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Development of oligonucleotide probes for genetic analysis

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ABSTRACT

FACULTY OF SCIENCE

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Development of oligonucleotide probes for genetic analysis

by Jonathan Paul May

Fluorescent oligonucleotide probes are of great value in genetic analysis. These include TaqMan® probes, molecular beacons and scorpion primers. The efficacy of an oligonucleotide probe is dependent on the specific detection of a target and the emission of an intense signal. In this thesis the optimisation and development of novel oligonucleotide probes are discussed.

Most fluorescent oligonucleotide probes consist of two key elements; a fluorescent reporter and a quenching moiety. Conventionally, non-fluorescent diazo-dyes, known as 'universal' quenchers, perform the role of the quencher. However, these do not nullify the fluorescence of longer wavelength dyes sufficiently. Hence, the use of alternative chromophores as quenchers, more efficient at long wavelengths, is discussed. We have designed and synthesised diaminoanthraquinone derivatives, which absorb in the 500-700nm region. The relative efficiency of quenching of a variety of fluorophores has been determined in a number of different probe systems.

Stabilisation of the interaction between an oligonucleotide and its specific complementary sequence, without increasing non-specific binding, is also investigated. The stabilities of oligonucleotide duplexes containing a variety of nucleoside modifications have been studied by UV-melting. Synthesis of a novel 2'-aminoethyl-2'-deoxyuridine nucleoside is discussed.

CONTENTS

Abbreviations

A	adenosine
Abs.	absorbance
Ac	acetyl
2'-aeo-dU	2'-aminoethoxy-2'-deoxyuridine
2'-ae-dU	2'-aminoethyl-2'-deoxyuridine
AIBN	azobisisobutyronitrile
AMA	ammonium hydroxide/methylamine solution
Anhyd.	anhydrous
aq.	aqueous
Ar	aryl
BHQ	black hole quencher
bp	base pair
^{i}Bu	*iso*-butyl
^{t}Bu	*tert*-butyl
$^{t}BuNH_2$	*tert*-butylamine
C	cytidine
c.	Concentrated
$CDCl_3$	deuterated chloroform
CD_3OD	deuterated methanol
conc.	concentrated
CPG	controlled pore glass
Cy	cyanine dye
dA	2'-deoxyadenosine
DAP	diaminopurine (2-amino-2'deoxyadenosine)
DAQ	diaminoanthraquinone
DB	disperse blue 3
dC	deoxycytosine
^{acetyl}dC	acetyl protected deoxycytosine
DCM	dichloromethane
DEAD	diethyl azodicarboxylate
DEPT	distortionless enhancement phase transfer
dG	2'-deoxyguanosine
^{dmf}dG	dimethylformamide protected deoxyguanosine

DIBAL	diisobutylaluminium
DIPEA	diisopropylethylamine
DMF	*N*,*N*-dimethylformamide
DMAP	4-dimethylaminopyridine
DMSO	dimethyl sulfoxide
DMSO-d_6	deuterated dimethyl sulfoxide
DMT	4,4'-dimethoxytrityl
DMTCl	4,4'-dimethoxytrityl chloride
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
DMAP	4-*N*,*N*-dimethylaminopyridine
dR	2'-deoxyribose
dT	2'-deoxythymidine
dU	2'-deoxyuridine
EDC	*N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide
EDTA	ethylenediamine tetraacetic acid
Elem. Anal.	C,H,N elemental analysis
Em	emission
ES^+	electrospray mass spectroscopy (positive)
ES^-	electrospray mass spectroscopy (negative)
Et_3N	triethylamine
Et_2O	diethyl ether
EtOAc	ethyl acetate
EtOH	ethanol
eq	equivalents
Ex	excitation
FAM	6-carboxyfluorescein
FET	fluorescence energy transfer
Fmoc	9-fluorenylmethoxycarbonyl
FRET	fluorescence resonance energy transfer
G	guanosine
HEG	hexaethylene glycol
HEX	hexachlorofluorescein
HOBT	1-hydroxybenzotriazole

HPLC	high performance liquid chromatography
HRMS	high resolution mass spectrometry
hrs	hours
Int	intensity
IR	infra red
JOE	6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein
LCAA-CPG	long chain alkyl amine controlled pore glass
LNA	locked nucleic acids
M^+	molecular ion
MALDI-TOF	matrix-assisted laser desorption ionisation time of flight
Me	methyl
MeCN	acetonitrile
$MeNH_2$	methylamine
MeOH	methanol
MeRed	methyl red
Mins.	minutes
mmol	millimoles
MMT	4-monomethoxytrityl
Mpt.	melting point
MR	methyl red
mRNA	messenger RNA
NaOMe	sodium methoxide
NBS	*N*-bromosuccinimide
NEt_3	triethylamine
NMNO	*N*-methylmorpholine *N*-oxide
NMR	nuclear magnetic resonance
NH_4OAc	ammonium acetate
NHS	*N*-hydroxysuccinimide
N-type	northern ring pucker
OD	optical density unit
Oligo	oligonucleotide
PCR	polymerase chain reaction
p-dU	propyne-deoxyuridine
pa-dU	propargylamino-deoxyuridine

PG	protecting group
Ph	phenyl
PhOCSCl	phenyl chlorothioformate
PNA	peptide nucleic acid
POM	pyrrolidine-amide oligonucleotide mimic
PPh_3	triphenylphosphine
^{i}Pr	isopropyl
Pyr	pyridine
RNA	ribonucleic acid
ROX	carboxy-X-rhodamine
$Rh_2(OAc)_4$	dirhodium(II) tetraacetate
$Rh_2(TPA)_4$	dirhodium(II) tetra(triphenylacetate)
RP-HPLC	reverse phase high performance liquid chromatography
Sat.	saturated
sh	shoulder (in UV/Vis spectrum)
SNPs	single nucleotide polymorphisms
S-type	southern ring pucker
T	thymidine
TAMRA	tetramethylrhodamine
Taq	taq polymerase isolated from thermus aquaticus
TBAF	tetrabutylammonium fluoride
TBDMS	*tert*-butyldimethylsilyl
TBDMSCl	*tert*-butyldimethylsilyl chloride
TBDPS	*tert*-butyl diphenylsilyl
TBDPSCl	*tert*-butyl diphenylsilyl chloride
Temp.	temperature
TET	tetrachlorofluorescein
TFA	trifluoroacetate
THF	tetrahydrofuran
TIPS	tetraisopropyldisiloxane
TLC	thin layer chromatography
TMSCl	trimethylsilylchloride
T_m	melting temperature (temp. at which 50% duplex dissociation occurs)
TPA	tripropylamine

tRNA	transfer RNA
UV	ultraviolet light
w/v	weight to volume ratio
∴	therefore

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Finally a big thank you to Emma who has been very patient and understanding.

1. INTRODUCTION

1.1 Nucleic acids background

1.1.1 Nucleic acid structure

Nucleic acids are polymers of monomeric units called nucleotides. Nucleotides consist of three main components: a pentose sugar, a nitrogen containing heterocyclic base and a phosphate residue. Compounds containing just the sugar and base moieties are refered to as nucleosides. The sugar usually takes the form of either ribose or 2'-deoxyribose, hence the names: ribonucleic acid (RNA) and deoxyribonucleic acid (DNA). There are four nucleotides found naturally in DNA, each differing by the structure of the base moiety (figure 1.1.1).

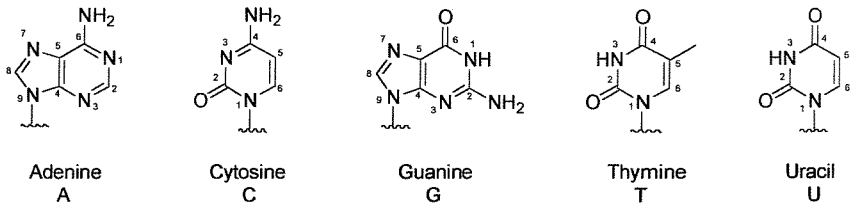


Figure 1.1.1: Nucleic acid bases. Thymine and uracil are found in DNA and RNA repectively.

The bases are functionalised pyrimidine and purine heterocycles. Adenine, cytosine and guanine are found in both RNA and DNA, but thymine, which is found in DNA, is replaced by uracil in RNA. The nucleotides are linked to form a nucleic acid chain *via* phosphodiester linkages at the 3' and 5' positions on the sugar (figure 1.1.2) and this gives the oligonucleotide its directionality. At physiological pH the phosphate moieties are deprotonated; hence nucleic acids are highly negatively charged and exist as salts.

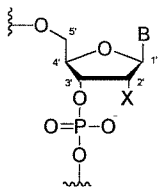


Figure 1.1.2: The sugar-phosphate backbone repeat unit, where B = base.

In the nucleoside structure, the furanose ring twists out of plane to minimise energy from steric and electronic interactions between atoms (figure 1.1.3). This 'puckering' is

described as either C2'-endo (Southern, S-type) or C3'-endo (Northern, N-type). The equilibrium between these two extremes of conformation is influenced by the preference of electronegative groups at C2' and C3' for an axial conformation, thus DNA adopts an S-type and RNA prefers an N-type conformation.

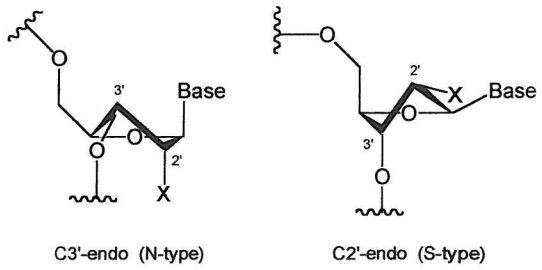


Figure 1.1.3: Furanose ring conformations. X = H (DNA); OH (RNA).

Two separate complementary strands of DNA will wind around each other following a helical pathway to form a "double helix".[1] The presence of this unique structure in DNA was determined by Watson and Crick, who famously published their work in 1953. In this double helix each DNA strand runs in the opposite direction to the other, i.e. the strands are anti-parallel (figure 1.1.4). The phosphates form a negatively charged external backbone, surrounding a core of stacked heterocylic bases.

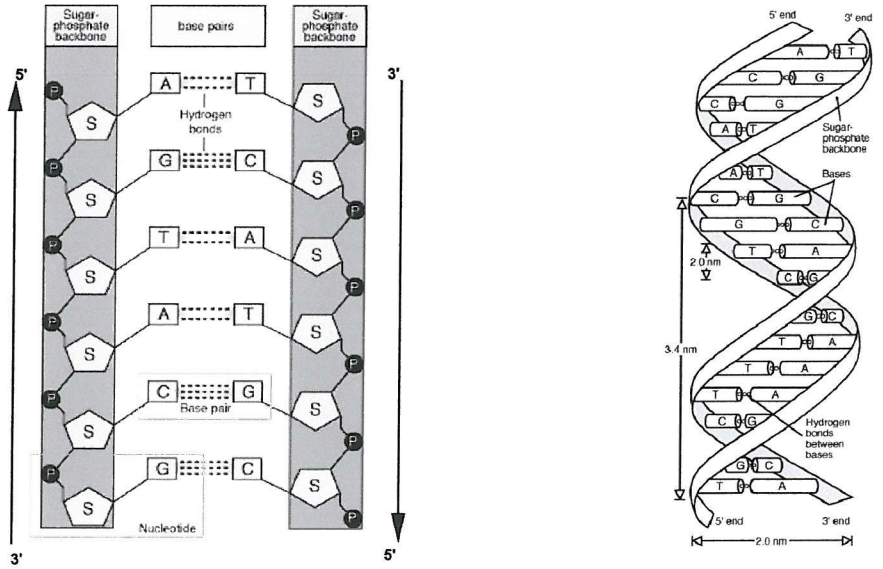


Figure 1.1.4: Schematic diagrams of DNA (B-type), showing the base pairing, anti-parallel strands and the double helix with major and minor grooves.

The helix coils in a "right-handed" fashion and generates major and minor grooves between the helical phosphate backbone. Bases of the two strands recognise each other in complementary sequences by hydrogen bonding to form "base pairs". Two types of base pair occur in DNA: G.C and A.T. (figure 1.1.5) as deduced from Chargaff's work in the early 1950's.[2]

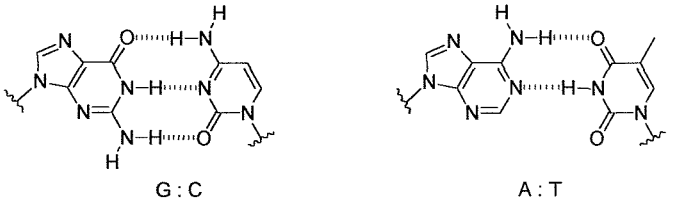


Figure 1.1.5: The G.C and A.T base pairs.

There are between ten and eleven base pairs in one complete turn of the DNA helix. The stability of this structure is not only due to these hydrogen bonds, but is also dependent on a number of contributory factors including stacking, electrostatic and hydrophobic interactions of the bases, both inter and intra-strand.[3]

X-ray diffraction studies have shown that there are two predominant conformations of DNA/RNA helices, although there are others that are less common.[4] These two forms are denoted as A-type and B-type conformations. In general RNA will adopt an A-type duplex and DNA will adopt a B-type duplex. This is related to the furanose ring puckers of each. A C3'-endo pucker is found in A-type and a C2'-endo conformation is observed in B-type helices.[5] Although there is certainly a relationship between helix and sugar conformation, it may not be the ring pucker that determines the helical conformation. It has been suggested that the conformation of the helix is partly determined by the energetics of base-stacking and so thatthe sugar conformations are largely inconsequential.[3]

1.1.2 Oligonucleotide synthesis

Oligonucleotides must be synthesised using very mild reaction conditions to avoid side reactions. Specific coupling of two nucleosides to form an internucleoside 3' to 5' phosphodiester linkage can occur, providing there is good protection of all other nucleophilic sites (i.e. the 1° amines in the bases of dA, dC and dG). The 5'-hydroxyl group of the sugar must be protected and the most widely used protecting group is the 4,4'-

dimethoxytrityl group. This can easily be removed by acidic treatment with only a small degree of depurination.

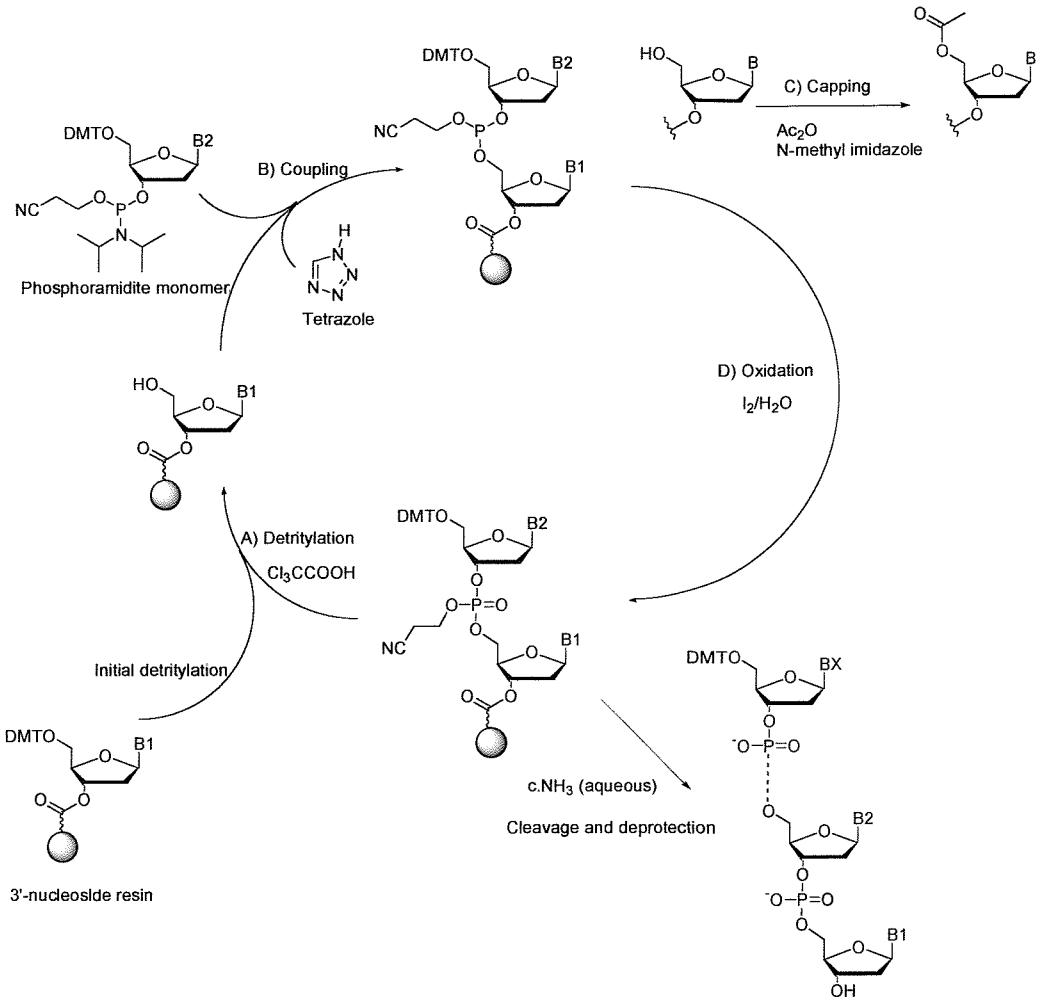


Figure 1.1.6: Oligodeoxynucleotide synthesis cycle. DMT = dimethoxytrityl.

