = 9.10 (s, 1H, N**H**^{3}), 8.00 (d, J = 9.0 Hz, 1H, **H**^{6}), 7.68 (s, 1H, **H**^{14}), 7.12-7.39 (m, 9H, **H**^{21}, **H**^{26}, **H**^{27}, **H**^{28}), 6.99 (s, 1H, **H**^{17}), 6.71-6.80 (m, 4H, **H**^{22}), 6.17 (q, J = 6.0 Hz, 1H, **H**^{10}), 5.90 (s, 1H, **H**$^{1'}$), 5.95-6.01 (m, 2H, **H**^{18}), 5.82 (d, J = 9.0 Hz, 1H, **H**^{5}), 3.31-4.29 (m, 14H, **H**$^{2'}$, **H**$^{3'}$, **H**$^{4'}$, **H**$^{5'}$, **H**^{7}, **H**^{8}, **H**^{11}, **H**^{31}), 2.67 (dt, J = 1.8, 6.2 Hz, 1H, **H**^{29}), 2.54 (m, 1H, **H**^{29}), 2.36 (m, 2H, **H**^{32}), 1.54 (d, J = 6.0 Hz, **H**^{11}), 0.91-1.22 (m, 12H, **H**^{30})

^{13}C NMR (100MHz, $CDCl_3$): δ = 173.83 (**C**^{4}), 163.44 (**C**^{23}), 158.69 (**C**^{13}), 154.36 (**C**^{2}), 152.48 (**C**^{9}), 150.14 (**C**^{16}), 147.34 (**C**^{15}), 144.18 (**C**^{25}), 141.56 (**CH**^{6}), 140.08 (**C**^{12}), 134.94 (**C**^{20}), 130.28, 128.26, 127.96, 127.19 (**CH**^{21}, **CH**^{26}, **CH**^{27}, **CH**^{28}), 117.69 (**C**^{33}), 113.22 (**CH**^{22}), 105.79 (**CH**^{17}), 105.15 (**CH**^{14}), 103.11 (**CH$_2$**^{18}), 102.00 (**CH**^{5}), 88.84 (**CH**^{19}), 88.53 (**CH**$^{1'}$), 86.86 (**CH**$^{4'}$), 81.82 (**CH**$^{2'}$), 72.17 (**CH**^{10}), 68.50 (**CH**$^{3'}$), 67.06 (**CH$_2$**^{8}), 60.40 (**CH$_2$**^{7}), 58.20 (**CH$_2$**^{31}), 58.11 (**CH$_2$**$^{5'}$), 55.23 (**CH$_3$**^{24}), 24.68, 24.64 (**CH**^{29}), 22.99, 22.96, 22.90, 22.87 (**CH$_3$**^{30}), 20.14 (**CH$_2$**^{32}), 14.19 (**CH$_3$**^{11})

^{31}P NMR ($CDCl_3$): δ = 150.50 (P III)

IR (film, cm^{-1}): υ_{max} = 2969 (br), 2354 (w), 2245 (m), 1744 (m), 1687 (s), 1602 (s)

LRMS (Scan CI): m/z = 1029 ($(M+H+1)^+$, 30%)



26

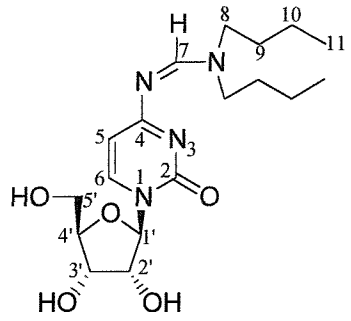
$C_{11}H_{25}NO_2$

***N,N*-Dibutylformamide dimethyl acetal (26).** Dibutylamine (50.0ml, 297.00mmol) and *N,N*-dimethylformamide dimethyl acetal (48.0ml, 326.70mmol) were stirred together in a flask at 100°C for 3 days. The product was purified by distillation to give the title compound **26** as a colurless liquid (28.45g, *47%*).

Boiling Point :- 120-130°C at 15mmHg

^{1}H NMR (400MHz, $CDCl_3$): δ = 4.50 (s, 1H, $\mathbf{H}^1$), 3.30 (s, 6H, $\mathbf{H}^2$, $\mathbf{H}^3$), 2.53-2.64 (m, 4H, $\mathbf{H}^4$), 1.21-1.53 (m, 8H, $\mathbf{H}^5$, $\mathbf{H}^6$), 0.85-1.00 (m, 6H, $\mathbf{H}^7$)

^{13}C NMR (100MHz, $CDCl_3$): δ = 112.79 ($\mathbf{C}H^1$), 54.01, 53.78 ($\mathbf{C}H_3{}^2$, $\mathbf{C}H_3{}^3$), 49.89, 47.22 (t, $\mathbf{C}H_2{}^4$), 32.47, 30.85 (t, $\mathbf{C}H_2{}^5$), 20.28 (t, $\mathbf{C}H_2{}^6$), 13.92 (q, $\mathbf{C}H_3{}^7$)



27

$C_{18}H_{30}N_4O_5$

6-*N*-((Di-*n*-butylamino)-methylene)-cytidine (27). Under a nitrogen atmosphere, to a solution of cytidine (30.00g, 123.50mmol) in DMF (300.0ml) was added *N,N*-dibutylformamide dimethylacetal (30.00g, 148.00mml). After stirring for 30 hours *N,N*-dibutylformamide dimethylacetal (10.00g, 50.00mmol) was added. After stirring for a further 18 hours the solvent was removed *in vacuo*. The residue was purified by recrystallisation from boiling methanol to give the title compound **27** as a white crystaline solid (41.83g, *88%*). [R_F =0.3 (10% methanol/ DCM), U.V., ammonium molybdate].

Melting Point: 140-142°C

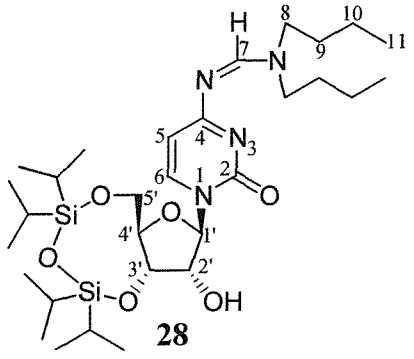
^{1}H NMR (400MHz, DMSO-d_6): δ = 8.75 (s, 1H, **H**^{7}), 8.20 (d, J = 7.4 Hz, 1H, **H**^{6}), 6.05 (d, J = 7.4 Hz, 1H, **H**^{5}), 5.91 (d, J = 2.5 Hz, 1H, **H**$^{1'}$), 5.50 (d, J = 5.0 Hz, 1H, O**H**$^{2'}$), 5.23 (t, J = 5.0 Hz, 1H, O**H**$^{5'}$), 5.12 (d, J = 5.5 Hz, 1H, O**H**$^{3'}$), 4.05 (m, 2H, **H**$^{2'}$, **H**$^{3'}$), 3.94 (m, 1H, **H**$^{4'}$), 3.78 (m, 1H, **H**$^{5'}$), 3.68 (m, 1H, **H**$^{5'}$), 3.48-3.62 (m, 4H, **H**^{8}), 1.59-1.70 (m, 4H, **H**^{9}), 1.30-1.44 (m, 4H, **H**^{10}), 1.00 (t, J = 7.2 Hz, 6H, **H**^{11})