The most common form of oligonucleotide synthesis is the phosphoramidite method, introduced by Marv Caruthers.[6-9] Good coupling yields (~99%) can be achieved due to the sterically unhindered phosphorous(III) chemistry involved in the coupling step (later oxidised to P(V)). The phosphoramidite group contains a diisopropylamino moiety, which has an ideal balance of reactivity and stability. This compound is stable at neutral pH, but when protonated at the amino nitrogen, the phosphorus becomes very reactive due to the strong electron withdrawing effect of the protonated amine. Displacement of the di-isopropylamine group by tetrazole can also occur, giving rise to a reactive tetrazolide.

Phosphoramidite monomers are used for large-scale synthesis using automated, solid-phase methods (figure 1.1.6).

1.1.3 The central dogma of molecular biology

Human DNA is found in 23 chromosomes and contains ca. of 3 x 10^9 base pairs. 95% of this material is found in the regions known as introns and does not code for proteins, whilst the other 5% is found in regions called exons. These exons form what are known as "genes" and it is these regions which code for proteins. A single gene can be as much as 2 million base pairs long.

The flow of genetic information is normally from DNA to RNA to protein. This is described as the central dogma of molecular biology and is represented in the schematic below (figure 1.1.7).

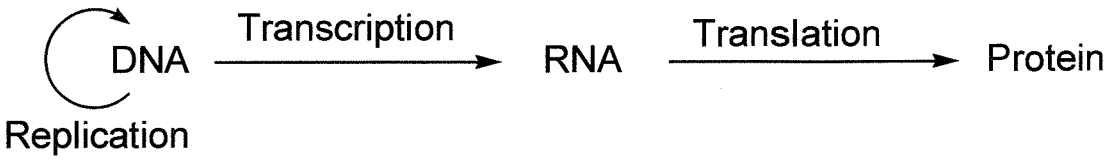


Figure 1.1.7: The flow of genetic information from DNA to protein.

DNA acts as the storage medium and can be replicated easily for cell division or repair. This DNA can also be "transcribed" to produce lengths of single-stranded mRNA (messenger RNA), which in turn can be recognised by tRNA (transfer RNA) at the ribosomes in the process known as "translation", leading to the formation of proteins. Each amino acid is coded by a group of three nucleotides, collectively called a codon. There are 64 non-overlapping codons which constitute the "genetic code" (61 code for amino acids, 3 are stop codons). There are only 20 natural amino acids, so there are many codons that are degenerate.

1.1.4 Genetic disorders and diagnostics

There are many diseases that can be identified by screening at the genetic level. These fall into two categories: 1) Mutations to the host genome (e.g. sickle cell anaemia,[10] cystic fibrosis,[11-16] phenylketonuria[17,18] and Huntington's disease[19]); 2) Detection of the genetic of a pathogenic organism[20,21] (e.g. measles, rubella, HIV,[22] Hepatitis A & B, salmonella,

candida). The first group are all genetic disorders originating from mutations during cell division. These can then be passed from parent to child. When cell division takes place the DNA is replicated.[23] Although this process is very accurate, errors are made and many mutations may occur in an individual genome during a lifetime. These mutations can be beneficial (resistance to a disease) and/or detrimental. These mutations may cause cancer[24,25] or cause modifications to germ cells, leading to inherited genetic conditions, which take the form of autosomal dominant, autosomal recessive or X-linked disorders. Every human has two copies of each chromosome, one inherited from each parent. These 22 chromosome pairs are called autosomes. There are also two sex chromosomes, X and Y. A female has two copies of X, whilst a male has a copy of each of X and Y. If a person inherits a single copy of a chromosome containing a dominant disorder then the disease will develop. In the case of a recessive disorder, two copies of the faulty chromosome are needed, i.e. both parents must have a copy of the faulty chromosome. If just one chromosome containing a recessive disorder is inherited then the progeny will become a carrier. X-linked chromosomal disorders usually affect males, because they only have a single copy of the X chromosome.

Utilising the power of a single strand of DNA to recognise its complementary copy, genetic probes have been developed, which are capable of identifying nucleic acid sequences associated with certain diseases or disorders. In order for DNA probes to be effective they should be highly specific, have low detection limits and be simple to prepare.[26] In addition, DNA probe technology has been used in forensic applications and criminal investigation, employing the technique of "DNA fingerprinting". This exploits the highly variable and hence almost unique minisatellite regions of the genome to yield a "bar code" for a particular individual.[27] This information can be used instead of blood group or conventional fingerprint details for identification purposes.

1.1.5 The polymerase chain reaction (PCR)[28-32]

The polymerase chain reaction (PCR) is an *in-vitro* method of DNA amplification. It is almost always the case that a sample of DNA is not enough material to study, so this method of amplification has proved essential for genetic, diagnostic and forensic applications.

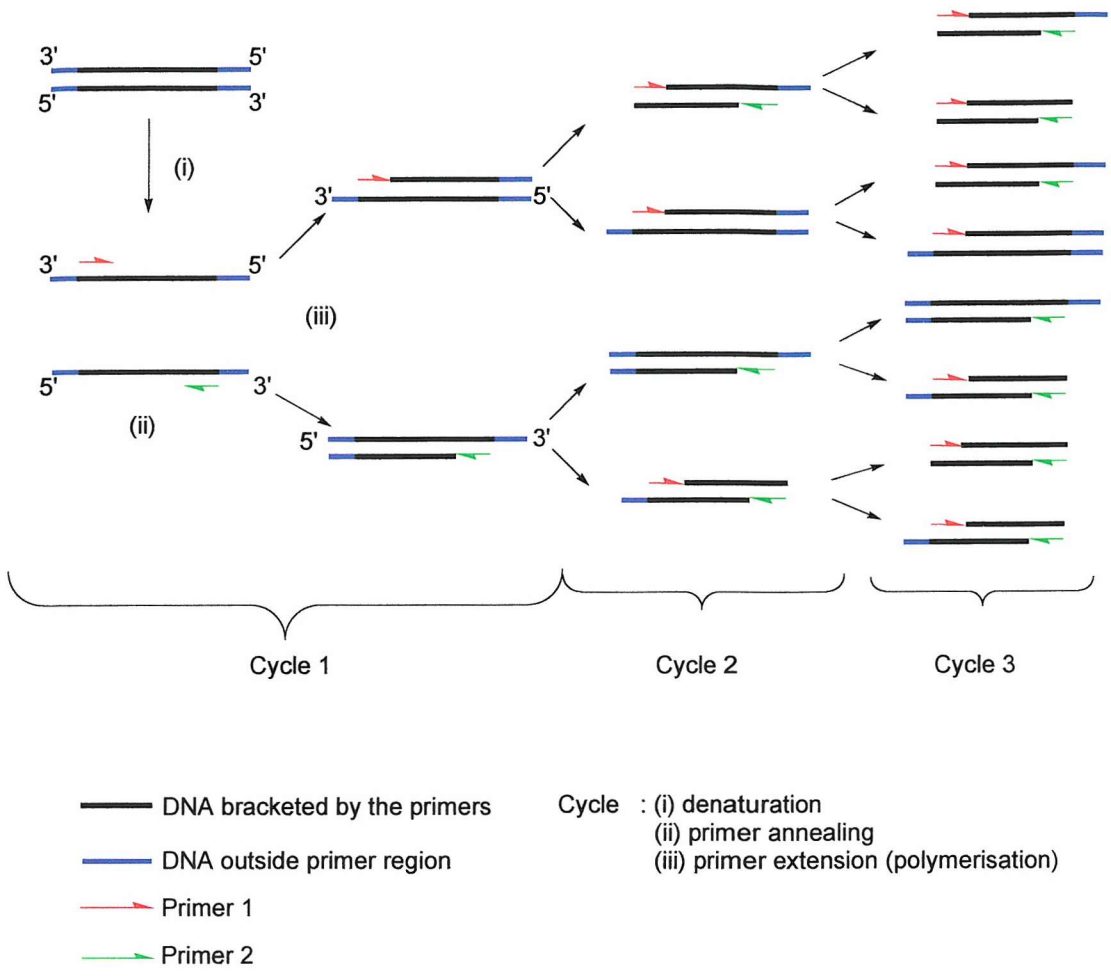


Figure 1.1.8: Schematic diagram of the polymerase chain reaction.

The PCR cycle (figure 1.1.8) begins by heating the target DNA duplex to 95°C (i). The temperature is then lowered to 55°C to allow the primers (two short oligonucleotides which flank the area to be amplified) to anneal to the target strands (one primer per strand) (ii). The temperature is then increased to 72°C allowing the thermostable DNA polymerase, *Taq* (isolated from *thermus aquaticus*),[33] to extend the target region utilising the deoxynucleoside triphosphates present (iii). The second cycle then begins and the newly synthesised strands denature at 95°C. Again annealing and extension steps take place, but extension can only go as far as the other primer in all newly synthesised strands. This is repeated and after three cycles, two double stranded sections of amplified target DNA will have been synthesised. Providing the extension product is long enough, it will include the sequence complementary to the primer at the other end of the sequence. Hence, each extension product can act as a template for the next cycle. This leads to an exponential increase in the number of strands of the PCR product with each cycle and after

20 cycles there are more than a million times the number of copies of target sequence. PCR amplification is very rapid and highly efficient.

1.2 Oligonucleotide probes

Oligonucleotide probes can be used to detect a specific sequence of nucleic acids, such as that of a particular genetic disorder or disease.[26] Probes must be specific enough to detect single nucleotide polymorphisms (SNPs).[34] The probe must contain a label (often a radioisotope or a luminescent tag), which will allow identification of the recognised sequence. A label must release a signal, allowing differentiation between hybridised and non-hybridised states of the probe. Labels should also be cheap, easy to attach to the oligonucleotide, stable to long term storage and conveniently disposable.

A typical label consists of three units:

1) a signal moiety – often luminescent molecule or radioisotope
2) a linker – must not interfere with signal moiety and controls flexibility,[35] e.g. alkyl chain
3) a reactive group for attachment – forms a covalent linkage to the oligonucleotide under mild conditions, for example:
    - NHS (*N*-hydroxysuccinimide) esters,[36] isothiocyanates,[37] – react with amines in oligonucleotide, post-synthesis
    - Nucleotide triphosphates – incorporated enzymatically[38]
    - Phosphoramidites – incorporated during oligonucleotide synthesis[39]

There are two methods of labelling: direct labelling and indirect labelling. The direct labelling method attaches a reporter directly to the oligonucleotide. This label must not interfere with the binding of the oligonucleotide to its target (figure 1.2.1).

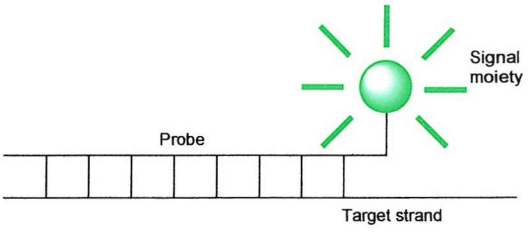


Figure 1.2.1: Direct labelling.

Indirect labelling requires an oligonucleotide to be functionalised with a moiety that will bind to a highly specific binding protein (e.g. hapten, antibody, biotin/streptavidin).[40,41] In turn, the protein is linked to a signaling moiety. A signal is produced at the probe, post-

hybridisation (figure 1.2.2). However, a problem with this method is the high level of non-specific binding of the protein and therefore background signal.

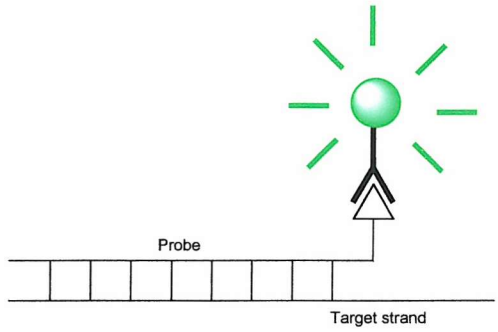


Figure 1.2.2: Indirect labelling.

The signal moiety is the most important part of a label. The most commonly used signalling moieties are radioisotopes and fluorescent dyes. The earliest oligonucleotide probes were tagged with radioactive labels.[42] These tags often took the form of radiolabelled dNTPs which were incorporated enzymatically.[43,44] Many isotopes have been used, including ^{3}H, ^{125}I, ^{14}C, ^{32}P, ^{35}S.[45,46] A major advantage of this technique is high sensitivity, but disadvantages include expense, short isotope half-lifes, hazards in handling and disposal.

To avoid these drawbacks other labelling methods were developed including colourimetric,[40] chemiluminescent,[47] bioluminescent[48] and in particular fluorescent labels.[49] Fluorescent labels, although not as sensitive, avoid the problems associated with hazardous radio-isotopes and in addition, allow many different labels to be used in one experiment simultaneously. [50,51]

1.3 Fluorescence[52]

1.3.1 Introduction to fluorescence

Fluorescence is a three-stage process that certain atoms and molecules (fluorophores) go through when light of a particular wavelength is incident upon them (figure 1.3.1). This process is:

- Excitation (1) – a photon of energy $h\nu_{EX}$ is absorbed by the fluorophore and promotes an electron to an excited state ($S_0 \rightarrow S_1'$).
- Relaxation (2) – the excited state exists for a finite time ($< 10^{-8}$s). During this period the fluorophore may rearrange or interact with its environment to produce a relaxed excited state ($S_1' \rightarrow S_1$) (2).
- Fluorescence emission (3) – a photon of energy $h\nu_{EM}$ is emitted and in doing so the fluorophore returns to the ground state ($S_1 \rightarrow S_0$)

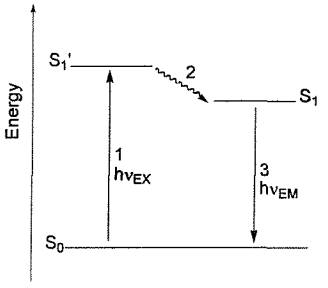


Figure 1.3.1: Jablonski diagram.

During the lifetime of the excited state there are many alternative ways the energy may be dissipated other than by fluorescence. These include collisional quenching, fluorescence energy transfer and inter-system crossing to produce phosphorescence. The degree to which these other processes take place controls the fluorescence quantum yield of the fluorophore (i.e. ratio of the number of photons emitted by fluorescence, to the number of photons absorbed). The energy of the emitted photon will be less than that of the one initially absorbed, because of the relaxation in excited state and partial dissipation of the excited energy. Hence the energy $h\nu_{EM}$ is always less than $h\nu_{EX}$ and correspondingly, the emission wavelength is always longer than that of the absorption wavelength. This difference in energy is called a Stoke's shift and fluorescent dyes with a large Stoke's shift allow detection of their fluorescence emission above a low background (i.e. no excitation photon's present).

1.3.2 Fluorescence resonance energy transfer [53-56]

Fluorescence resonance energy transfer (FRET) is the transfer of energy between two molecules by induced-dipole interactions, without the emission of a photon (figure 1.3.2). Hence molecule A will absorb the incident photon and an electron will be promoted to the excited state S_1. The energy of this excited state is then transferred to an excited state in molecule B. Molecule B then emits the energy of its excited state as a photon to return it to its ground state and in doing so fluorescence is observed.

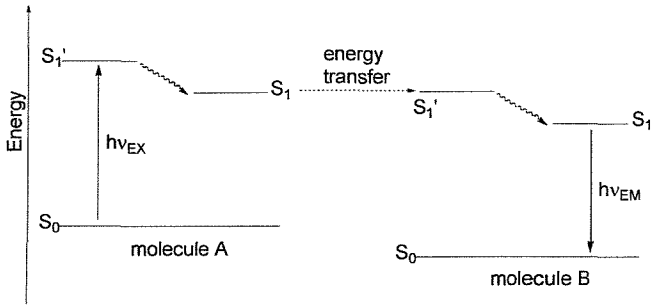


Figure 1.3.2: FRET. Molecule A = donor, molecule B = acceptor.

For FRET to take place there are a few criteria which must be met:

- The donor and acceptor molecules must be in close proximity (typically 10-100Å)
- The absorption spectrum of the acceptor must overlap with the fluorescence emission spectrum of the donor
- The donor and acceptor transition dipole orientations must be approximately parallel

The efficiency of FRET falls off rapidly ($1/r^6$) with separation between the donor and acceptor. The distance at which energy transfer is 50% efficient is called the Förster radius (R_0). These terms are related in the following equation:

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

where: k_T = rate of energy transfer; τ_d = lifetime of the donor in the absence of the acceptor; R_0 = Förster distance; r = separation between donor and acceptor.

Thus energy transfer can be used to study biological phenomena which produce changes in molecular proximity.[57,58 59] This includes study of the DNA duplex[60] and triplex[61] and protein denaturation.[62]

1.3.3 Fluorescence quenching[63]

Fluorescence quenching refers to processes that decrease the fluorescence intensity of a substance and hence the quantum yield. There are several routes (figure 1.3.3) that quenching of fluorescence may take:

- Excited state reactions
- Energy transfer
- Complex (static) quenching
- Collisional quenching

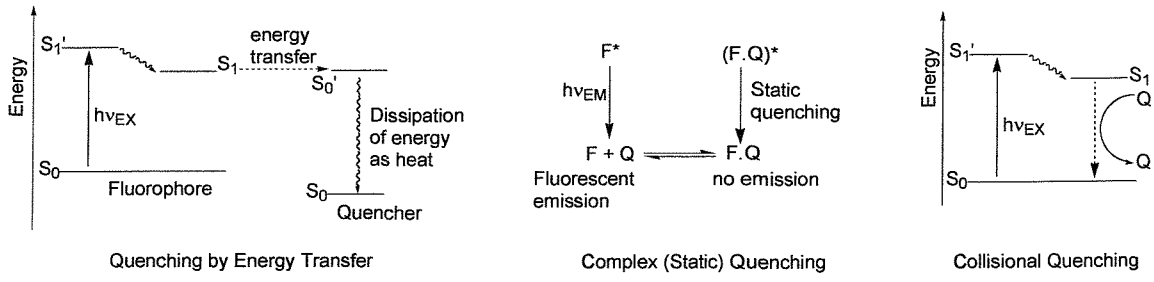


Figure 1.3.3: Schematics representing three methods of quenching: i) Quenching by energy transfer; ii) Complex (static) quenching; iii) Collisional quenching.

Excited state reactions are self-explanatory, in that electrons in an excited state may react chemically to form a new compound and in doing so lose their excited state energy and thus the possibility of fluorescence is diminished.[64,65] Quenching by energy transfer is similar to the FRET process. The excited state energy is transferred to another molecule, but instead of this being re-emitted as fluorescence, the energy is dissipated by non-radiative pathways. Complex or static quenching involves the fluorophore and quencher forming a non-fluorescent complex; hence this acts as a quenching mechanism. Finally, collisional quenching is where the fluorophore and quencher collide during the lifetime of the fluorophore excited state. In doing so, the excited state energy is dissipated as heat and the fluorophore returns to its ground state.

1.3.4 Fluorophores for use with oligonucleotides

There are many fluorescent dyes on the market, but the most common fall into four basic types, dependent on their structure. Some of the most common fluorescent dyes are those based on fluorescein (FAM, 6-carboxyfluorescein).[66,67] There are many derivatives (figure 1.3.4), including the chlorinated analogues TET, HEX and JOE (4',5'-dichloro-2',7'-dimethoxy-6-carboxyfluorescein). In the rhodamine family anilines replace the phenols: 6-ROX (6-carboxy-X-rhodamine),[68] 6-TAMRA (6-carboxytetramethylrhodamine)[69]. These fluorescent dyes have extinction coefficients in the region of 70,000$cm^{-1}M^{-1}$, but are pH dependent.[70]

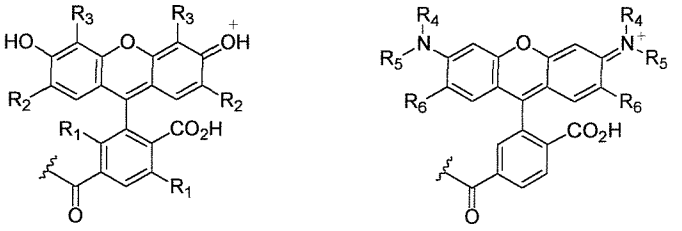


Figure 1.3.4: Left: Fluorescein structure. FAM (R_1, R_2, R_3 = H), TET (R_1, R_2 = Cl, R_3 = H) HEX (R_1, R_2, R_3 = Cl), JOE (R_1 = H, R_2 = OMe, R_3 = Cl). Right: Rhodamine structure: ROX (R_4 = H, R_5 = Et, R_6 = Me), TAMRA (R_1, R_5 = Me, R_6 = H).

An alternative to fluorescein and its derivatives are the cyanine family of dyes.[38,71] These all have the generic structure in figure 1.3.5, but by altering the length of the conjugated alkenyl chain and changing the functionality on the two aromatic moieties, the properties can be tailored accordingly. An advantage of these dyes is the large extinction coefficients (~200,000$cm^{-1}M^{-1}$) and relative stability to pH.

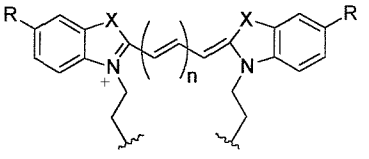


Figure 1.3.5: Structure of a cyanine dye. Cy3 (R = H, X = $C(Me)_2$, n = 1), Cy5 (R = H, X = $C(Me)_2$, n = 2), Cy5.5 (R = fused Ph, X = $C(Me)_2$, n = 2).

BODIPY dyes (figure 1.3.6) are also available with a range of fluorescent properties.[51,72] These dyes can be functionalised with a variety of different groups (at position R), allowing derivatives to cover the majority of the visible spectrum (figure 1.3.6). Extinction coefficients range from 60,000-140,000$cm^{-1}M^{-1}$ and these dyes are not sensitive to pH.

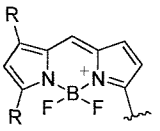


Figure 1.3.6: Structure of the BODIPY dye skeleton. R = Me, Ph, Thiazole, MeOPh.

Molecular Probes, Inc. have recently introduced a whole new range of fluorophores known as the Alexa dyes. These fourteen dyes span the entire visible spectrum, with emission wavelengths from 400nm to 775nm and extinction coefficients ranging from 16,000-240,000 $cm^{-1}M^{-1}$. However they are relatively expensive and are only available as NHS esters for amino modification. Their structures are related to the fluorescein and rhodamine family, with a large variety of additional substitutions to the conjugated ring systems.

The absorption and emission wavelengths together with extinction coefficients are shown for a selection of the fluorescent dyes discussed (figure 1.3.7).

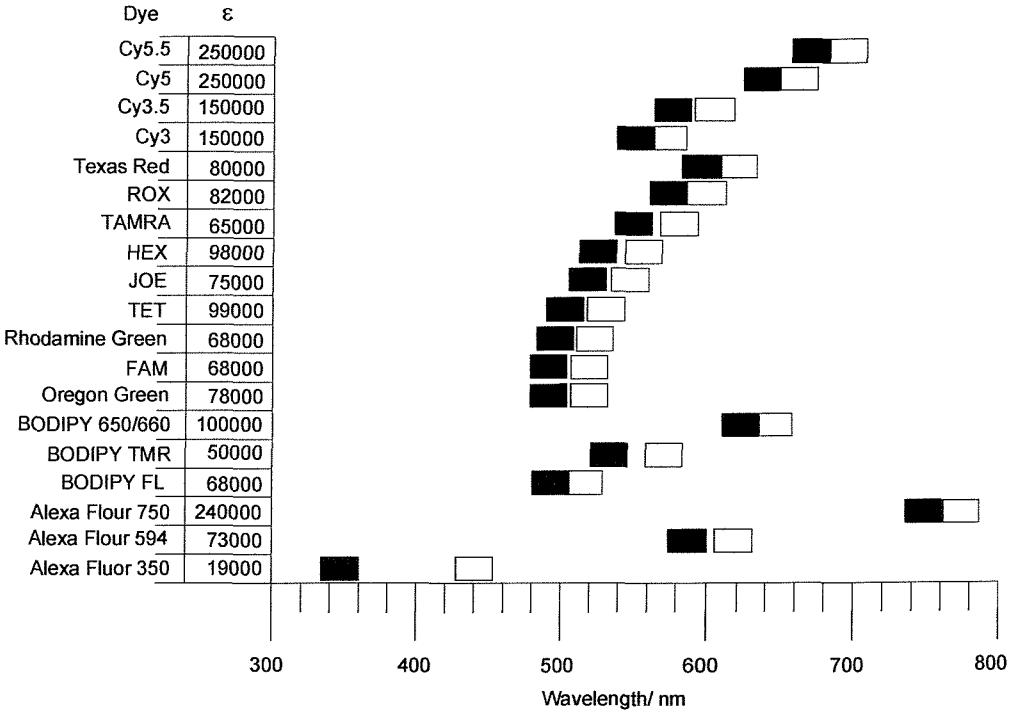


Figure 1.3.7: Fluorescent dyes with their extinction coefficients ($cm^{-1}M^{-1}$) and wavelengths of absorption and emission. ■ = Absorption wavelength; □ = emission wavelength.

1.3.5 Quenchers for use with oligonucleotides

Until recently the diazo dye DABCYL has usually been used as a "universal quencher" in oligonucleotide probes.[73] Long-wavelength fluorescent dyes, such as TAMRA, have also been used as quenchers in some probe systems, but these dyes still emit fluorescence (at a long wavelength) so are not true "dark" quenchers (i.e. no native fluorescence).[74] In the last few years there have been more developments in this emerging field and now many new quenchers are available, which cover the entire visible spectrum.

DABCYL (*p*-(dimethylaminophenylazo)benzoic acid) and methyl red are both diazo-dyes and are the original quenchers for molecular beacons.[73] The only difference between these is that DABCYL is a *para* derivative and methyl red is the *ortho* derivative (figure 1.3.8).

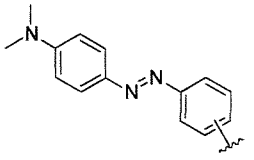


Figure 1.3.8: DABCYL / methyl red. Abs_{max}: 453nm; $\varepsilon = 32000 cm^{-1}M^{-1}$.

Molecular Probes, Inc. provide the QSY series of quenchers.[36,72] These are based upon a fluorescein-type structure (figure 1.3.9), but by varying the substituents, the dyes have been engineered to be non-fluorescent and absorb at a range of wavelengths.[75]

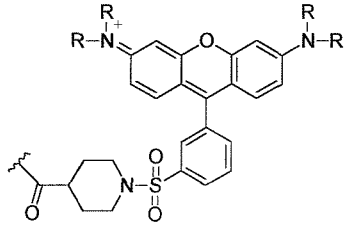


Figure 1.3.9: QSY quenchers. $\varepsilon = 23000 - 92000 cm^{-1}M^{-1}$.

Finally, Biosearch Technologies have released a series of quenchers called Black Hole Quenchers (BHQ). Although there are three quenchers in this family, which together span the entire visible spectrum, only BHQ-1 and BHQ-2 are generally available at the time of writing (figure 1.3.10).[76]

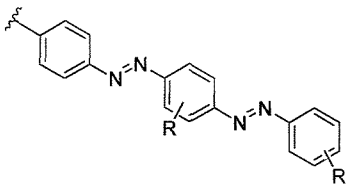


Figure 1.3.10: Common backbone structure to Black Hole Quenchers. ε = n/a.

1.3.6 Other luminescent labels

Lanthanides have been used to generate luminescent emissions with large Stoke's shifts, in addition to being time resolved (i.e. a slight time delay between excitation and emission). The time resolved nature of this emission leads to lower levels of background interference. Lanthanides are attached to DNA by a chelating ligand of some kind. This can be an EDTA, bipy, porphyrin or a simple poly-carboxylate or amino branch structure.[77,78]

Quantum dots[79] are another type of fluorescent label. They consist of a particle of cadmium selenide of nanometer dimensions, which is coated with a shell of zinc sulfide. Quantum dots have a large absorption band and narrow emission, which can be tuned according to the size of the CdSe particle. Emissions in the range of 500-650nm are most common. Advantages include pH stability and resistance to photobleaching, but there may be problems due to their size (relatively large) in some applications.

1.4 Fluorescent probe systems

1.4.1 Hybridisation probes

Hybridisation probes have been used for many years to detect specific oligonucleotide sequences.[80-82] Generally they take the form of two oligonucleotides which are complementary to a target strand. The two oligonucleotides hybridise to the target in such a way as to create a relatively small gap (~ 4 b.p.) between the 3'-end of one probe strand and the 5'-end of the other (figure 1.4.1). These ends are functionalised with either a donor or acceptor molecule (e.g. fluorescein, rhodamine). When a complementary target is present the hybridisation probes bind and fluorescence energy transfer takes place between the donor and acceptor molecules. This hybridisation event can be studied as a decrease in donor fluorescence intensity and an increase in acceptor fluorescence intensity. Hybridisation probes have been used to study the real-time formation of PCR product.[83]

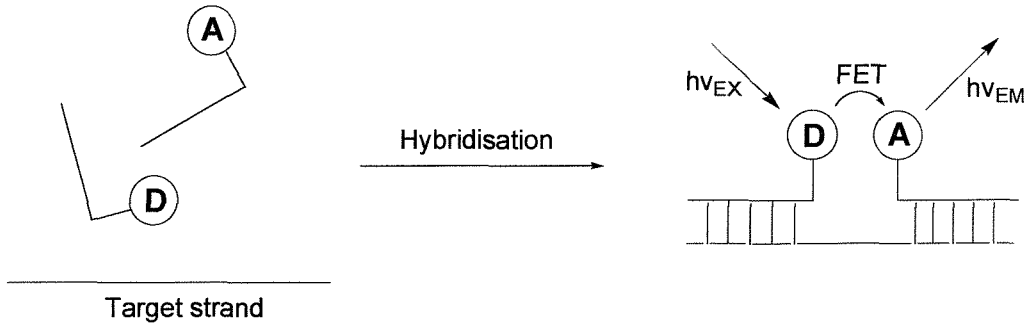


Figure 1.4.1: Hybridisation probes method of action.

1.4.2 Molecular beacons

Molecular beacons are single stranded oligonucleotide probes that possess a stem-loop structure.[73,84] The loop section contains a complementary sequence to the desired target strand. The stem consists of two complementary arm sequences (at either end of the loop section) which anneal together to produce a characteristic "hairpin" structure (figure 1.4.2). At either end of this oligonucleotide strand a fluorescent moiety and quencher molecule are attached. Hence, whilst in a hairpin conformation, the close proximity of the fluorophore and quencher allows energy of the fluorophore to be transferred to the quencher and to be dissipated as heat, so that no fluorescence is observed. When a target strand is introduced the loop section is able to form a duplex which is longer and more stable than that of the stem. Hence, the stem melts and the two arms are separated by the

hybridised loop section. In turn this separates the fluorophore and quencher moieties, allowing the fluorescence from the fluorophore to be observed.

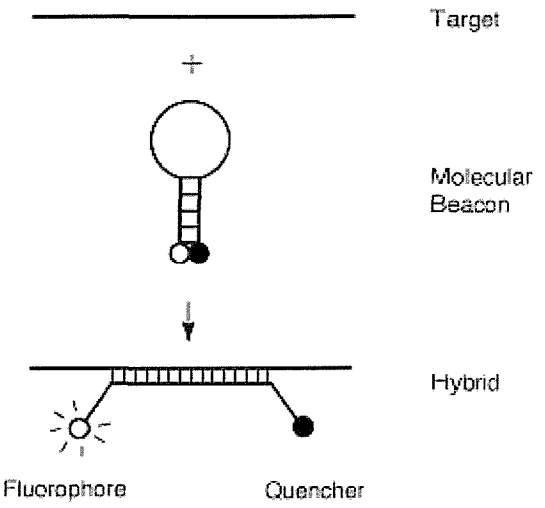


Figure 1.4.2: Molecular beacon mechanism.

Molecular beacons must be designed so that the duplex formed by the stem section is less stable than that formed by the probe loop section annealing to its complementary target. However, it must be stable enough to retain the hairpin loop structure in the absence of a complementary target sequence. Highly specific beacons may be designed in this way, usually with stems in the range of 4-12 base pairs long and loop sections from 17 to 35 nucleotides in length.

Molecular beacons are suitable for homogeneous detection of single point mutations and by employing several beacons each with a unique fluorescent label, multiplexing assays can be performed. Molecular beacons have been used to detect RNAs within living cells[85] and for genotyping single-nucleotide variations in DNA.[86-90] They have also been used in multiplex PCR studies.[91] Molecular beacons have been attached to a solid-phase for immobilised hybridisation studies[92,93] and have been used to study protein-DNA interactions.[94]

1.4.3 TaqMan® probes

The TaqMan® assay is a popular method for detection of real-time PCR product.[74,95,96] It gets its name from the *Thermus aquaticus* (*Taq*) DNA polymerase enzyme used in the PCR reaction, with its inherent 5'→ 3' exonucleolytic activity. A TaqMan® probe consists of an oligonucleotide modified at the 5'-end with a fluorophore and the 3'-end with a quencher. This oligonucleotide is complementary to a section of the PCR template strand. The probe is designed so that the fluorophore is quenched when the probe is intact. During PCR, the probe will hybridise to the template strand and *Taq* polymerase will cleave the probe due to its exonucleolytic activity (figure 1.4.3). This cleavage results in the separation of the fluorophore and quencher moieties, and an increase in fluorescence is observed. As more PCR product is generated, more TaqMan® probes will be cleaved hence an increase in fluorescence builds up over a number of PCR cycles, which directly corresponds to the formation of specific PCR product.