^{13}C NMR (100MHz, DMSO-d_6): δ = 171.69 (**C**^{4}), 162.94 (**C**H^7), 158.32 (**C**^{2}), 142.94 (**C**H^6), 102.01 (**C**H^5), 90.22 (**C**H$^{1'}$), 84.55 (**C**H$^{4'}$), 74.84 (**C**H$^{2'}$), 69.87 (**C**H$^{3'}$), 60.74 (**C**H$_2$$^{5'}$), 51.62 (**C**H$_2$^{8}), 45.15 (**C**H$_2$^{8}), 31.21 (**C**H$_2$^{9}), 29.39 (**C**H$_2$^{9}), 19.98, 19.53 (**C**H$_2$^{10}), 14.10, 13.99 (**C**H$_3$^{11})

IR (Film, cm^{-1}): υ_{max} = 2954 (br), 2147 (w), 1765 (m), 1684 (s)

LRMS (Scan CI): m/z = 383 ((M+H)$^+$, 100%)

Microanalysis: Expected (C, 56.53; H, 7.91; N, 14.64); Found (C, 55.90; H, 7.94; N, 14.62)



28

$C_{30}H_{56}N_4O_6Si_2$

3',5'-*O*-1,1,3,3-Tetraisopropyldisiloxan-1,3-diyl-6-*N*-((di-*n*-butylamino)-methylene)-cytidine (28). Under a nitrogen atmosphere, to a soution of the nucleoside **27** (30.00g, 78.50mmol) and imidazole (5.90g, 86.40mmol) in pyridine (300.0ml) was added 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (30.0ml, 94.20mmol). After stirring for 9 hours the product was extracted with ethyl acetate (3x100ml), washed with 2M $HCl_{(aq)}$ (3x200ml), dried over sodium sulfate and concentrated *in vacuo*. The residue was purified by recrystalisation from boiling ethyl acetate to give the title compound **28** as a white crystaline solid (31.09g, *63%*). [R_F = 0.4 ethyl acetate, U.V., ammonium molybdate].

Melting Point: 178-180°C

^{1}H NMR (400MHz, d_5-pyridine): δ = 9.05 (s, 1H, **H**^{7}), 8.42 (d, J = 7.0 Hz, 1H, **H**^{6}), 6.49 (s, 1H, **H**$^{1'}$), 6.40 (d, J = 7.0 Hz, 1H, **H**^{5}), 4.95 (s, 1H, **OH**$^{2'}$), 4.83 (d, J = 4.0 Hz, 1H, **H**$^{2'}$), 4.62 (d, J = 9.5 Hz, 1H, **H**$^{4'}$), 4.52 (dd, J = 9.5, 4.0 Hz, 1H, **H**$^{3'}$), 4.38 (d, J = 13.1 Hz, 1H, **H**$^{5'}$), 4.07 (dd, J = 13.1, 2.0 Hz, 1H, **H**$^{5'}$), 3.51 (t, J = 8.0 Hz, 4H, **H**^{8}), 3.16 (t, J = 8.0 Hz, 4H, **H**^{9}), 1.40 (m, 2H, **H**^{10}), 1.27 (m, 2H, **H**^{10}), 1.10 (m, 28H, **^{i}Pr-CH**, **^{i}Pr-CH$_3$**), 0.95 (m, 6H, **H**^{11})

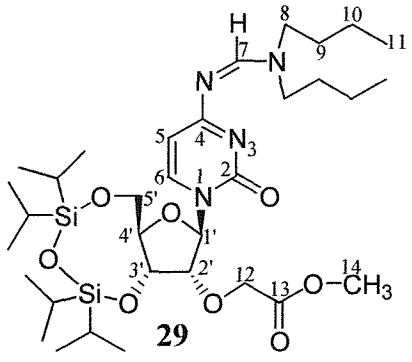
^{13}C NMR (100MHz, d_5-pyridine): δ = 173.58 (**C**^{4}), 159.18 (**CH**^{7}), 157.24 (**C**^{2}), 142.68 (**CH**^{6}), 102.70 (**CH**^{5}), 93.63 (**CH**$^{1'}$), 82.74 (**CH**$^{4'}$), 76.62 (**CH**$^{2'}$), 70.18 (**CH**$^{3'}$), 61.35

($CH_2^{5'}$), 52.87, 46.48 (CH_2^{8}), 31.88, 30.18 (CH_2^{9}), 21.12, 20.67 (CH_2^{10}), 18.57, 18.47, 18.36, 18.29, 18.02, 17.93, 17.88 (^{i}**Pr-CH$_3$**), 14.71, 14.54, 14.48, 14.19, 14.01, 13.61 (^{i}**Pr-CH**; q, CH_3^{11})

IR (Film, cm^{-1}): υ_{max} = 2978 (br), 2876 (m), 2089 (w), 1674 (s), 1549 (s)

LRMS (Scan CI): m/z = 626 ($(M+H+1)^+$, 100%)

Microanalysis: Expected (C, 57.66; H, 9.03; N, 8.96); Found (C, 57.34; H, 9.07; N, 8.90)



29

$C_{33}H_{60}N_4O_8Si_2$

3',5'-*O*-1,1,3,3-Tetraisopropyldisiloxan-1,3-diyl-6-*N*-((di-*n*-butylamino)-methylene)-2'methylethanoate-cytidine (29). Under a nitrogen atmosphere, to a solution of the nucleoside **28** (10.00g, 16.00mmol) in DMF (100.0ml) was added methyl bromoacetate (9.1ml, 96.00mmol) at -5°C (methanol/ice). After stirring for 15 minutes, sodium hydride (3.20g, 80.00mmol) was added portionwise over a period of 2 hours. Saturated potassium chloride (50.0ml) was added and the product was extracted with ethyl acetate (3x100ml). The organic layer was washed with brine (3x50ml), dried over sodium sulfate and concentrated *in vacuo*. The residue was purified by column chromatography (ethyl acetate/hexane 2:1) to give the title compound **29** as a colourless oil (7.78g, *69%*). [R_F = 0.2 (ethyl acetate/hexane 2:1), U.V., ammonium molybdate].