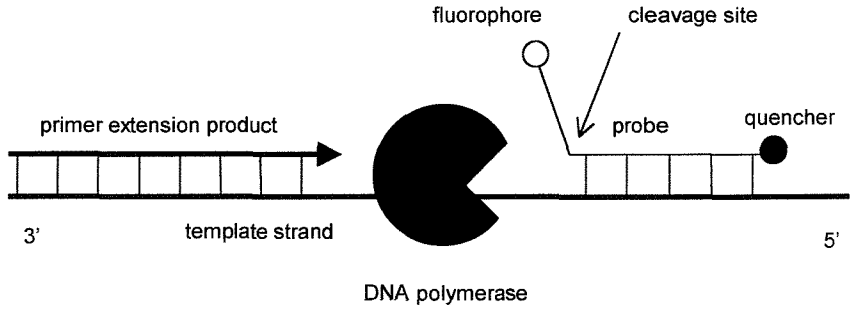


Figure 1.4.3: TaqMan® schematic.

TaqMan® systems have been used to study quantitative PCR[97,98] and the lower limits of detection on the nanolitre scale.[99]

1.4.4 Scorpion primers

These are unimolecular fluorescent probes, consisting of primer and probe elements, designed for efficient, homogeneous detection of PCR amplicons.[100,101] The basic design can be described as a fluorescent probe (similar to a molecular beacon), attached to the 5'-end of a PCR primer, *via* a linker/blocker section. This linker prevents copying through the probe element of the scorpion. The probe is designed to hybridise to its target only

when the target has been incorporated into the same molecule by PCR extension of the primer element of the scorpion (figure 1.4.4).

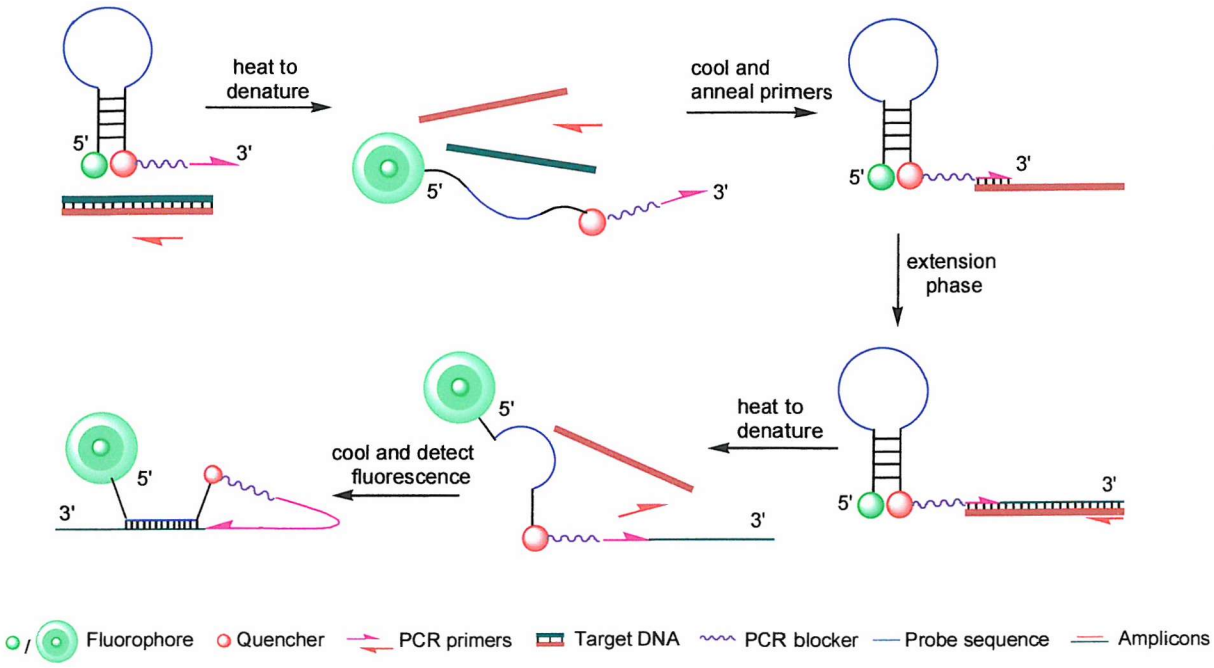


Figure 1.4.4: Mechanism of action for a stem-loop scorpion primer.

The intramolecular nature of the scorpion primer probing mechanism leads to more favourable kinetics than for bimolecular probes such as molecular beacons. The speed of this unimolecular process lends itself well to rapid PCR assays, where equilibration times are short.[102] Scorpion primers have low background levels of fluorescence, because while unextended they are unable to fluoresce. A precise 1:1 ratio of probe to target is guaranteed. In addition scorpions are highly specific and can discriminate at the level of a single base mismatch.[102]

Recent developments in this field have lead to a new version of scorpion primer, adopting a duplex format (figure 1.4.5). These retain the intramolecular probing mechanism, but instead of the stem-loop hairpin structure, there is simply a duplex consisting of the fluorogenic probe/primer in one strand and a complementary strand containing the quencher. The sensitivity of the probe can be adjusted by changing the length (stability) of the quencher oligonucleotide.

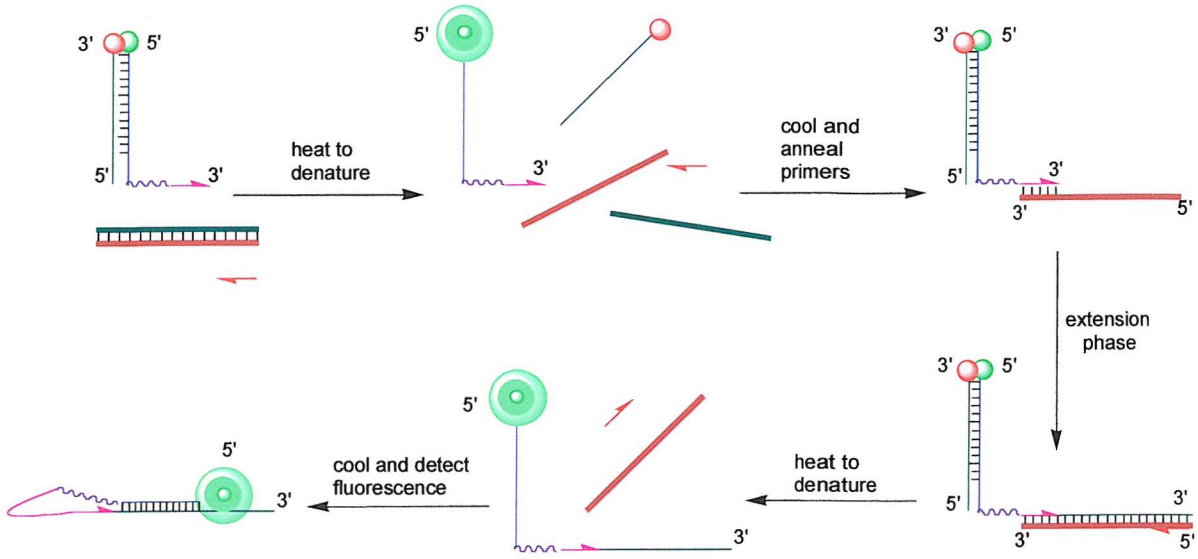


Figure 1.4.5: Mechanism of action of a duplex scorpion primer.

There are two main advantages of the duplex format: greater fluorescence signal and ease of synthesis. The intensity of the fluorescent signal is much greater, due to the vastly increased separation between fluorophore and quencher in the "on" state, and they are simpler to synthesise, because the quencher and fluorophore are in separate oligonucleotides.[103]

1.5 Duplex stabilisation

The stabilisation of DNA and RNA hybrids is an important area in nucleic acid research and has been for many years. Alterations to the stability of duplexes have been achieved using modifications of the nucleotide base, sugar and phosphate backbone. In addition, the introduction of intercalators and minor groove binders have been used, displaying great changes in duplex stability. Stabilisation of duplex and triplex structures has been important for antisense[104] and antigene[105] strategies. In a similar manner duplex stabilisation can introduce benefits to oligonucleotide probe technologies. Increasing the stability of an oligonucleotide probe means that the probe can be made shorter for the same melting temperature. In turn, a shorter length probe would cause a mismatch to have a greater destabilisation effect and thereby the specificity of the probe can be increased. The fidelity of the probe is also of fundamental importance, so any modifications must not increase non-specific binding of the oligonucleotide, but purely increase binding to a complementary target. In other words, a balance is needed between outright duplex stability and specific recognition of an oligonucleotide by a complementary sequence.

1.5.1 UV-melting

This is a spectroscopic technique used to study the stability of nucleic acid duplexes. It is reliant on the absorption of nucleic acids in the ultraviolet region of the electromagnetic spectrum. The heterocyclic base moieties absorb in the ultraviolet region between 240-260nm. The extinction coefficient varies according to the local environment. In an ordered nucleic acid duplex structure the bases are stacked efficiently, with greater π-π interactions, leading to lower UV absorption than that observed in unhybridised single strands. This effect is known as hypochromicity and can be used as a measure of the base pairing between two oligonucleotides. Hypochromicity is due to electronic interactions between neighbouring bases: when the chromophore is excited, a dipole forms as a result of the electronic transition. In turn this dipole induces opposing dipoles in neighbouring chromophores. This effect is much greater in a highly rigid base stacked duplex structure than in a highly disordered single nucleic acid strand (hypochromicity is proportional to the inverse cube of the base separation). These induced dipoles oppose the inducing dipole and create an overall reduction in the transition dipole and hence a lower absorbance is observed (lower extinction coefficient).

In the technique of UV melting the phenomenon of hypochromicity is put to use to signal a change from double stranded to single stranded nucleic acids. Increasing the temperature causes the duplex to gradually unwind and melt and in doing so the UV absorbance increases.

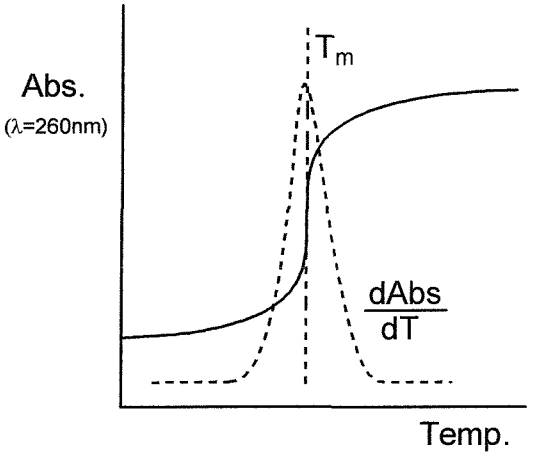


Figure 1.5.1: Diagram of a UV-melting curve (solid curve), showing the first derivative (dotted curve) and T_m (dAbs/dT = 0).

A plot of absorbance versus temperature yields a sigmoidal curve (figure 1.5.1), the midpoint (first derivative maximum) of which signifies the melting temperature (T_m), defined as the temperature at which half of the duplexes have dissociated. Melting temperature values (for a particular buffer) are used to compare relative duplex stabilities.

1.5.2 Base modifications

There have been many attempts at modifying nucleic acid bases to achieve duplex stabilisation. Some of these are discussed below.

One of the simplest modifications to a pyrimidine base is the introduction of a methyl group at the C5 position. Indeed, in the case of uracil, *nature* has already introduced this modification in the form of thymine (found solely in DNA). However, the equivalent modification can be made to cytosine (figure 1.5.2) and a small stabilisation of the duplex melting temperature (1.3°C) is observed (5-methyl-dC is relatively rare in nature).[106] A further increase in stabilisation is observed for the incorporation of propyne at the C5 position of pyrimidines (figure 1.5.2).[107] This propyne moiety has two positive effects: 1)

Increased base stacking through additional conjugation of the heterocyclic base π electron cloud; 2) Increased entropy by release of water molecules from the duplex into the bulk solvent. However, if longer chain lengths are used there is a noticeable destabilisation of the duplex due to the increased steric bulk of the larger groups.[108,109]

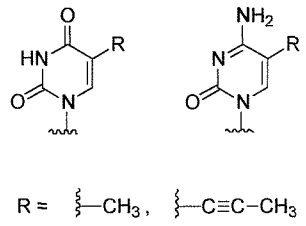


Figure 1.5.2: C5 methyl and propyne modifications to pyrimidine bases.

Propynyl modifications have also been investigated with the purine bases. 8-Propynyl-2'-deoxyguanosine and 8-propynyl-2'-deoxyadenosine (figure 1.5.3) have been synthesised and their melting temperatures have been determined. A marked destabilisation (>20°C) was noted, which is thought to be due to the propyne moiety clashing sterically with the sugar phosphate backbone in the duplex structure, causing a large degree of strain.[110] However, 7-propyne-7-deaza purines stabilise DNA duplexes in a similar manner to the 5-propyne-pyrimidines previously mentioned (figure 1.5.3).[111]

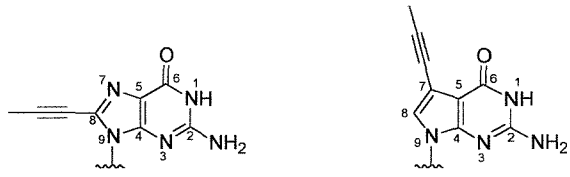


Figure 1.5.3: Structure of the bases for 8-propynyl-dG and 7-propynyl-7-deaza-dG.

Amine containing moieties have been introduced into nucleosides to stabilise duplex structures. Amino groups (pK_a: ~8) will be partially protonated at physiological pH and hence reduce the repulsion between two oligonucleotides arising from the otherwise highly negatively charged sugar phosphate backbones. It is not absolutely necessary for amino groups to be attached to a heterocyclic base, but because this is relatively straightforward to do, these modifications are popular. Functionalisation of C5 with alkyl amines (figure 1.5.4), such as 5-(aminopropyl)-deoxythymidine show little increase[112] if any.[113] Indeed, amines at the end of longer alkyl chains show a large degree of destabilisation,[114]

indicating that any possible increase due to the cationic amine is countered by steric effects of the aliphatic side chain.[115-117]

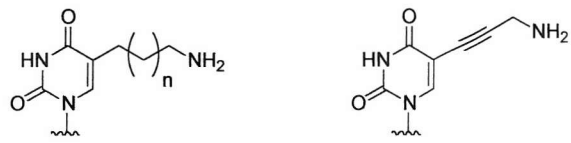
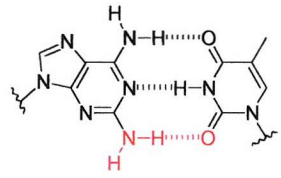


Figure 1.5.4: Amino modifications to thymine.

Significant stabilisation is observed for 5-(3-aminopropyn-1-yl)-2'-deoxyuridine (figure 1.5.4), exceeding the stabilisation observed for a neutral propyne side chain.[113] The lack of sensitivity of these oligonucleotides to ionic strength (i.e. salt concentration) demonstrates that the cationic amine is having a significant effect. For normal nucleic acid duplexes T_m increases with increased salt concentration. In addition, studies have been made to test the base pairing fidelity of this modification which shows significant destabilisation for all possible mismatches at a site opposite the modified base.[113] It is believed that the terminal amino group of the aminopropynyl moiety interacts with the non-bridging oxygen of the 5'-phosphate.[113] This modification is also effective in triplex stabilisation.[118,119]

Another amino modification to the base of a nucleoside is 2-amino-2'-deoxyadenosine (diaminopurine, DAP). Normally 2'-deoxyadenosine only forms two hydrogen bonds with its complement 2'-deoxythymidine, compared to a G.C base pair which has three hydrogen bonds. By introducing another amino group in the 2-position of adenine, a third hydrogen



DAP : T

bond can be formed.

Figure 1.5.5: A base pair between 2-amino-2'-deoxyadenosine and deoxythymidine. Additional hydrogen bond is shown in red.

However, despite three hydrogen bonds DAP.T is still not as stable as a G.C base pair.[120] Diaminopurine has been used to increase melting temperature of duplexes[121,122] and in short oligonucleotides it has been shown to increase the thermal stability by as much as

2°C per modification.[122] [123] It has been suggested that the additional amino group projects into the minor groove[124] and there are reports that this disrupts the hydration spine which contributes to the stabilisation of the B-DNA structure.[5]

1.5.3 Sugar modifications

Sugar modifications are also numerous and tend to operate by affecting the conformation of the ring pucker. By making the sugar adopt a particular conformation more easily, the energy barrier is reduced and so a particular duplex may form more readily. This results in an effective stabilisation of that duplex (increase in ΔG) because the decrease in entropy (ΔS) upon duplex formation is less ($\Delta G = \Delta H - T\Delta S$, where ΔH remains constant).