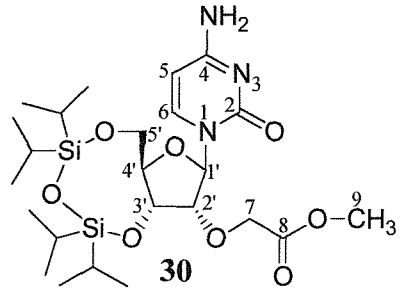
^{1}H NMR (400MHz, $CDCl_3$): δ = 8.80 (s, 1H, **H**^{7}), 7.95 (d, J = 7.1 Hz, 1H, **H**^{6}), 5.92 (d, J = 7.1 Hz, 1H, **H**^{5}), 5.82 (s, 1H, **H**$^{1'}$), 4.69 (d, J = 16.6 Hz, 1H, **H**^{12}), 4.42 (d, J = 16.6 Hz, 1H, **H**^{12}), 4.21 (d, J = 13.6 Hz, 1H, **H**$^{5'}$), 4.19 (dd, J = 9.8, 2.1 Hz, 1H, **H**$^{4'}$), 4.12 (dd, J = 9.8, 4.3 Hz, 1H, **H**$^{3'}$), 3.99 (d, J = 4.3 Hz, 1H, **H**$^{2'}$), 3.91 (dd, J = 13.6, 2.1 Hz, 1H, **H**$^{5'}$), 3.66 (s, 3H, **H**^{14}), 3.52 (dt, J = 13.2, 7.6 Hz, 1H, **H**^{8}), 3.42 (dt, J = 13.2, 7.6 Hz, 1H, **H**^{8}), 3.25 (t, J = 7.4 Hz, 2H, **H**^{12}), 1.55 (m, 4H, **H**^{9}), 1.20 (m, 4H, **H**^{10}), 1.05 (m, 28H, ^{i}**Pr-CH**, ^{i}**Pr-CH$_3$**), 0.95 (m, 6H, **H**^{11})

^{13}C NMR (100MHz, $CDCl_3$): δ = 171.22 (**C**^{4}), 169.75 (**C**^{13}), 157.38 (**CH**^{7}), 155.12 (**C**^{2}), 139.80 (**CH**^{6}), 101.50 (**CH**^{5}), 88.15 (**CH**$^{1'}$), 81.65 (**CH**$^{2'}$), 80.36 (**CH**$^{4'}$), 66.83 (**CH**$^{3'}$), 66.28 (**CH$_2$**^{12}), 58.53 (**CH$_2$**$^{5'}$), 51.11 (**CH$_2$**^{8}), 50.68 (**CH$_3$**^{14}), 44.42 (**CH$_2$**^{8}), 30.01, 28.10 (**CH$_2$**^{9}), 19.08, 18.79 (**CH$_2$**^{10}), 16.53, 16.46, 16.34, 16.32, 15.99, 15.95, 15.76 (^{i}**Pr-CH$_3$**), 12.77, 12.67, 12.43, 12.14, 11.93, 11.22 (^{i}**Pr-CH**; q, **CH$_3$**^{11})

IR (Film, cm^{-1}): υ_{max} = 2942 (br), 2847 (m), 2079 (w), 1997 (w), 1678 (s), 1477 (s)

LRMS (Scan CI): m/z = 698 ($(M+H+1)^+$, 100%)

HRMS (FTMS):m/z = Found (697.4038 $(M+H)^+$, 100%); Expected 697.4029



30

$C_{24}H_{43}N_3O_8Si_2$
C, 51.68; H, 7.77; N, 7.53; O, 22.95; Si, 10.07

3',5'-*O*-1,1,3,3-Tetraisopropyldisiloxan-1,3-diyl-2'-methylethanoate-cytidine (30). To a solution of the nucleoside **29** (10.00g, 14.40mmol) in methanol (50.00ml) was added concentrated aqueous ammonia (50.0ml). After being stirred overnight at 70°C the product was extracted with DCM (3x100ml), washed with 2M $HCl_{(aq)}$ (3x50ml), dried over sodium sulfate and the solvent removed *in vacuo*. The residue was purifed

by column chromatography (ethyl acetate to 10% methanol/ethyl acetate) to give the title compound **30** as a colourless oil (4.37g, *54%*). [R_F = 0.5 ethyl acetate, U.V., ammonium molybdate].

Melting Point: 99-101°C

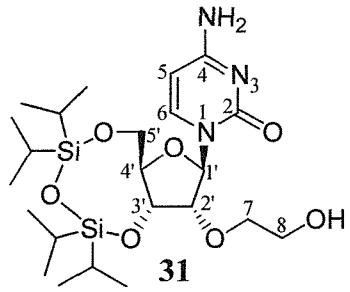
^{1}H NMR (400MHz, $CDCl_3$): δ = 7.97 (d, J = 8.1 Hz, 1H, **H^6**), 6.22 (d, J = 8.1 Hz, 1H, **H^5**), 5.73 (s, 1H, **H$^{1'}$**), 4.59 (d, J = 16.2 Hz, 1H, **H^7**), 4.41 (d, J = 16.2 Hz, 1H, **H^7**), 4.24 (m, 1H, **H$^{5'}$**), 4.15 (dd, J = 4.4, 9.6 Hz, 1H, **H$^{4'}$**), 4.02 (d, J = 4.4 Hz, 1H, **H$^{2'}$**), 3.95 (m, 1H, **H$^{3'}$**), 3.71 (m, 4H, **H$^{5'}$**, **H^9**), 1.05 (m, 28H, **^{i}Pr-CH**, **^{i}Pr-CH$_3$**)

^{13}C NMR (100MHz, $CDCl_3$): δ = 173.98 (**C^4**), 170.42 (**C^8**), 162.90 (**C^2**), 141.14 (**CH^6**), 95.22 (**CH^5**), 88.84 (**CH$^{1'}$**), 82.23 (**CH$^{2'}$**), 81.67 (**CH$^{4'}$**), 68.04 (**CH$^{3'}$**), 67.39 (**CH$_2$^{7}**), 59.24 (**CH$_2$$^{5'}$**), 52.58 (**CH$_3$^{9}**), 19.63, 17.44, 17.40, 17.28, 17.25, 16.98, 16.94, 16.73, (**^{i}Pr-CH$_3$**), 13.79, 13.64, 13.43, 12.97 (**^{i}Pr-CH**)

IR (Film, cm^{-1}): υ_{max} = 2958 (br), 2113 (w), 2093 (w), 1688 (s), 1604 (s)

LRMS (Scan CI): m/z = 558 ($(M+H)^+$, 40%)

HRMS (FTMS): m/z = Found (558.2661 $(M+H)^+$, 100%); Expected 558.2668



31

$C_{23}H_{43}N_3O_7Si_2$

3',5'-*O*-1,1,3,3-Tetraisopropyldisiloxan-1,3-diyl-2'-ethoxy-cytidine (31). Under a nitrogen atmosphere, to a solution of the nucleoside **30** (4.20g, 7.50mmol) in THF (32.0ml) and methanol (8.0ml) was added lithium borohydride (0.70g, 30.00mml). After stirring for 6 hours the product was extracted with DCM (2x100ml), washed with saturated brine (3x50ml), dried over sodium sulfate and the solvent removed *in vacuo*. The residue was purified by column chromatography (5% methanol/ DCM) to give the title compound **31** as a yellow solid (3.64g, *91%*). [R_F = 0.3 (10% methanol/ DCM), U.V., ammonium molybdate].