Locked nucleic acids (LNA) have their conformation locked by the formation of a second ring fused to the furanose ring of the ribose sugar.[125] There are four main types which have been studied (figure 1.5.6).[126] The first LNA structure (i) has been widely studied. The furanose ring adopts a C3'-endo (or N-type) conformation and hence gives the highest degree of stabilisation with RNA (T_m = +3.0-11.0°C).[127] The second type is known as α-L-LNA (ii), which consists of an inversion of the configuration in carbons C2', C3' and C4' with respect to LNA (i).[128] This form stabilises duplex formation but not as significantly as the previous LNA. Type (iii) adopts a high energy conformation which is less stable than the C3'-endo conformation seen already and corresponding decreases in the melting temperature have been observed.[129] The fourth form has a backbone modification with a nitrogen replacing the 3'-oxygen. This modification adopts an S-type conformation as opposed to the previous N-type furanoses. However, decreases in duplex stability were seen with both RNA and DNA complements.[130]

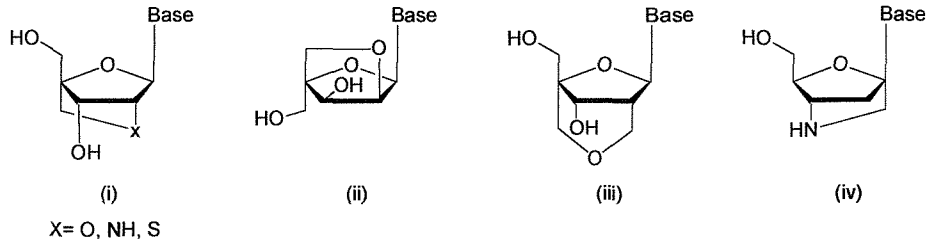


Figure 1.5.6: The various forms of locked nucleic acids.

The dominant form of LNA (i) has been the subject of many structural and stability studies which not only suggest a large stabilisation for RNA, but also DNA with a southern ring pucker. The LNA:DNA duplex shows a degree of unwinding and a widening of the minor groove, and melting temperatures are stabilised by as much as 5°C per modification.[131]

The 2'-position of the sugar is unsubstituted in deoxynucleotides and when this is modified with a 2'-*O*-alkyl or 2'-fluoro substitution, increased binding affinity towards RNA has been observed (figure 1.5.7).[132] This has been attributed to the preference of these nucleotides to adopt a C3'-endo (northern) conformation leading to thermally stable A-type duplexes.[132] It is believed this conformation is adopted due to the *gauche* effect between O2' and O4' (along the C1'-C2' bond). For the 2'-*O*-alkyl substitution, small alkyl groups are best, because of reduced steric interactions.[133] For 2'-*O*-methyl substitution an increase in duplex stability (with RNA target) of ~1°C per modification is observed, but with DNA targets there is no stabilisation.[134] Other 2'-modifications are the 2'-aminoalkoxy moieties. These show no stabilisation in duplex structures, but impressive stabilities have been observed in triplexes.[135] Meanwhile 2'-*O*-aminooxyethyl and 2'-*O*-methoxyethyl modifications show increased stabilities with RNA targets, of 1-2°C per modification.[132] A 2'-amino modification displays a strong destabilising influence.[136] Finally studies into 2'-*C*-alkyl substitutions (figure 1.5.7) have led to destabilisation of duplexes with both RNA and DNA targets.[137,138]

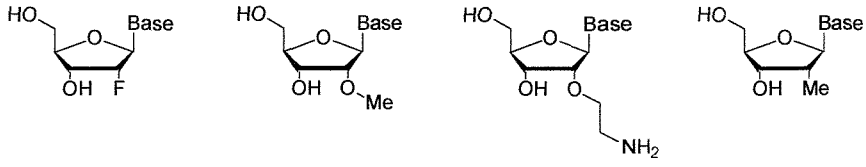


Figure 1.5.7: A selection of 2'-substituents for stabilisation of duplexes and triplexes.

Substitutions at positions other than 2'-position of the furanose ring are less common, but there are reports of small increases in DNA binding affinity for some 4'-substituents.[139] The best of these are the 4'-aminomethyl and 4'-methoxymethyl substituents which show stabilisations of up to 1.8°C with DNA targets.[140] Increasing length of the 4'-substitution causes slight destabilisation for all complementary strands.[141] A slight destabilisation has been observed with RNA complements and it is thought that a C2'-endo ring pucker is responsible.[142,143] 3'-substituents on the other hand, show decreased stability.[138,144] Arabino

nucleic acids, possess an inversion of stereochemistry at the 2'-position (figure 1.5.8). Oligonucleotides containing these modifications show intermediate duplex structures between A-type and B-type duplexes. Stabilisation is observed with the fluoro-derivative and an RNA target.[145,146]

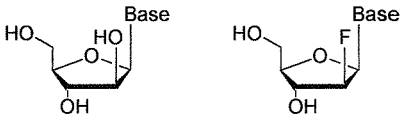


Figure 1.5.8: Arabino nucleic acids.

Other sugar modifications include replacing the furanose ring altogether. Hexitol nucleic acids are based on a pyranose ring and adopt N-type furanose conformations (figure 1.5.9). This shows significant stabilisation with RNA complements.[147,148] Cyclohexene nucleic acids are another furanose replacement. These are relatively flexible mimics, which adopt different conformations according to their complement (figure 1.5.9). When binding to DNA an S-type mimic is formed and when hybridised to RNA an N-type mimic is preferred. Slight increases in duplex stability are observed, between 0.8-1.7°C per modification.[149,150]

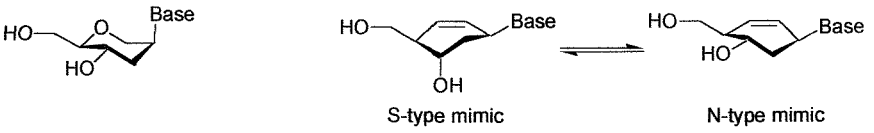


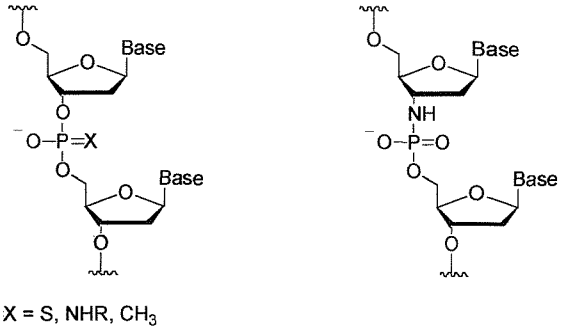
Figure 1.5.9: Left: Hexitol nucleic acid. Right: Cyclohexene nucleic acid showing equilibrium between the two conformations.

1.5.4 Backbone modifications

Many backbone modifications have been described, generally developed for antisense research. Resistance to nuclease activity has been a key requirement of these rather than duplex stability. Examples include phosphorothioates,[151] phosphoramidates and methyl phosphonates (figure 1.5.10) all of which destabilise duplex structures. These and many other destabilising backbones are discussed by Freier *et al.*[112]

N3'→P5'-Phosphoramidates (figure 1.5.10) do show an increase in binding affinity with RNA targets ~2.4°C per modification. The increase in stability has been attributed to the

3'-nitrogen causing the furanose ring to adopt an N-type (C3'-endo) conformation and correspondingly A-type duplexes with RNA have been observed.[152,153] However, binding



affinities to DNA (~1°C per modification)[154] are less than for RNA, although a 2'-fluoro phosphoramidate does show an increase of ~2°C per modification.[155]

Figure 1.5.10: Left: Phosphothioate(X=S), phosphoramidate (X=NHR), and methyl phosphonate ($X=CH_3$) backbone structures. Right: N3'→P5' phosphoramidate backbone modification.

Peptide nucleic acids (PNA) involve a complete replacement of the phosphate linkage and sugar moieties. PNA consists of an N-(2-aminoethyl)glycine polyamide structure with base moieties attached through methylenecarbonyl bridges (figure 1.5.11). These structures are neutral and yet mimic the conformation of nucleotides, leading to large increases in duplex stability with DNA (1°C per mod.) and RNA (1.5°C per mod.).[156] PNA/DNA hybrids can form in either a parallel or antiparallel manner, though antiparallel conformations are more stable. PNA hybridisation is highly specific and shows good discrimination of mismatch structures (up to 20°C difference in T_m). However they suffer from insolubility and aggregation.[156]

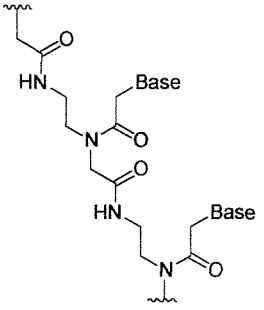


Figure 1.5.11: The structure of PNA.

Related to the PNA structure is a relatively new backbone construct, POM (pyrrolidine-amide oligonucleotide mimic, figure 1.5.12). This is still very much in its infancy, but

initial studies have suggested extremely high increases in duplex stability (as much as 10°C per mod.).[157]

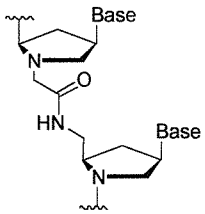


Figure 1.5.12: Structure of POM (pyrrolidine-amide oligonucleotide mimic).

1.5.5 Intercalators

Planar aromatic molecules, often with a positive charge are able to bind to DNA in a process known as intercalation. Widening of the neighbouring base pair distance and a slight unwinding of the helix is observed, allowing the intercalator to bind within the duplex. Intercalators generally have a large degree of bond conjugation, hence stacking in the same way as the bases maximises π-π interactions. Intercalation usually results in a large increase in duplex stability,[158,159] although there may be some sequence dependency observed.[160] For example, phenazine (similar to acridine) has been attached to oligonucleotides and increases in T_m of up to 14.4°C were observed for DNA complementary targets.[161]

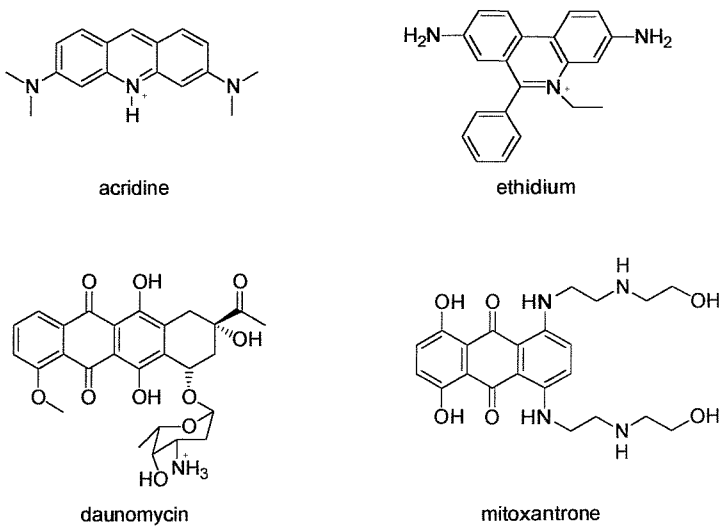


Figure 1.5.13: Examples of intercalating agents.

It has been shown that by attaching intercalators to an oligonucleotide, a probe with high affinity for its complementary strand can be obtained.[158-162]

1.5.6 Minor groove binders

B-form DNA has two grooves that run the length of the double helix structure. One of these is wide, called the major groove and the other is smaller – the minor groove. The minor groove is a suitable size to accommodate relatively narrow poly-aromatic structures and molecules that bind in this groove are known as minor groove binders.

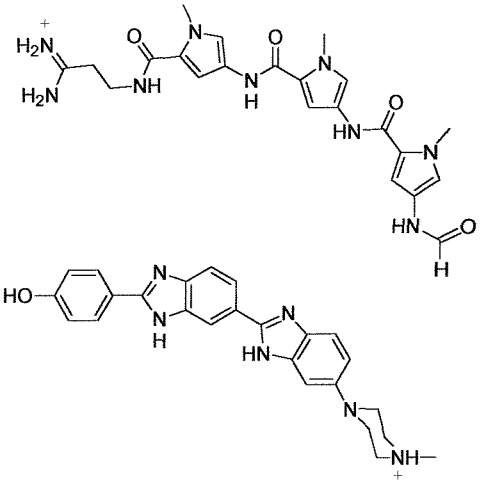


Figure 1.5.14: Minor Groove Binders distamycin (top) and Hoechst 33258 (bottom).

Minor groove binding molecules (figure 1.5.14) are often made up of several conjugated ring systems such as pyrrole, furan, or benzene, connected by bonds with limited torsional freedom[163] Thus the compounds have a degree of flexibility and are able to twist and fit into the curve of the helix. A molecule of this type is able to fit snugly into A.T rich tracts and form van der Waals contacts with the helix. Hydrogen bonds are also formed between the minor groove binder and the duplex, stabilising the interaction further.[164,165] Large increases in duplex stability are seen with the appropriate molecule, although minor groove binders do show a degree of sequence specificity. Recently, a probe system has been produced by EPOCH Pharmaceuticals Inc. This TaqMan® oligonucleotide probe incorporates a tripeptide minor groove binder (1,2-dihydro-(3*H*)-pyrrolo[3,2-e]indole-7-carboxylate) at the 3'-end, which displays increases in duplex stability of almost 20°C.[164] It is claimed that a 12mer modified probe of this type has the same T_m as an unmodified

27mer probe, leading to much improved single base mismatch discrimination. This decrease in length also has the bonus of bringing the fluorophore and quencher moieties closer together, leading to improved fluorescence quenching and hence better signal-to-noise ratio for fluorogenic probes.

2. DEVELOPMENT OF NOVEL QUENCHERS

2.1 Introduction to fluorescent oligonucleotide probes

Advances in genetic analysis have led to the development of numerous techniques for the detection of nucleic acid sequences in homogeneous solution. The most favoured signalling technology for such assays is fluorescence. Typically, fluorescent oligonucleotide probes which are employed are molecular beacons,[84] scorpion primers[100] and TaqMan® probes[95]. These probes are single stranded oligonucleotides with a fluorescent dye and a quencher molecule attached. In their "switched-off" form they adopt a conformation where the quencher is close enough to the fluorescent dye to nullify its fluorescence. On hybridisation to a target sequence the probes either undergo a comformational change (molecular beacons and scorpion primers) or an enzymatic cleavage (TaqMan® probes), thereby separating the quencher and fluorescent dye, allowing a fluorescent signal to be observed.

Development of optimal dye/quencher pairs is an important area of research, but some of those presently available suffer from drawbacks, including intrinsic fluorescence of the quencher itself and poor spectral overlap between the fluorescent dye and quencher molecule. Both of these result in a poor signal-to-noise ratio. There are limitations with currently available "universal" quenchers such as the non-fluorescent diazo-dyes (e.g. DABCYL and methyl red). They are able to quench the fluorescence of the shorter wavelength reporters e.g. FAM, but do not efficiently quench the fluorescence of longer wavelength dyes such as Cy5, Cy3 and TAMRA.

Described here are new quenchers based on diaminoanthraquinone derivatives, which absorb in the 450-700nm region of the visible spectrum. They are non-fluorescent, thus eliminating any complications of background signals, which can arise when fluorophores are used as quenchers.[95] These quenchers have been incorporated into different types of probe systems and the degree of quenching determined in a variety of assays.

2.2 Limitations of current quenchers

Originally, molecular beacons, scorpions and TaqMan® probes designed in our laboratory used the fluorophore FAM and the quencher methyl red. TaqMan® probes could also be made with a long-wavelength dye as a quencher moiety instead, such as TAMRA (FRET between the FAM and TAMRA leads to "quenching" of the FAM fluorescence and the emission of TAMRA is observed at a longer wavelength).[95] However, the need to synthesise oligonucleotide probes with alternative dyes of a longer-wavelength has become necessary, particularly with the development of microarrays. This technology uses a range of fluorophores, including the longer wavelength cyanine dyes. Microarray and multiplexing techniques require many oligonucleotide probes to be used in a single assay, each utilising a different fluorophore. Hence a broad spectrum of dyes was necessary and quenchers were needed to cover the broad spectrum of their emissions.

Study of the long-wavelength dyes in oligonucleotide probes using the conventional methyl red quencher displayed limitations in its quenching ability. It was previously thought to be a "universal" quencher, suitable for all fluorescent dyes, but experiments with Cy5 and Cy5.5 showed that this was not the case (refer to figure 2.5.2, section 2.5.1). Signal-to-noise ratios were significantly poorer for probes containing methyl red with long wavelength dyes and further investigation indicated that this was due to the high level of background noise (probe in "switched-off" state). The level of background noise is inversely proportional to the efficiency of the quenching, so consequently it was decided that methyl red was not a suitable quenching moiety for long wavelength dyes. This prompted investigation into the design of a new fluorescence quencher for oligonucleotide probes.

2.3 Development of the diaminoanthraquinone quencher

2.3.1 Finding a suitable chromophore

Our aim was to develop a quencher for long wavelength dyes. The initial step therefore was to find a suitable chromophore for this purpose. Necessary criteria included: high extinction coefficient; long wavelength of absorption (ideally 500-700nm); minimal intrinsic fluorescence; ready availability; compatibility with organic synthesis. Potentially suitable chromophores included anthraquinones, phthalocyanines and diazo derivatives, all of which have a long history in the dye industry. However, 1,4-diaminoanthraquinone (DAQ) was chosen (figure 2.3.1) because it was cheap and readily available. It also has two primary amines suitable for functionalisation, which could be used as an attachment point to oligonucleotides.

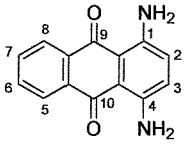


Figure 2.3.1: 1,4-Diaminoanthraquinone (DAQ).