Melting Point: 120-122°C

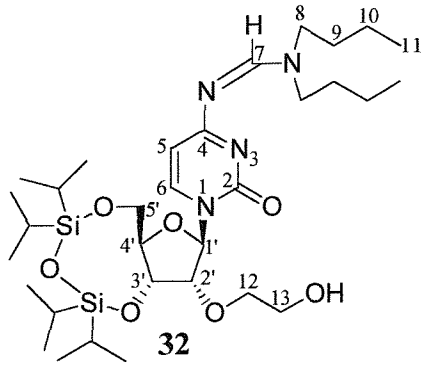
^{1}H NMR (400MHz, $CDCl_3$): δ = 7.80 (d, J = 7.5 Hz, 1H, **H^6**), 5.74 (d, J = 7.5 Hz, 1H, **H^5**), 5.65 (s, 1H, **H$^{1'}$**), 4.18 (d, J = 13.1 Hz, 1H, **H$^{5'}$**), 4.05 (m, 2H, **H$^{3'}$**, **H$^{4'}$**), 3.70 (m, 4H, **H$^{2'}$**, **H$^{5'}$**, **H^7**), 3.67 (m, 2H, **H^8**), 0.95 (m, 28H, **^{i}Pr-CH**, **^{i}Pr-CH$_3$**)

^{13}C NMR (100MHz, $CDCl_3$): δ = 172.09 (**C^4**), 154.66 (**C^2**), 139.25 (**CH^6**), 93.45 (**CH^5**), 89.11 (**CH$^{1'}$**), 81.62 (**CH$^{2'}$**), 80.49 (**CH$^{4'}$**), 72.05 (**CH$_2$^{7}**), 67.29 (**CH$^{3'}$**), 60.69 (**CH$_2$^{8}**), 58.42 (**CH$_2$$^{5'}$**), 16.45, 16.39, 16.29, 16.25, 16.07, 15.99, 15.97, 15.82 (**^{i}Pr-CH$_3$**), 12.42, 11.88, 11.57, 11.32 (**^{i}Pr-CH**)

IR (Film, cm^{-1}): υ_{max} = 2967 (br), 2543 (m), 2117 (m), 1664 (s), 1548 (s)

LRMS (Scan CI): m/z = 530 ($(M+H)^+$, 100%), 552 ($(M+Na)^+$, 40%)

HRMS (FTMS): m/z = Found (530.2725 $(M+H)^+$, 100%); Expected 530.2719



32

$C_{32}H_{60}N_4O_7Si_2$

3',5'-*O*-1,1,3,3-Tetraisopropyldisiloxan-1,3-diyl-6-*N*-((di-*n*-butylamino)-methylene)-2'-ethoxycytidine (32). Under a nitrogen atmosphere, to a solution of the nucleoside **31** (3.50g, 6.60mmol) in DMF (35.0ml) was added *N,N*-dibutylformamide dimethyl acetal (1.60g, 8.00mml). After stirring for 12 hours the solvent was removed *in vacuo*. The residue was purified by column chromatography (5% methanol/ DCM) to give the title compound **32** as a colourless oil (3.52g, *80%*). [R_F =0.6 (10% methanol/ DCM), U.V., ammonium molybdate].

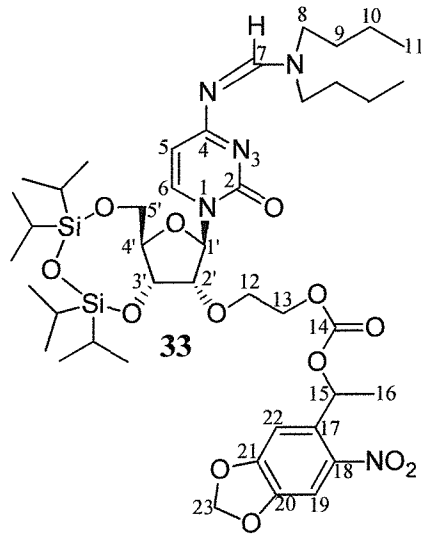
^{1}H NMR (400MHz, $CDCl_3$): δ = 8.78 (s, 1H, **H**^{7}), 7.93 (d, J = 7.5 Hz, 1H, **H**^{6}), 5.98 (d, J = 7.5 Hz, 1H, **H**^{5}), 5.69 (s, 1H, **H**$^{1'}$) 4.20 (d, J = 13.6 Hz, 1H, **H**$^{5'}$), 4.10 (m, 2H, **H**$^{3'}$, **H**$^{4'}$), 3.90 (m, 4H, **H**$^{2'}$, **H**$^{5'}$, **H**^{12}), 3.70 (m, 2H, **H**^{13}), 3.34-3.58 (m, 2H, **H**^{8}), 3.25 (t, J = 7.5 Hz, 2H, **H**^{8}), 1.52 (m, 4H, **H**^{9}), 1.25 (m, 4H, **H**^{10}), 0.82-1.07 (m, 34H, **^{i}Pr-CH**, **^{i}Pr-CH$_3$**, **H**^{11})

^{13}C NMR (100MHz, $CDCl_3$): δ = 171.24 (**C**^{4}), 161.67 (**CH**^{7}), 157.38 (**C**^{2}), 139.42 (**CH**^{6}), 101.68 (**CH**^{5}), 89.37 (**CH**$^{1'}$), 81.51 (**CH**$^{2'}$), 80.49 (**CH**$^{4'}$), 72.06 (**CH$_2$**^{12}), 67.18 (**CH**$^{3'}$), 60.75 (**CH$_2$**^{13}), 58.46 (**CH$_2$**$^{5'}$), 51.15, 44.44 (**CH$_2$**^{8}), 30.00, 28.40 (**CH$_2$**^{9}), 19.08, 18.72 (**CH$_2$**^{10}), 16.49, 16.40, 16.32, 16.27, 16.04, 15.97, 15.82 (**^{i}Pr-CH$_3$**), 12.77, 12.67, 12.41, 12.02, 11.92, 11.60 (**^{i}Pr-CH**; q, **CH$_3$**^{11})

IR (Film, cm^{-1}): υ_{max} = 2967 (br), 2367 (w), 2189 (w), 1647 (s), 1603 (s)

LRMS (Scan CI): m/z = 670 ($(M+H+1)^+$, 100%)

HRMS (FTMS): m/z = Found (669.4087 $(M+H)^+$, 100%); Expected 669.4080



33

$C_{42}H_{67}N_5O_{13}Si_2$

3',5'-*O*-1,1,3,3-Tetraisopropyldisiloxan-1,3-diyl-6-*N*-((di-*n*-butylamino)-methylene)-2'-ethoxy-1-(6-nitro-1,3-benzodioxol-5-yl)-cytidine (33). Under a nitrogen atmosphere, to a solution of carbonyldiimidazole (2.00g, 12.0mmol) in nitromethane (20.0ml) was added methyl triflate (2.50ml, 24.00mmol) dropwise at 5°C (ice bath). The reaction was warmed to room temperature and after stirring for 30 minutes the solution was transferred to a flask containing the alcohol **21** (2.50g, 12.00mmol) and stirred for 2 hours. Under a nitrogen atmosphere to a solution of the nucleoside **32** (1.50g, 2.20mmol) in pyridine (15.0ml) was added the above solution (6.8ml). After stirring for 8 hours the solvent was removed *in vacuo*. The residue was purified by column chromatography (hexane:ethyl acetate 1:1 to ethyl acetate) to give the title compound **33** as a yellow foam (1.05g, *54%*). [R_F = 0.5 ethyl acetate, U.V., brown upon heating, ammonium molybdate].

^{1}H NMR (400MHz, $CDCl_3$): δ = 8.80 (s, 1H, $\mathbf{H^7}$), 8.13 (d, J = 7.5 Hz, 1H, $\mathbf{H^6}$), 7.40 (s, 1H, $\mathbf{H^{22}}$), 7.01 (s, 0.5H, $\mathbf{H^{19}}$), 6.99 (s, 0.5H, $\mathbf{H^{19}}$), 6.30 (m, 1H, $\mathbf{H^5}$), 6.18 (q, J = 6.0 Hz, 1H, $\mathbf{H^{15}}$), 6.05 (m, 2H, $\mathbf{H^{23}}$), 5.66 (d, J = 5.0 Hz, 1H, $\mathbf{H^{1'}}$), 4.26 (m, 2H, $\mathbf{H^{13}}$), 4.18 (m, 1H, $\mathbf{H^{5'}}$), 4.03-4.13 (m, 2H, $\mathbf{H^{3'}}$, $\mathbf{H^{4'}}$), 4.00 (m, 2H, $\mathbf{H^{12}}$), 3.90 (m, 1H, $\mathbf{H^{5'}}$), 3.84 (m, 1H, $\mathbf{H^{2'}}$), 3.60 (m, 2H, $\mathbf{H^8}$), 3.49 (t, J = 7.5 Hz, 2H, $\mathbf{H^8}$), 1.56 (d, J = 6.0 Hz, 3H, $\mathbf{H^{16}}$),

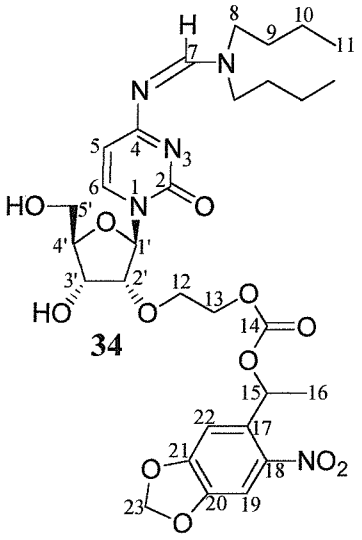
1.59 (m, 4H, $\mathbf{H^{9}}$), 1.30 (m, 2H, $\mathbf{H^{10}}$), 1.18 (t, J = 7.0 Hz, 2H, $\mathbf{H^{10}}$), 0.80-1.03 (m, 34H, $\mathbf{^{i}Pr\text{-}CH}$, $\mathbf{^{i}Pr\text{-}CH_3}$, $\mathbf{H^{11}}$)

^{13}C NMR (100MHz, $CDCl_3$): δ = 160.97 ($\mathbf{C^{4}}$), 155.59 ($\mathbf{CH^{7}}$), 153.09 ($\mathbf{C^{2}}$), 151.83 ($\mathbf{C^{18}}$), 151.48 ($\mathbf{C^{21}}$), 146.32 ($\mathbf{C^{14}}$), 142.56 ($\mathbf{CH^{6}}$), 140.42 ($\mathbf{C^{20}}$), 134.09 ($\mathbf{C^{17}}$), 104.81 ($\mathbf{CH^{19}}$), 104.10 ($\mathbf{CH^{22}}$), 102.12 ($\mathbf{CH_2^{23}}$), 101.43 ($\mathbf{CH^{5}}$), 88.93 ($\mathbf{CH^{1'}}$), 81.18 ($\mathbf{CH^{2'}}$), 80.78 ($\mathbf{CH^{4'}}$), 71.09 ($\mathbf{CH^{15}}$), 67.81 ($\mathbf{CH_2^{12}}$), 66.98 ($\mathbf{CH^{3'}}$), 66.07 ($\mathbf{CH_2^{13}}$), 58.28 ($\mathbf{CH_2^{5'}}$), 53.18, 46.47 ($\mathbf{CH_2^{8}}$), 29.57, 27.91 ($\mathbf{CH_2^{9}}$), 21.05 ($\mathbf{CH_3^{16}}$), 19.00, 18.76 ($\mathbf{CH_2^{10}}$), 16.60, 16.45, 16.33, 16.28, 16.21, 16.09, 15.95, 15.82 ($\mathbf{^{i}Pr\text{-}CH_3}$), 12.66, 12.56, 12.36, 12.21, 11.89, 11.83 ($\mathbf{^{i}Pr\text{-}CH}$; q, $\mathbf{CH_3^{11}}$)

IR (Film, cm^{-1}): υ_{max} = 2971 (br), 2779 (m), 2114 (w), 2018 (w), 1691 (s), 1456 (s)

LRMS (Scan CI): m/z = 907 ($(M+H+1)^+$, 50%)

HRMS (FTMS): m/z = Found (906.4359 $(M+H)^+$, 100%); Expected 906.4353



34

$C_{30}H_{41}N_5O_{12}$

6-*N*-((Di-*n*-butylamino)-methylene)-2'-ethoxy-1-(6-nitro-1,3-benzodioxol-5-yl)-cytidine (34). A solution of the nucleoside **33** (2.00g, 2.20mmol) in 1M tetrabutylammonium fluoride in THF (20.0ml) was stirred at room temperature for 2 hours. The product was extracted with DCM (2x100ml), washed with saturated brine

(3x50ml), dried over sodium sulfate and the solvent removed *in vacuo*. The residue was purified by column chromatography (5% methanol/ DCM to 10% methanol/ DCM) to give the title compound **34** as a yellow foam (1.20g, *80%*). [R_F = 0.3 (10% methanol/ DCM), U.V., brown upon heating, ammonium molybdate].