2.3.2 Synthesis of a 5'-labelling DAQ phosphoramidite monomer

The synthetic route (figure 2.3.3) was designed to yield an appropriate phosphoramidite monomer for 5'-terminal labelling of oligonucleotides. The initial alkylation reaction (i)[166] was low yielding and generated **1** (20%), plus many by-products, including the bisalkylated product **2** (1%), and recovered starting material (60%). Many variants of this reaction have been attempted, using different solvent systems (DMF, NEt_3/DMF mix, pyridine) and a range of alkylation methods with different bases, ie. NaH, K_2CO_3 and pyridine. None were particularly clean or high yielding. The alkylation was also attempted with the hydroxyl group of 6-aminohexanol protected as a DMT ether (**6,** figure 2.3.2), with no significant improvement in efficiency.

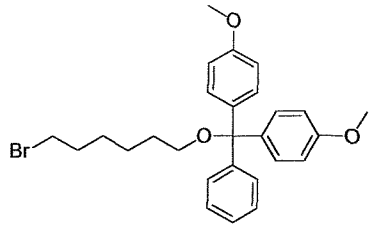


Figure 2.3.2: 4,4'-Dimethoxytrityl 1-bromohexyl ether (**6**)

Further optimisation of this reaction would be required if a larger scale synthesis was to be attempted, but the route was continued on this smaller scale to confirm its viability. Acetylation proceeded well, giving clean diacetylated product **3,** in good yield (92%). The ester was then selectively cleaved yielding the alcohol **4** (41%), whilst the amine remained protected. This product was then phosphitylated in the usual way,[6,8] however this step was not as efficient as normal because of the high sensitivity of the phosphitylated product **5** to oxidiation (yield: 89%). The reason for this decrease in stability is not known, but it is suspected that decreased steric hindrance relative to a nucleoside phosphoramidite might be a contributary factor. Interaction of an amine of the diaminoanthraquinone with the phosphoramidite moiety could also be affecting the stability. Further studies are needed to determine the true nature of this lack in stability.

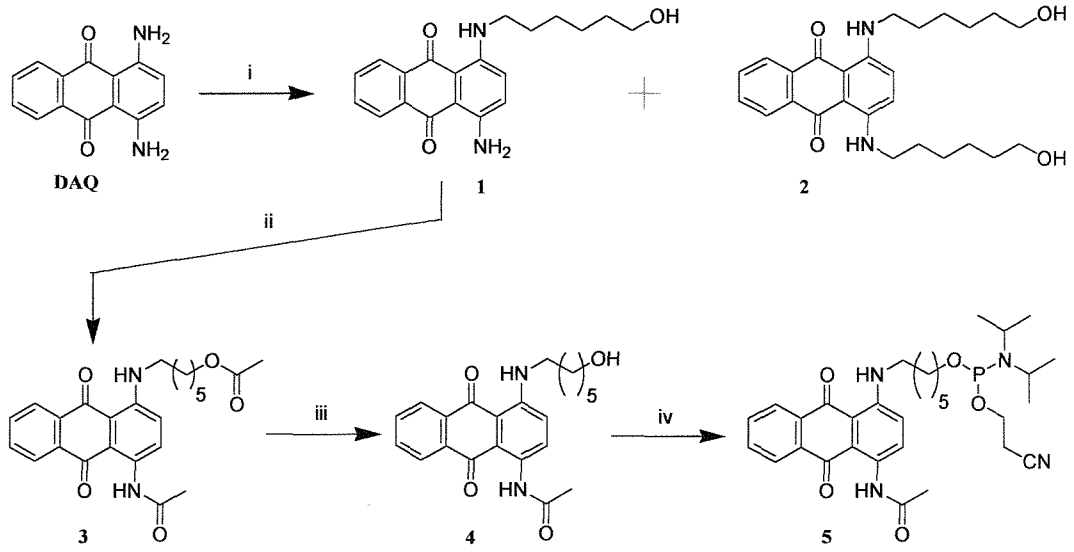


Figure 2.3.3: Reagents and conditions: i) 1-Bromohexanol (2.2eq), DMF, 130°C, 36hrs (**1**:20% + **2**:1%); ii) Acetic anhydride (200eq), pyr, 50°C, 15hrs (92%); iii) NaOMe (1.4eq), MeOH, rt, 5hrs (41%); iv) 2-Cyanoethoxy-*N,N*-diisopropylamine chlorophosphine (1.1eq), DIPEA (2eq), THF, rt, 2hrs (89%).

One limitation of this monomer is that it can only be placed at the 5'-end of oligonucleotides, because it only has one point for attachment (the phosphoramidite moiety). Consequently, further analogues were designed for incorporation internally within the oligonucleotide backbone.

2.3.3 Synthesis of a DAQ monomer for internal incorporation

A second derivative of DAQ was synthesised, this time using a linker based on the hexan-1,2-diol structure (figure 2.3.4). Hexan-1,2,6-triol was converted into the isopropyl acetal **7** (76%)[167] and the remaining hydroxyl group was reacted with *N*-bromosuccinimide to form the bromo compound **8** (64%).[168] This was attached to the dye *via* alkylation of the DAQ as before to yield **9**,[166] but similarly poor yields resulted. The separation of the product from the starting material proved challenging. Therefore the crude product was reacted with acetic anhydride to yield the acetylated amine (**10**), and purification was carried out after deprotection of the acetal to form the highly polar diol **11** (3 steps: 4% - due to low yield of initial alkylation step). The diol contains both primary and secondary alcohol functionalities, this allowing sufficient discrimination to protect the primary alcohol as a DMT ether (**12**, 85%), leaving the secondary alcohol free for phosphitylation to yield **13** (80%).[6] This monomer could be used to incorporate diaminoanthraquinone anywhere along the length of the oligonucleotide backbone.

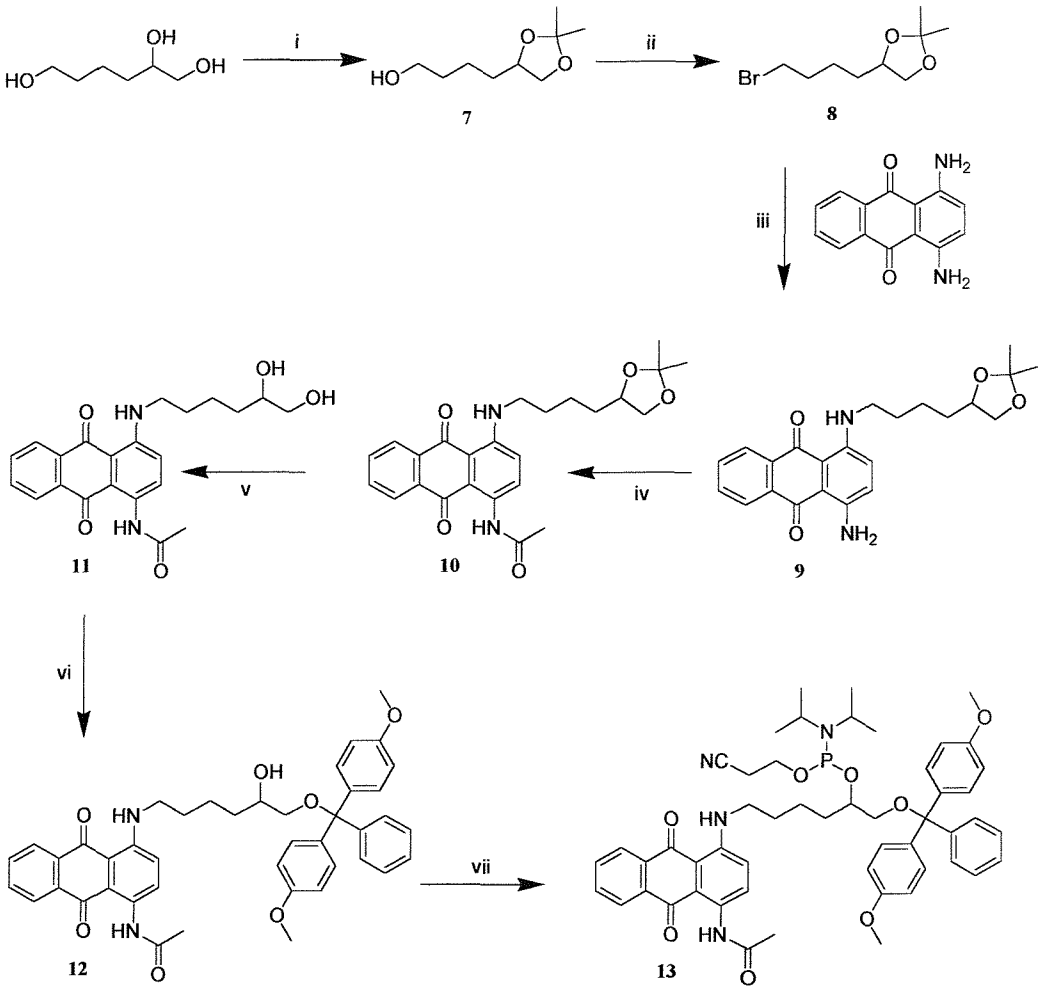


Figure 2.3.4: Reagents and conditions: i) Acetone, c.HCl, Na_2SO_4 (anhyd.), mol. sieves, rt, 68hrs (76%); ii) NBS (3eq), PPh_3 (2.8eq), pyr (1.2eq), CH_2Cl_2, rt, 5hrs (64%); iii) 1,4-diaminoanthraquinone (DAQ), NEt_3, 130°C, 24hrs; iv) Acetic anhydride (10eq), imidazole (1.5eq), pyr, 50°C, 2.5hrs; v) c.HCl, H_2O, THF, rt, 1hr (3 step: 4%); vi) DMTCl (1.1eq), pyr, rt, 15hrs (85%); vii) 2-Cyanoethoxy-*N,N*-diisopropylamine chlorophosphine (1.2eq), DIPEA (2eq), THF, rt, 1.5hrs (80%).

2.3.4 Determination of suitable deprotection conditions for monomer 5

Compound **5** was successfully incorporated into oligonucleotides. Initially some 5'-labelled poly-dT oligonucleotides (**ODN1,2**) were synthesised. These were used to assess the compatibility of the monomer **5** with oligonucleotide synthesis and purification.

ODN1 5-TT

ODN2 5-TTT TTT TTT T

ODN2 was split into three and to each sample different deprotection conditions were applied (figure 2.3.5). Following deprotection the samples were studied by RP-HPLC (figure 2.3.6), UV/vis spectroscopy and mass spectrometry.

Experiment	Temperature	Time	Reagents
1	25°C	1hr	$NH_{3\ (aq)}$/EtOH (3:1)
2	55°C	1hr	$NH_{3\ (aq)}$
3	55°C	5hr	$NH_{3\ (aq)}$

Figure 2.3.5: Deprotection conditions studied with **ODN2**.

The conditions in experiment 1 gave a single major peak on HPLC (figure 2.3.5), which had a mass of 4031 by electrospray mass spectrometry. On analysis of the second experiment by HPLC, two peaks were observed (figure 2.3.5), corresponding to the two masses: 3989 and 4031. The harshest conditions, in experiment 3, produced a single major peak on HPLC (figure 2.35) with a mass of 3989. There was no significant change in absorption wavelength: all samples showed a large UV/vis chromophore from 450-600nm.

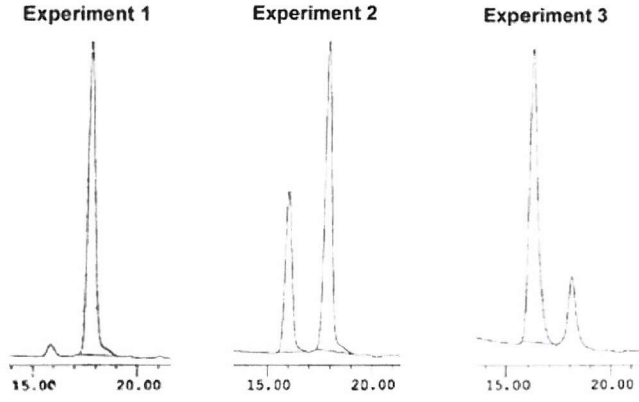


Figure 2.3.6: RP-HPLC chromatograms for DAQ deprotection studies. X-axes: Abs. (264nm); Y-axes: retention time (s).

The mass of 4031 was the expected mass for the oligonucleotide containing the 5'-DAQ modification (**5**). The mass of 3989 corresponded to removal of the acetyl protecting group on the amine by the harsher deprotection conditions. Hence, suitable deprotection conditions were ascertained (mild: $NH_{3\ (aq)}$; <1hr; rt). The acetylated product was used for all other studies, although both acetylated and deacetylated products would be suitable for our purposes because there is not a significant change in absorption maxima.

2.3.5 Fluorescence quenching properties of the DAQ derivatives

Molecular beacons were synthesised with both methyl red (**ODN3**) and the novel diaminoanthraquinone monomer **5** (**ODN4**) as the quencher moiety, for sequences:

Molecular beacon: **Q**-GCA GCC GTT TCC TCC ACT GTT GCG CTG C-**F**

Target (ODN5): GCA ACA GTG GAG GAA ACG

where **Q** = quencher: MR (**ODN3**) or DAQ monomer **5** (**ODN4**); **F** = fluorophore: Cy5.

These beacons were designed to hybridise at relatively low temperatures and hence were studied at 30°C on a PE LS 50B luminescence spectrometer. Samples were excited at the Cy5 absorption wavelength of 650nm and the emission was observed from 660-700nm. A 100mM phosphate buffer (0.5M NaCl, 1mM EDTA, pH 7) was used throughout and the relative quenching of Cy5 (λ_{em} 670nm) was assessed (figure 2.3.7).

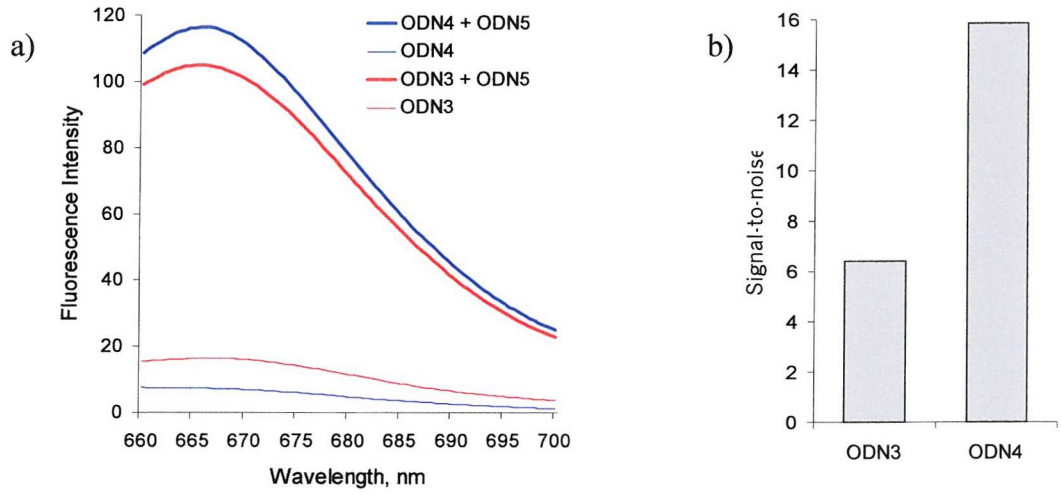


Figure 2.3.7: a) Fluorescence of beacons demonstrating the increase in fluorescence on addition of target (**ODN5**) to both molecular beacons **ODN3** and **ODN4**. b) Comparison of signal-to-noise ratios for molecular beacons **ODN3** and **ODN4**.

The fluorescence study of the molecular beacons **ODN3** (0.1nmol) and **ODN4** (0.1nmol), is shown in figure 2.3.7a. Fluorescence increased for both molecular beacons upon addition of target **ODN5** (5eq.). The maximum fluorescence was similar for both molecular beacons, but the initial fluorescence (i.e. background noise) for **ODN3** was almost double that for **ODN4**. The superior quenching properties of the

diaminoanthraquinone for the Cy5 dye could explain this. To demonstrate the improvements this novel quencher offers, the relative signal-to-noise ratios for these two beacons were calculated (figure 2.3.7b). The signal-to-noise ratio for the beacon containing the DAQ quencher **5** (**ODN4**) was double that of the beacon containing the conventional methyl red quencher (**ODN3**). This result clearly demonstrated that optimising the fluorescence quenching is important in order to obtain the best signal-to-noise ratios for fluorescent probes of this type. The diaminoanthraquinone moiety appeared to be a significantly better quencher for the Cy5 dye than the conventional methyl red quencher.

2.3.6 Limitations of the DAQ quenchers 5 and 13

Initial studies with the DAQ quencher **5** in oligonucleotides displayed very good fluorescence quenching properties, but there were drawbacks with the synthetic yields and sensitive phosphoramidite monomers. The greatest problem was the alkylation step, which gave low yields, unsuitable for large scale synthesis. Investigation of the reaction of 1-bromo-4-aminoanthraquinone with 6-aminohexan-1-ol or the amino compound **15** (*via* the phthalimide **14**) was attempted using the Ullmann condensation method,[169] but this was unsuccessful (figure 2.3.8).

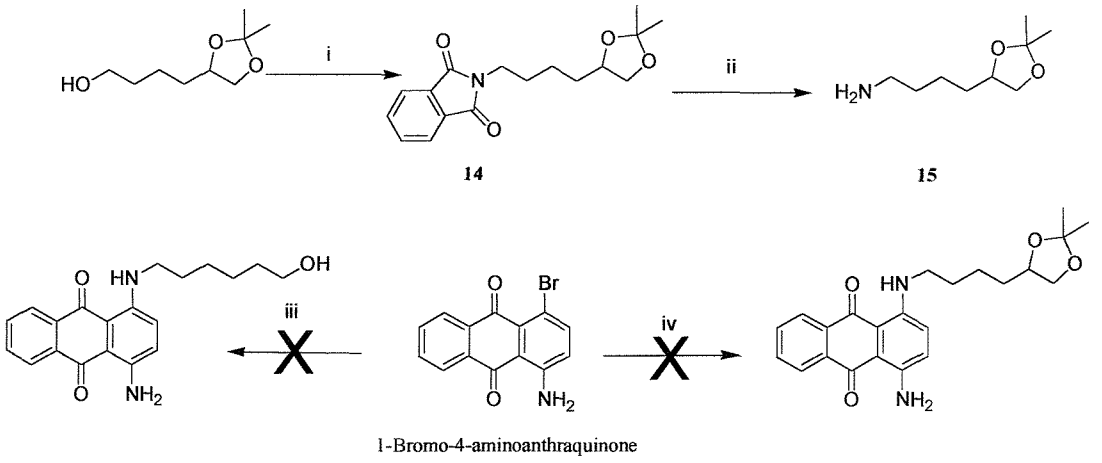


Figure 2.3.8: Reagents and conditions: i) Phthalimide, PPh_3, DEAD, THF, rt, 1.5hr; ii) $N_2H_4.H_2O$, MeOH, rt, 15hr (2 steps: 64%); iii) aminohexanol (2eq), Cu(I)Br (0.5eq), THF, 60°C, 24hrs; iv) **15** (10eq), Cu(I)Br (0.5eq), THF, 60°C, 24hrs.

Instead of pursuing this further it was decided to find a new starting material, which might allow easier functionalisation.

2.4 Development of the disperse blue quencher

A starting material was found, known as disperse blue 3 (1-(2-hydroxyethylamino)-4-(methylamino)-anthraquinone) (figure 2.4.1). This dye has a slightly longer wavelength than 1,4-diaminoanthraquinone (λ_{max} = 640nm) and is available from Aldrich at a low price, but unfortunately only as a mixture containing 20% of the desired material.

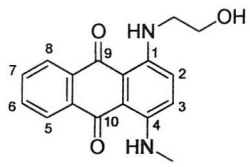


Figure 2.4.1: Disperse blue 3.

Disperse blue 3 was purified by silica gel column chromatography eluting with Hexane/EtOAc (1:1). A second column was needed to purify the material sufficiently from the other materials with similar R_f values. Pure 1-(1-hydroxyethylamino)-4-(methylamino)-anthraquinone was obtained as a blue solid (10%).

The UV/vis absorption spectrum of this purified material was studied and compared to the absorption spectrum of methyl red. The absorption maximum was about 150-200nm longer for disperse blue than methyl red (figure 2.4.2), indicating that this material should be ideal for quenching longer wavelength dyes.

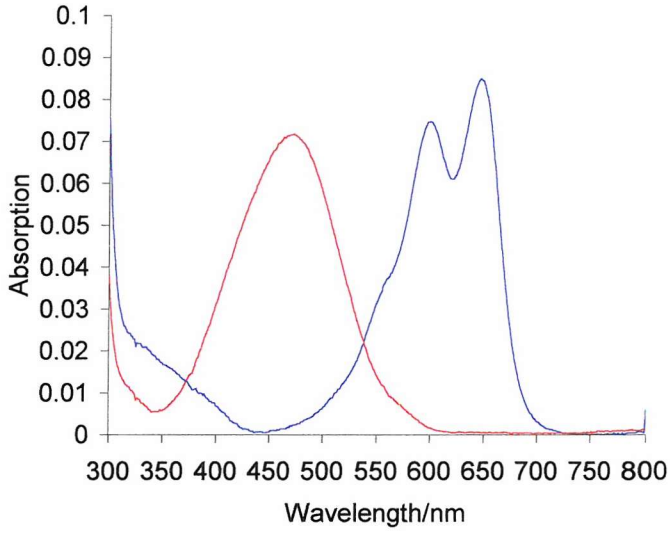
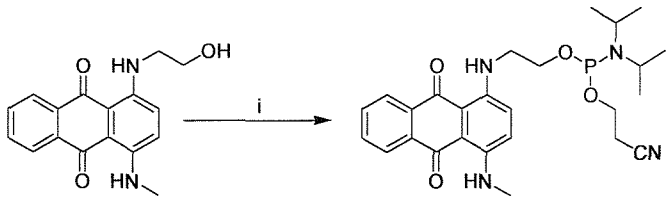


Figure 2.4.2: Absorption spectra of purified disperse blue (—) and methyl red (—).

2.4.1 Synthesis of a disperse blue phosphoramidite monomer



16

Figure 2.4.3: Reagents and conditions: i) 2-Cyanoethoxy-*N,N*-diisopropylamine chlorophosphine (1.2eq), DIPEA (5eq), CH_2Cl_2, rt, 3hrs (63%).

Pure 1-(1-hydroxyethylamino)-4-(methylamino)-anthraquinone was phosphitylated in the normal way[6,8] to yield the phosphoramidite monomer **16** (figure 2.4.3). This monomer was suitable for initial investigations and was used to determine the properties of disperse blue as a quencher. However, as with the DAQ monomer synthesised earlier (**5**), having a quencher which can only be attached to the 5'-end is limited and so plans were made to synthesise derivatives which could be incorporated internally within the DNA backbone.

Previously, the internal DAQ monomer **13** seemed relatively unstable to air oxidation (P(V) oxidiation state was confirmed by ^{31}P NMR), so an alternative linker was adopted for this quencher. Two backbone/linker structures were chosen: a C5-modified-deoxyuridine and a 2'-deoxyribose (figure 2.4.4).[170,171] Although these structures were more complex than the hexan-1,2-diol linker used previously, they have a number of advantages. The dR monomers are easier to handle, being of similar stability to conventional nucleotide phosphoramidites. The deoxyribose backbone might improve the stability of the duplex formed by the oligonucleotide because it possesses a more uniform spacing, in keeping with that of natural nucleotides. To this end, the β anomer could be used for an internal phosphoramidite monomer (where base-pairing is not important) and the α anomer could be used to make a modified-CPG solid-phase resin for functionalising the terminal 3'-position (where a β conformation is not necessary).

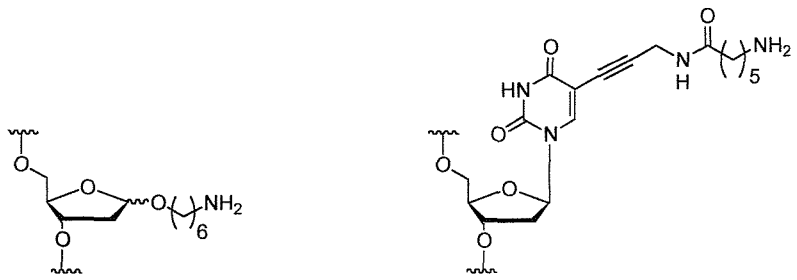


Figure 2.4.4: Backbone/linker constructs. Quencher can be attached to 1° amine.

The dU monomer was a further advance on the dR monomer for internal functionalisation. The chromophore could be attached at the C5 position of the uracil base, so that the monomer would still be able to base pair with a complementary adenosine. The formation of these hydrogen bonds in the duplex structure would not be affected by the C5 modification.

2.4.2 Synthesis of disperse blue dU phosphoramidite monomer

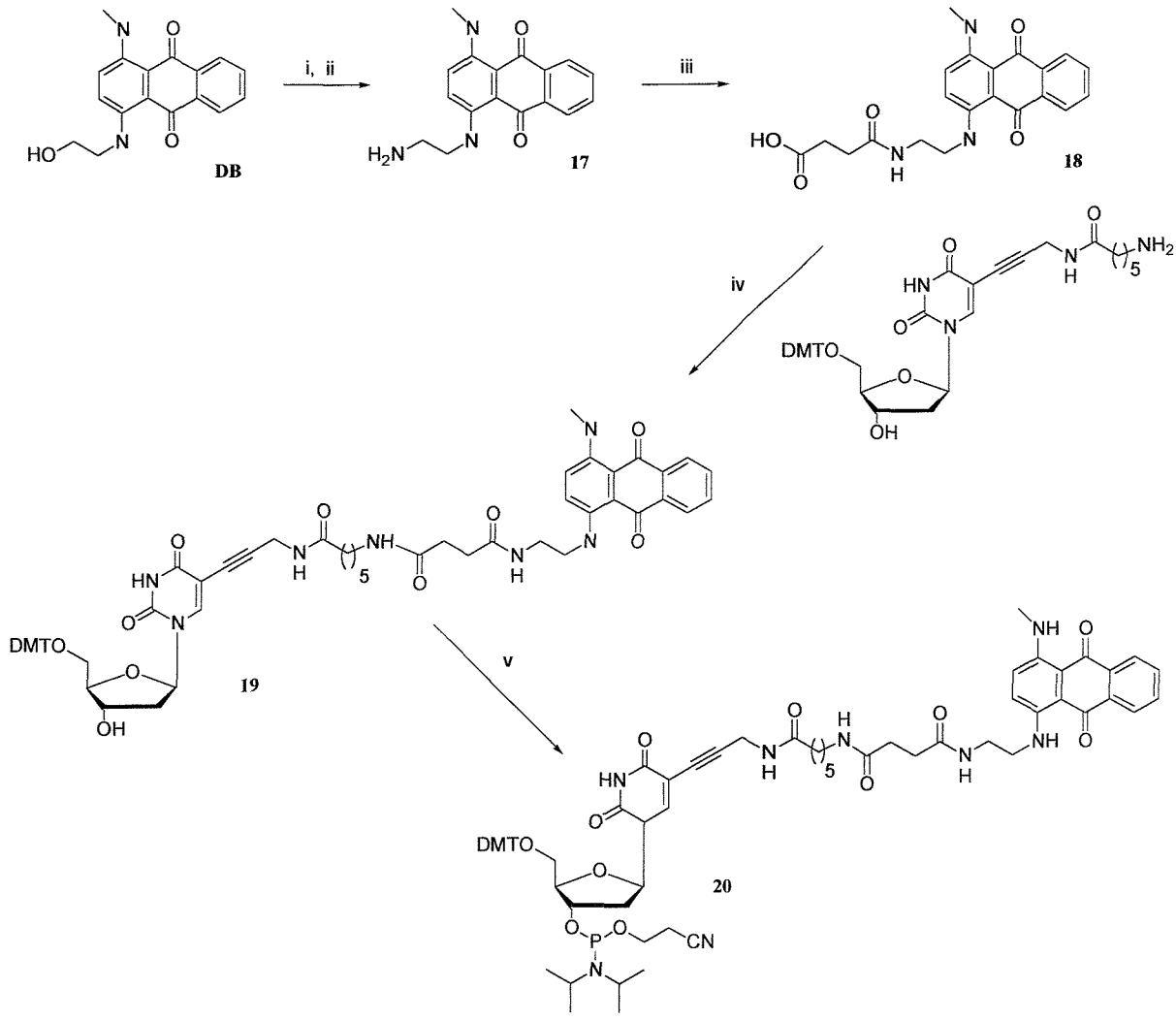


Figure 2.4.5: Reagents and conditions: i) Phthalimide (1.1eq), PPh_3 (1.1eq), DEAD (1.1eq), THF, rt, 1.5hrs; ii) Hydrazine hydrate (2eq), MeOH, rt, 15hrs (2 steps, 90%); iii) Succinic anhydride (1.5eq), DMAP (0.1eq), pyridine, rt, 1.5hrs (90%); iv) EDC (1.1eq), HOBT (1.1eq), DIPEA, DMF, rt, 24hrs, (46%); v) 2-Cyanoethoxy-*N,N*-diisopropylamine chlorophosphine (1.2eq), DIPEA (5eq), THF, rt, 2.5hrs (93%).

The hydroxyl group of disperse blue was converted to an amino group (**17**) *via* the phthalimide in a Mitsunobu reaction. The amino group was reacted further with succinic anhydride to yield the succinic acid derivative **18**. This was coupled to 5-(*N*-(2-propynyl)-6-amino-hexanamide)-5'-*O*-(4,4'-dimethoxytrityl)-2'-deoxyuridine[ψ][170,171] to form DMT-DB-dU **19**. The 3'-hydroxyl group was then phosphitylated in the usual manner,[6,8] to yield the phosphoramidite **20** (figure 2.4.5).

[ψ] This compound was provided by Oswel Research Products Ltd

2.4.3 Synthesis of disperse blue functionalised CPG resin

The design was based upon a deoxyribose sugar with a linker to the chromophore (figure 2.4.6).

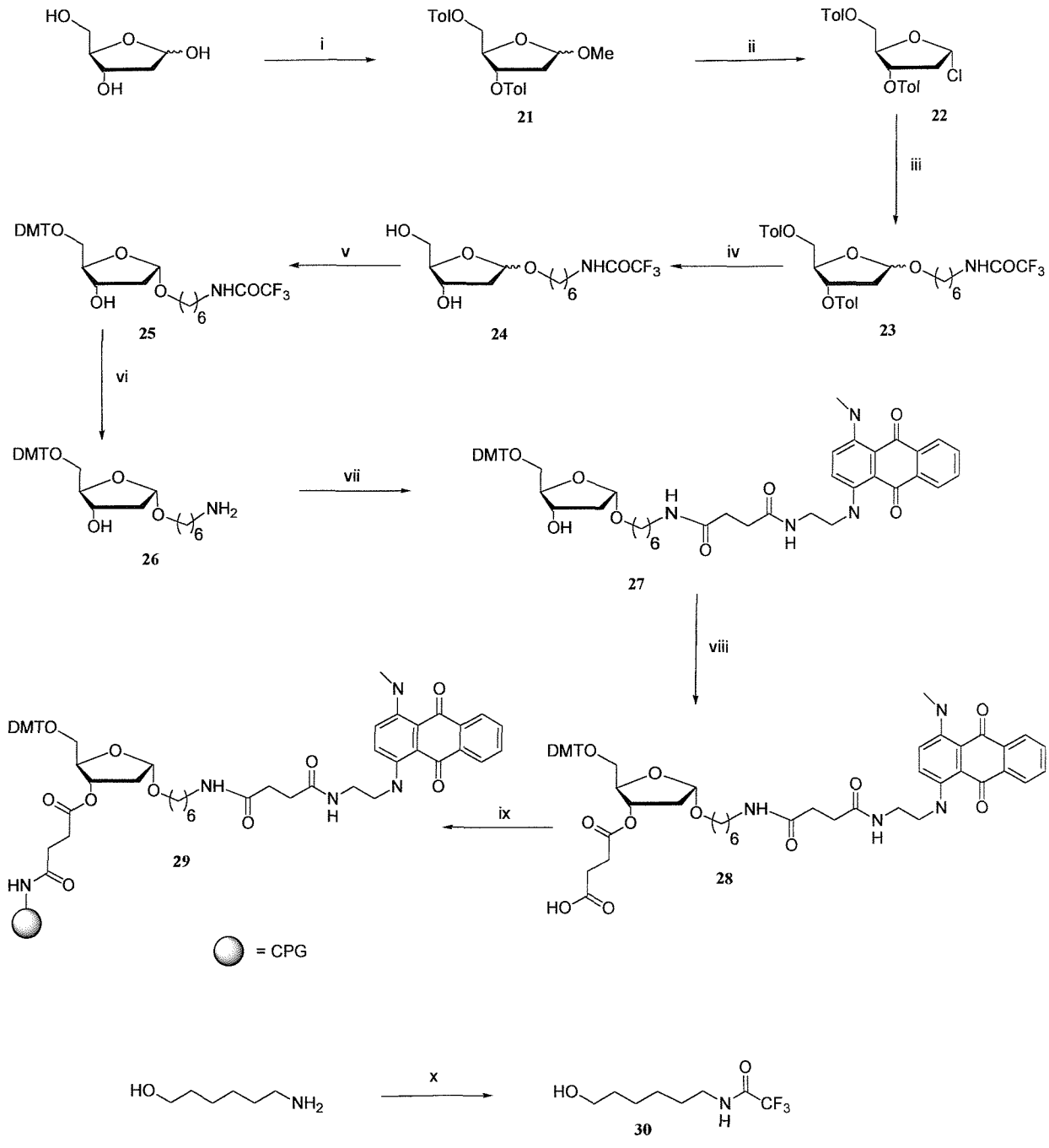


Figure 2.4.6: Reagents and conditions: i) 0.1% HCl/MeOH, 0.5hrs, rt, then, p-toluoyl chloride (2.2eq), pyridine, 0°C, then rt, then 65°C, 6hrs, (71%); ii) Acetyl chloride, AcOH, rt, 10min, (79%); iii) **30** (0.9eq), DMAP (0.2eq), THF, rt, 3hrs, (80%); iv) 0.5M NaOMe/MeOH, rt, 1hr, (53%); v) DMTCl (1.1eq), pyridine, rt, 6hrs; (α 30%, β 20%); vi) NaOH (5eq), MeOH:H_2O (5:1), rt, 2hrs, (60%); vii) **18**, EDC (1.1eq), HOBT(1.1eq), DIPEA, DMF, rt, 12hrs, (30%); viii) Succinic anhydride (2eq.), DMAP (0.1eq), pyridine, rt, 12hrs, (94%); ix) Amino CPG resin, EDC (5eq), HOBT, DIPEA, CH_2Cl_2, rt, 2hrs; x) Ethyltrifluoroacetate (1.5eq), CH_2Cl_2, NEt_3, rt, 24hrs, (70%).