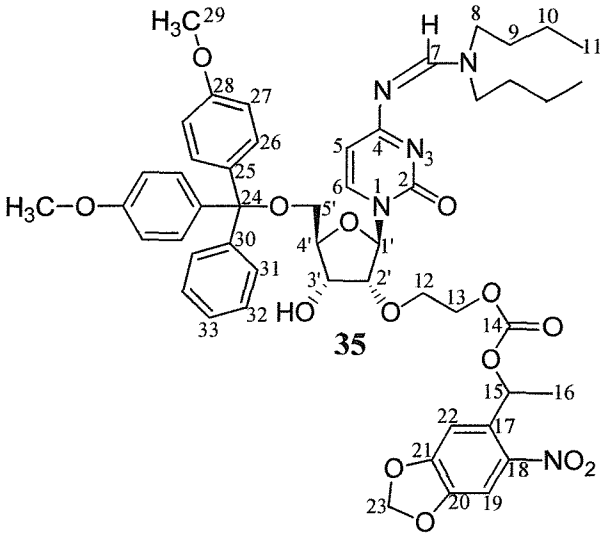
^{1}H NMR (400MHz, $CDCl_3$): δ = 8.83 (s, 1H, $\mathbf{H^7}$), 8.19 (d, J = 7.5 Hz, 1H, $\mathbf{H^6}$), 6.69 (s, 0.5H, $\mathbf{H^{22}}$), 6.68 (s, 0.5H, $\mathbf{H^{22}}$), 7.30 (s, 0.5H, $\mathbf{H^{19}}$), 7.28 (s, 0.5H, $\mathbf{H^{19}}$), 6.34 (d, J = 7.5 Hz, 1H, $\mathbf{H^5}$), 6.12 (q, J = 6.0 Hz, 1H, $\mathbf{H^{15}}$), 6.05 (d, J = 7.0 Hz, 1H, $\mathbf{H^{23}}$), 6.04 (d, J = 7.0 Hz, 1H, $\mathbf{H^{23}}$), 5.92 (s, 1H, $\mathbf{H^{1'}}$), 4.24-4.35 (m, 2H, $\mathbf{H^{13}}$), 4.12-4.20 (m, 2H, $\mathbf{H^{12}}$), 3.89-4.02 (m, 2H, $\mathbf{H^{2'}}$, $\mathbf{H^{4'}}$), 3.82 (m, 1H, $\mathbf{H^{3'}}$), 3.60 (t, J = 7.0 Hz, 2H, $\mathbf{H^8}$), 3.53 (t, J = 7.0 Hz, 2H, $\mathbf{H^8}$), 3.29 (m, 2H, $\mathbf{H^{5'}}$), 1.62-1.74 (m, 5H, $\mathbf{H^9}$, $\mathbf{H^{16}}$), 1.38-1.48 (m, 4H, $\mathbf{H^{10}}$), 0.98-1.09 (m, 6H, $\mathbf{H^{11}}$)

^{13}C NMR (100MHz, $CDCl_3$): δ = 171.75 ($\mathbf{C^4}$), 158.04 ($\mathbf{CH^7}$), 155.50 ($\mathbf{C^2}$), 154.10 ($\mathbf{C^{18}}$), 152.72 ($\mathbf{C^{21}}$), 147.79 ($\mathbf{C^{14}}$), 142.53 ($\mathbf{CH^6}$), 142.01 ($\mathbf{C^{20}}$), 133.90 ($\mathbf{C^{17}}$), 106.17 ($\mathbf{CH^{19}}$), 104.99 ($\mathbf{CH^{22}}$), 104.04 ($\mathbf{CH_2^{23}}$), 101.52 ($\mathbf{CH^5}$), 88.34 ($\mathbf{CH^{1'}}$), 84.40 ($\mathbf{CH^{2'}}$), 82.74 ($\mathbf{CH^{4'}}$), 71.62 ($\mathbf{CH^{15}}$), 68.10 ($\mathbf{CH_2^{12}}$), 67.57 ($\mathbf{CH^{3'}}$), 67.44 ($\mathbf{CH_2^{13}}$), 58.01 ($\mathbf{CH_2^{5'}}$), 51.64 ($\mathbf{CH_2^8}$), 45.18 ($\mathbf{CH_2^8}$), 30.85 ($\mathbf{CH_2^9}$), 29.05 ($\mathbf{CH_2^9}$), 23.54 ($\mathbf{CH_3^{16}}$), 19.66 ($\mathbf{CH_2^{10}}$), 19.57 ($\mathbf{CH_2^{10}}$), 13.92 ($\mathbf{CH_3^{11}}$)

IR (Film, cm^{-1}): υ_{max} = 2969 (br), 2273 (w), 2120 (w), 1687 (s), 1544 (s)

LRMS (Scan CI): m/z = 665 ($(M+H+1)^+$, 40%), 687 ($(M+Na+1)^+$, 50%)

HRMS (FTMS): m/z = Found (664.2826 $(M+H)^+$, 100%); Expected 664.2831



35

$C_{51}H_{59}N_5O_{14}$

5'-Dimethoxytrityl-6-*N*-((di-*n*-butylamino)-methylene)-2'-ethoxy-1-(6-nitro-1,3-benzodioxol-5-yl)-cytidine (35). Under a nitrogen atmosphere 4, 4'-dimethoxytrityl chloride (0.60g, 1.70mmol) and the nucleoside **34** (1.00g, 1.50mmol) were dissolved in pyridine (10.0ml). After stirring for 8 hours the product was extracted with ethyl acetate (3x50ml), washed with brine (3x25ml), dried over sodium sulfate, and the solvent removed *in vacuo*. The residue was purified by column chromatography (hexane/ethyl acetate 1:1) to give the title compound **35** as a yellow foam (1.58g, *81%*). [R_F = 0.2 ethyl acetate, U.V., brown upon heating, ammonium molybdate].

^{1}H NMR (400MHz, $CDCl_3$): δ = 8.75 (s, 0.5H, $\mathbf{H^7}$), 8.74 (s, 0.5H, $\mathbf{H^7}$), 8.08 (d, J = 7.0 Hz, 1H, $\mathbf{H^6}$), 7.68 (s, 0.5H, $\mathbf{H^{22}}$), 7.67 (s, 0.5H, $\mathbf{H^{22}}$), 7.32-7.55 (m, 9H, $\mathbf{H^{26}}$, $\mathbf{H^{31}}$, $\mathbf{H^{32}}$, $\mathbf{H^{33}}$), 7.29 (s, 0.5H, $\mathbf{H^{19}}$), 7.28 (s, 0.5H, $\mathbf{H^{19}}$), 7.01 (d, J = 8.5 Hz, 4H, $\mathbf{H^{27}}$), 6.33 (s, 1H, $\mathbf{H^{23}}$), 6.28 (d, J = 3.0 Hz, 1H, $\mathbf{H^{23}}$), 6.13 (q, J = 5.0 Hz, 1H, $\mathbf{H^{15}}$), 5.92 (d, J = 2.0 Hz, 0.5H, $\mathbf{H^{1'}}$), 5.90 (d, J = 2.0 Hz, 0.5H, $\mathbf{H^{1'}}$), 5.73 (d, J = 7.0 Hz, 1H, $\mathbf{H^5}$), 4.25-4.42 (m, 2H, $\mathbf{H^{13}}$), 3.90-4.15 (m, 5H, $\mathbf{H^{2'}}$, $\mathbf{H^{3'}}$, $\mathbf{H^{4'}}$, $\mathbf{H^{12}}$), 3.85 (s, 6H, $\mathbf{H^{29}}$), 3.61 (t, J = 7.0 Hz, 2H, $\mathbf{H^8}$), 3.54 (t, J = 7.0 Hz, 2H, $\mathbf{H^8}$), 3.25-3.30 (m, 2H, $\mathbf{H^{5'}}$), 1.60-1.72 (m, 7H, $\mathbf{H^9}$, $\mathbf{H^{16}}$), 1.32-1.48 (m, 4H, $\mathbf{H^{10}}$), 0.98-1.17 (m, 6H, $\mathbf{H^{11}}$)

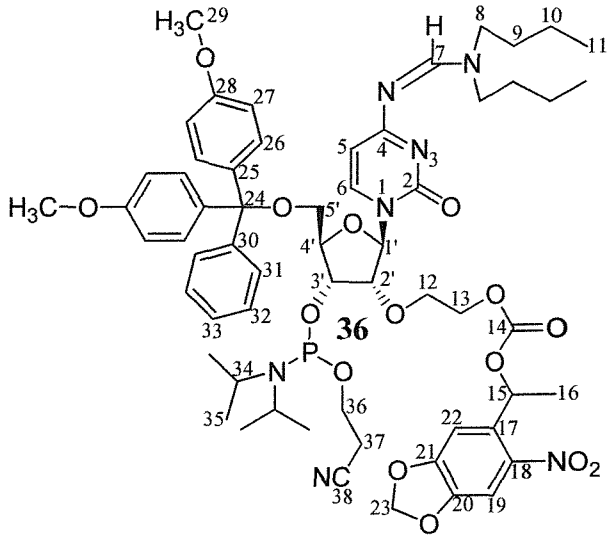
^{13}C NMR (100MHz, $CDCl_3$): δ = 170.09 ($\mathbf{C^4}$), 169.35 ($\mathbf{C^{28}}$), 158.63 ($\mathbf{CH^7}$), 158.15 ($\mathbf{C^2}$), 154.13 ($\mathbf{C^{18}}$), 152.73 ($\mathbf{C^{21}}$), 147.78 ($\mathbf{C^{14}}$), 145.03 ($\mathbf{C^{30}}$), 142.22 ($\mathbf{CH^6}$), 142.03 ($\mathbf{C^{20}}$), 135.64 ($\mathbf{C^{25}}$), 133.93 ($\mathbf{C^{17}}$), 130.31, 130.23, 128.36, 128.27 ($\mathbf{CH^{31}}$, $\mathbf{CH^{32}}$, $\mathbf{CH^{33}}$),

127.26 (CH^{26}), 113.73 (CH^{27}), 106.19 (CH^{19}), 105.02 (CH^{22}), 104.02 (CH^{23}), 101.71 (CH^{5}) 89.01 ($CH^{1'}$), 86.35 (C^{24}), 82.57 ($CH^{2'}$), 82.25 ($CH^{4'}$), 71.66 (CH^{15}), 68.58 ($CH^{3'}$), 68.31 (CH_2^{12}), 67.44 (CH_2^{13}), 58.01 ($CH_2^{5'}$), 55.51 (CH_3^{29}), 51.76 (CH_2^{8}), 45.30 (CH_2^{8}), 30.84 (CH_2^{9}), 29.04 (CH_2^{9}), 21.72 (CH_3^{16}), 19.97, 19.59 (CH_2^{10}), 13.99, 13.92 (CH_3^{11})

IR (Film, cm^{-1}): υ_{max} = 2969 (br), 2278 (w), 2265 (w), 2187 (w), 1651 (s), 1587 (s)

LRMS (Scan CI): m/z = 967 ($(M+H+1)^+$, 100%)

HRMS (FTMS): m/z = Found (996.4141 $(M+H)^+$, 100%); Expected 966.4138



$C_{60}H_{76}N_7O_{15}P$

5'-Dimethoxytrityl-6-*N*-((di-*n*-butylamino)-methylene)-2'-ethoxy-1-(6-nitro-1,3-benzodioxol-5-yl)-3'-(2-cyanoethyldiisopropylphosphoramidite)-cytidine (36). Under a nitrogen atmosphere 2-cyanoethyl diisopropylchlorophosphoramidite (0.1ml, 0.40mmol) and DIPEA (0.1ml, 3.60mmol) were added to a solution of the nucleoside **35** (0.40g, 0.40mmol) in THF (2.0ml). After stirring for 6 hours the reaction was quenched by addition of degassed ethyl acetate (10.0ml). The mixture was transferred to a RB flask and washed with aqueous saturated potassium chloride (3x25ml), dried over anhydrous sodium sulfate and the solvent removed *in vacuo*. The residue (0.30g) was purified by column chromatography (degassed ethyl acetate) to give the title

compound **36** as a white foam (0.15g, *36%*). [R_F = 0.2 ethyl acetate/hexane 3:1, U.V., ammonium molybdate, brown upon heating].

^{1}H NMR (400MHz, $CDCl_3$): δ = 8.73 (s, 1H, $\mathbf{H^7}$), 8.18 (d, J = 7.5 Hz, 0.5H, $\mathbf{H^6}$), 8.16 (d, J = 7.5 Hz, 0.5H, $\mathbf{H^6}$), 7.11-7.42 (m, 11H, $\mathbf{H^{19}}$, $\mathbf{H^{22}}$, $\mathbf{H^{26}}$, $\mathbf{H^{31}}$, $\mathbf{H^{32}}$, $\mathbf{H^{33}}$), 7.00 (d, J = 2.5 Hz, 1H, $\mathbf{H^{23}}$), 6.98 (d, J = 2.5 Hz, 1H, $\mathbf{H^{23}}$), 6.78 (d, J = 7.5 Hz, 2H, $\mathbf{H^{27}}$), 6.74 (d, J = 7.5 Hz, 2H, $\mathbf{H^{27}}$), 6.17 (q, J = 6.0 Hz, 1H, $\mathbf{H^{15}}$), 5.95-6.03 (m, 1H, $\mathbf{H^{1'}}$), 5.60 (d, J = 7.5 Hz, 0.5H, $\mathbf{H^5}$), 5.57 (d, J = 7.5 Hz, 0.5H, $\mathbf{H^5}$), 4.12-4.38 (m, 2H, $\mathbf{H^{13}}$), 3.76-3.96 (m, 5H, $\mathbf{H^{2'}}$, $\mathbf{H^{3'}}$, $\mathbf{H^{4'}}$, $\mathbf{H^{12}}$), 3.54 (s, 3H, $\mathbf{H^{29}}$), 3.52 (s, 3H, $\mathbf{H^{29}}$), 3.20-3.56 (m, 8H, $\mathbf{H^{5'}}$, $\mathbf{H^{34}}$, $\mathbf{H^{36}}$, $\mathbf{H^{37}}$), 2.52 (m, 2H, $\mathbf{H^8}$), 2.30 (t, J = 6.5 Hz, 2H, $\mathbf{H^8}$), 1.48-1.58 (m, 7H, $\mathbf{H^9}$, $\mathbf{H^{16}}$), 1.12-1.30 (m, 4H, $\mathbf{H^{10}}$), 1.00-1.10 (m, 12H, $\mathbf{H^{35}}$), 0.82-0.92 (m, 6H, $\mathbf{H^{11}}$)

^{13}C NMR (100MHz, $CDCl_3$): δ = 170.10 ($\mathbf{C^4}$), 169.25 ($\mathbf{C^{28}}$), 157.64 ($\mathbf{CH^7}$), 157.19 ($\mathbf{C^2}$), 153.06 ($\mathbf{C^{18}}$), 151.51 ($\mathbf{C^{21}}$), 148.07 ($\mathbf{C^{14}}$), 146.32 ($\mathbf{C^{30}}$), 143.27 ($\mathbf{CH^6}$), 140.28 ($\mathbf{C^{20}}$), 134.40 ($\mathbf{C^{25}}$), 134.00 ($\mathbf{C^{17}}$), 129.34, 129.24, 127.47, 126.89 ($\mathbf{CH^{31}}$, $\mathbf{CH^{32}}$, $\mathbf{CH^{33}}$), 126.04 ($\mathbf{CH^{26}}$), 116.75 ($\mathbf{C^{38}}$), 112.18 ($\mathbf{CH^{27}}$), 105.87 ($\mathbf{CH^{19}}$), 104.83 ($\mathbf{CH^{22}}$), 104.16 ($\mathbf{CH^{23}}$), 102.06 ($\mathbf{CH^5}$), 89.09 ($\mathbf{CH^{1'}}$), 85.88 ($\mathbf{C^{24}}$), 81.25 ($\mathbf{CH^{2'}}$), 80.44 ($\mathbf{CH^{4'}}$), 71.05 ($\mathbf{CH^{15}}$), 68.12 ($\mathbf{CH^{3'}}$), 67.10 ($\mathbf{CH_2}^{12}$), 66.26 ($\mathbf{CH_2}^{13}$), 59.36 ($\mathbf{CH_2}^{36}$), 57.53 ($\mathbf{CH_2}^{5'}$), 54.23 ($\mathbf{CH_3}^{29}$), 51.21 ($\mathbf{CH_2}^{8}$), 44.53 ($\mathbf{CH_2}^{8}$), 29.99, 28.07 ($\mathbf{CH_2}^{9}$), 23.65 ($\mathbf{CH^{34}}$), 23.43 ($\mathbf{CH_3}^{35}$), 20.99 ($\mathbf{CH_3}^{16}$), 19.28 ($\mathbf{CH_2}^{37}$), 19.28, 18.77 ($\mathbf{CH_2}^{10}$), 12.76, 12.67 ($\mathbf{CH_3}^{11}$)

^{31}P NMR ($CDCl_3$): δ = 150.50 (P III)

LRMS (Scan CI): m/z = 1167 ($(M+H+1)^+$, 100%)

5.3 Preparation of Synthetic Oligonucleotides

5.3.1 General Methods

All oligonucleotides were synthesised on an Applied Biosystems 394 solid phase DNA/RNA synthesiser using the standard assembly cycle of acid catalysed detritylation, coupling, capping and iodine oxidation procedures. Oligonucleotides were assembled on a 0.2μmol, or 1.0μmol scale, stepwise coupling efficiencies and overall yields were calculated using automated trityl cation conductivity monitoring. DNA phosphoramidites, solid supports and additional reagents were purchased from Applied Biosystems, unless otherwise stated.

Cleavage of the oligonucleotides from the solid support was achieved by exposure to concentrated ammonia, unless otherwise stated. All oligonucleotides requiring base deprotection were placed in concentrated ammonia or ammonia: ethanol (3:1) at 70°C for 4 hours and evaporated to dryness *in vacuo*.

Purifiaction was effected using reversed phase HPLC on a Gilson RPHPLC system, controlled by Gilson 7.12 software. Elution of oligonucleotides was monitored by UV detection at desired wavelengths (260-290nm). Buffer systems consisted of buffer A: 0.1M ammonium acetate, buffer B: acetonitrile: buffer A (4:6, 3:7 or 2.3:7.7), with a typical gradient programme as described in figure 4.3.1.1. Analytical injections were monitored at 265nm, 0.1 AUFS, preparative injections were monitored at 280-290nm, 1.0 AUFS. The oligonucleotides were collected manually.

All phosphoramidites synthesised were stored under argon, at -20°C, in bottles suitable for an ABI 394 DNA synthesiser. The phosphoramidites were dissolved in anhydrous acetonitrile immediately prior to use.

Time (in minutes)	% buffer B	Flow (ml/min)
0	0	4
2	0	4
15	70	4
17	100	4
20	100	4
22	0	4
22.9	0	4
23	0	0

Fig 5.3.1.1:- Typical RPHPLC Elution Gradient

5.4 Molecular Biology

5.4.1 General Methods

All molecular biological grade reagents were obtained from Sigma/Aldrich unless otherwise stated. Deionised water (MilliQ) was used in all experiments. NAP 10 Sephadex columns for desalting oligonucleotides were purchased from Pharmacia.

Fluorescence spectra were measured on a Perkin Elmer LS50B Luminescence Spectrometer using quartz glass 3ml and 0.4ml cells. The amount of fluorescence obtained is measured using arbitrary fluorescence intensity units.

Ultraviolet spectra and thermal melting experiments were carried out on a Perkin Elmer UV/Vis Lambda 2 spectrometer with PTP-1 temperature programmer.

Cleavage of the MeNPOC group was carried out using a multiband UV mineralight lamp at 365nm contained in a model UVGL-58 TLC lamp produced by UVP.

Rapid heating and cooling of samples was achieved using a Techne thermocycler PHC-3

Real time PCR experiments were carried out using a Roche LightCycler

5.4.2 Experimental

UV thermal melting of oligonucleotides

The two single stranded oligonucleotides to be melted were mixed (1:1, total 0.5OD) and diluted with buffer (sodium phosphate 10mM, EDTA 1mM, NaCL 0.1M or 1.0M, pH 7.0). The solution in a UV cevette (pathlength 10mm) was heated to 80°C and cooled slowly to room temperature to effect efficient strand annealing. The sample was melted at a heating rate of 1°C per minute, monitoring at 260nm on a PC with 10s intervals. The data curves were smoothed and the first derivatives obtained using the Perkin Elmer PECSS2 software. The time peaks were converted to temperature manually, in order to ascertain Tm values. The melt process was repeated to acquire consistent results.(±0.25°C)

Preparation of oligonucleotides for electrospray mass spectroscopy

The oligonucleotide sample was dissolved in water (1.0ml), loaded onto a NAP 10 Sephadex column and eluted with water (1.5ml). The water was removed *in vacuo* and the sample was dissolved in a solution of water:isopropanol (1:1) containing tripropylamine (0.2%) for electrospray mass spectral analysis.

Deprotection of the Photolabile Group

The resin columns containing the oligonucleotide bound to the resin were opened and the resin all transferred to one half of the column. The column were rested 5cm away from a UVGL-58 TLC lamp set at 365nm. The resin was agitated using a spatula every hour for a total of 10 hours. The column is sealed up again and put back on the DNA synthesiser.`

Chapter 6

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6.0 References

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