Initially a methoxy group was introduced at the 1'-position of the ribose sugar and the 3' and 5'-alcohols were protected with toluoyl groups (**21**). The methoxy group was then selectively displaced with chloride, to give the α anomer (R configuration) (**22**).[171] Meanwhile the primary amine of 6-aminohexan-1-ol was protected as a trifluoroacetate (**30**). The chloride of **21** could then be displaced by **30**, yielding racemate **23**.[171] The toluoyl groups were removed with NaOMe (**24**),[171] after which the 5'-hydroxyl was tritylated, leading to greater differentiation between the two anomers on silica gel, allowing them to be separated by column chromatography (**25**).[171] Taking solely the α anomer, the TFA protecting group was removed with sodium hydroxide to yield the free amine **26**. The acid **18** (figure 2.4.5) and the amine **26** were then coupled with EDC/HOBT to yield the disperse blue deoxyribose sugar derivative **27**. The 3'-alcohol was then succinylated to yield the acid **28**, before coupling to an amino CPG resin, again with EDC/HOBT, to yield the functionalised resin **29**. A CPG resin was used because this was the solid-support of choice for automated oligonucleotide synthesis.

The β form could be used for 5'-terminal, 3'-terminal or internal labelling of oligonucleotides. Hence the α anomer was used exclusively for CPG resin but the β anomer was retained for the synthesis of other phosphoramidite monomers.

2.4.4 Assessment of deprotection conditions

To assess the appropriate deprotection conditions for the disperse blue quencher, two poly-T oligonucleotides were synthesised (**ODN6,7**). One with a 5'-DB phosphoramidite (**16**) and one using a 3'-DB CPG resin (**29**):

ODN6 $^{5'}$**16**-TTT TTT TTT T $^{3'}$

ODN7 $^{5'}$TTT TTT TTT TTT TTT-**29** $^{3'}$

These oligonucleotides were cleaved from the resin with c. $NH_{3\,(aq)}$ (30min, rt) and purified by RP-HPLC. The sole major peak in each case was collected, which was blue in colour. An aliquot of each purified oligonucleotide was taken and heated in conc. $NH_{3\,(aq)}$ at 55°C for 4hrs and then studied by RP-HPLC. **ODN6** was shown to be one major peak (figure 2.4.7) and this was purified on a preparative scale and its molecular weight was confirmed

by mass spectrometry (expected: 3337; found: 3338). Hence conventional deprotection conditions were suitable for use with oligonucleotides containing **16**.

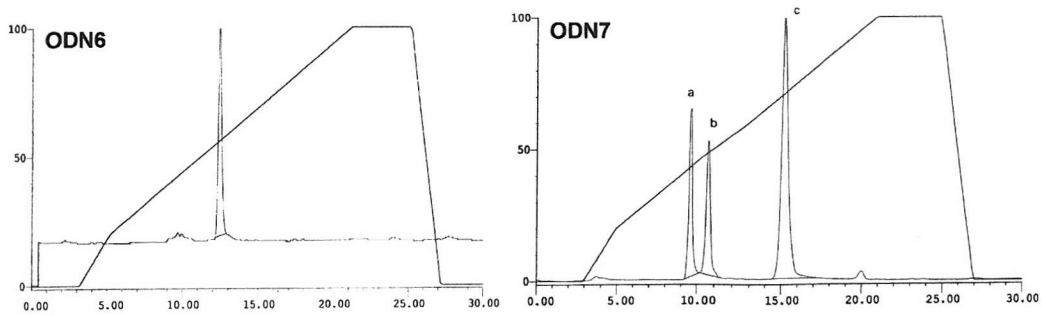


Figure 2.4.7: RP-HPLC traces of **ODN6** and **ODN7** after initial deprotection (c.$NH_{3\ (aq)}$).

However the chromatogram for **ODN7** (figure 2.4.7) showed three significant peaks, so further studies of the deprotection conditions were needed for this modification. This sample of **ODN7** was studied by mass spectroscopy (MALDI-TOF) revealing a number of degradation products.

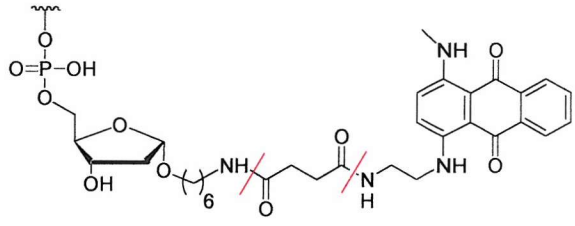
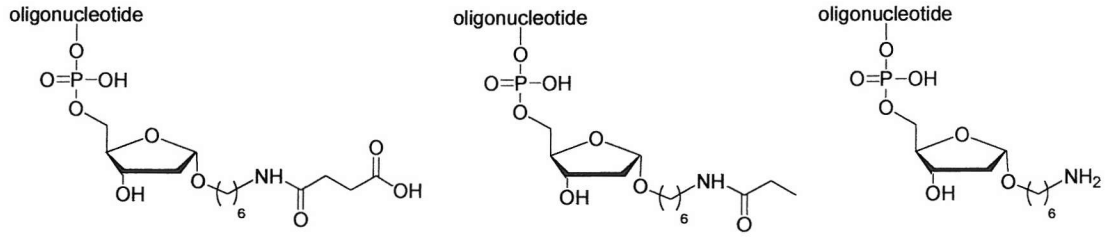


Figure 2.4.8: 3'-Disperse blue (**29**) in an oligonucleotide, showing cleavage sites in red.

Initial studies suggested that the two amide bonds in the linker were acting as points of cleavage (figure 2.4.8) and hence the free amines and acids were being formed. Masses corresponding to loss of the chromophore moiety, yielding three sugar derivatives were found by mass spectrometry (figure 2.4.9).



calculated: 3981.75
found: 3980

calculated: 3938.75
found: 3942

calculated: 3881.73
found: 3880

Figure 2.4.9: Three proposed structures to match the masses found by mass spectrometry.

Further studies were needed to develop a suitable deprotection protocol for use with the 3'-disperse blue. Hence, the remainder of **ODN7** was divided up into 4 aliquots and to each was applied different deprotection conditions (figure 2.4.10).

Experiment	Temperature	Time	Reagent
1	70°C	2 ½ hr	Conc. aq. NH_3
2	70°C	2 ½ hr	H_2O:MeOH:$^tBuNH_2$ (2:1:1)
3	25°C	24 hr	H_2O:MeOH:$^tBuNH_2$ (2:1:1)
4	25°C	1 ½ hr	NH_4OH:$MeNH_{2\,(aq)}$ (1:1)

Figure 2.4.10: Experimental details to ascertain optimal deprotection conditions.

After each experiment (figure 2.4.10), solutions were evaporated *in vacuo* and the residues were studied by RP-HPLC (figure 2.4.11).

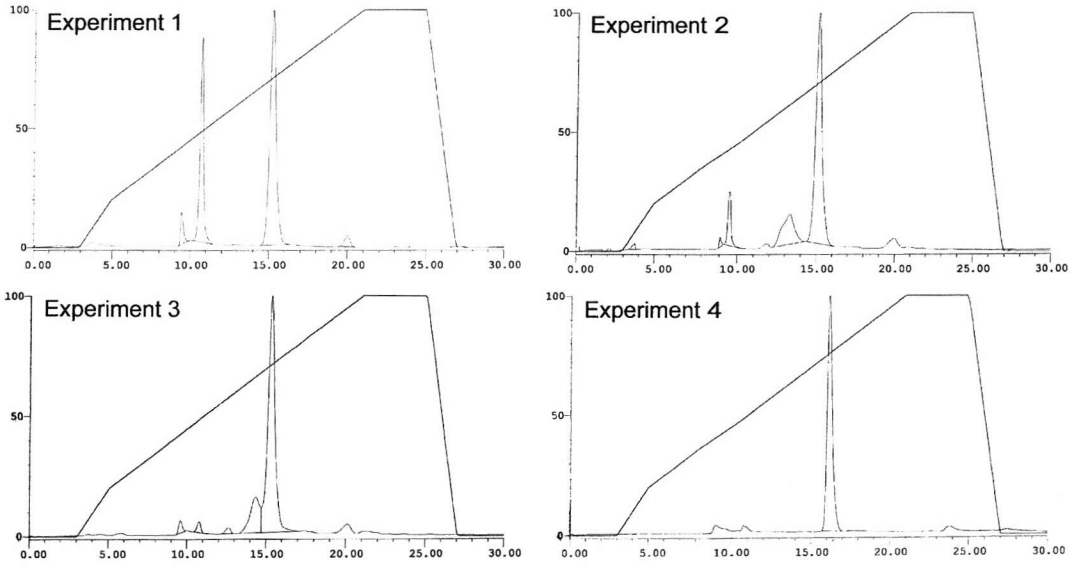
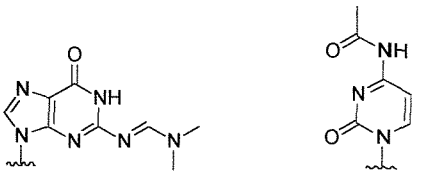


Figure 2.4.11: RP-HPLC chromatograms for experiments 1, 2, 3 and 4 (figure 2.4.10) to determine optimal deprotection conditions.

Any fraction containing the quencher moiety was blue in colour so assessment was relatively simple. It was clear that much improved deprotection could be achieved without using conc. aqueous ammonia. The monomer was more stable to the mixtures water:methanol:^{t}butylamine (2:1:1), and ammonium hydroxide:conc. aq. methylamine (1:1). Both of these deprotection solutions gave a much cleaner product, demonstrated by

the chromatograms (figure 2.4.10). It was evident that heating in ammonia was too harsh for this 3'-modification even for short periods of time. (It should be noted that if an oligonucleotide is to be deprotected with water:methanol:^{t}butylamine (2:1:1), ^{dmf}dG should be used and for use of AMA (ammonium hydroxide:conc. aq. methylamine) ^{acetyl}dC should be used (figure 2.4.12).)



^{dmf}dG ^{acetyl}dC

Figure 2.4.12: Structures of the base moieties for ^{dmf}dG and ^{acetyl}dC.

These studies with monomer **29** were also applicable to the deprotection of the internal monomer **20**, because both of these structures contained the same amide linker structure that appeared to be a weak point for cleavage during deprotection with concentrated aqueous ammonia. Consequently, precautions with deprotection reagent and temperature were taken with oligonucleotides containing either monomer. Indeed, these less harsh levels of deprotection are also necessary if Cy5 or TAMRA are used.

2.5 Physical studies of disperse blue quenchers

2.5.1 Fluorescence studies of DB containing oligonucleotides

Several oligonucleotides were synthesised to assess the relative quenching properties of the disperse blue functionality with respect to methyl red. Five oligonucleotides representing a broad range of wavelengths were made, incorporating the fluorescent dyes: FAM (**ODN8**); Cy3 (**ODN9**); TAMRA (**ODN10**); Cy5 (**ODN11**) and Cy5.5 (**ODN12**). Two quencher oligonucleotides were also synthesised containing either methyl red (**ODN13**), or disperse blue monomer **16** (**ODN14**). The oligonucleotides were designed so that the quencher oligonucleotides were complementary to the oligonucleotides containing a fluorophore. All fluorophores were attached at the 3'-end and the quenchers at the 5'-end, so that the fluorophore and quencher moieties would be in close proximity when the two oligonucleotides were hybridised as shown:

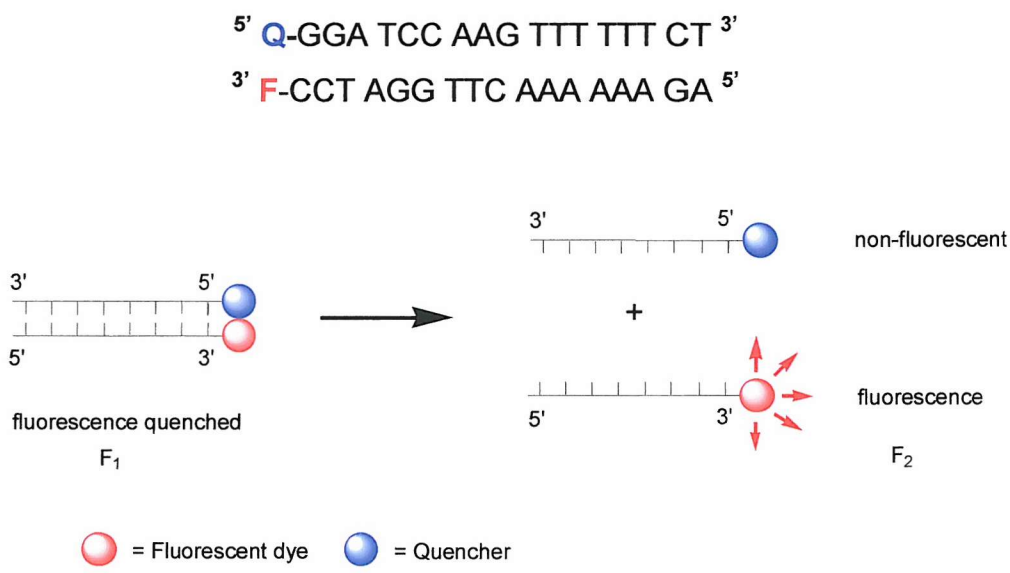


Figure 2.5.1: Sequences of hybridised fluorophore-quencher oligonucleotide duplex and schematic of fluorimetry melting assay. Signal-to-noise = F_2/F_1.

Fluorescence melting studies were then performed (figure 2.5.1), producing a series of curves displaying relative fluorescence over a range of temperature (0.1M Sodium phosphate buffer, 0.1M NaCl, 1mM EDTA, pH7). In each case the signal-to-noise ratio (maximum fluorescence/initial fluorescence) was calculated, allowing direct comparison of each fluorophore/quencher pair. The results (figure 2.5.2) demonstrated that disperse blue was a superior quencher to methyl red over the range of fluorophores studied. This was

particularly clear for the longer wavelength dyes (eg. Cy5, Cy5.5), where it seemed that methyl red was rather ineffective relative to disperse blue.

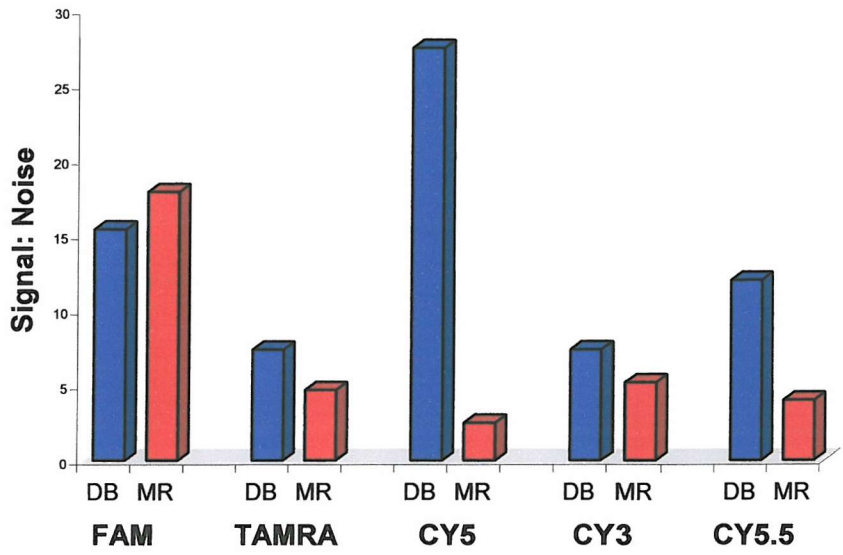


Figure 2.5.2: Signal-to-noise ratios for disperse blue (DB) and methyl red (MR) quenchers with 5 different fluorophores.

Disperse blue was also superior for the midrange visible fluorophores (Cy3, TAMRA). However, FAM (the shortest wavelength dye tested) was quenched slightly better by methyl red than disperse blue. This was expected because methyl red had an absorption maximum closer to the FAM emission wavelength than disperse blue.

2.5.2 Fluorescence study with doubly labelled oligonucleotides

Four doubly labelled oligonucleotides were designed (**ODN15-18**) with the common sequence:

$$^{3'}\,\mathbf{F}\text{-CCT AGG TTC AAA AAA GA-}\mathbf{Q}\,^{5'}$$

where **F** = fluorophore: FAM (**ODN15**), TAMRA (**ODN16**), Cy3 (**ODN17**), Cy5 (**ODN18**) and **Q** = quencher: DB monomer **16**

These were studied by fluorescence melting from 25-75°C. The melting characteristics were studied with two different complementary strands. One was an unmodified complement (**ODN19**) and the other was a 5'-DB modified (monomer **16**) complementary oligonucleotide (**ODN14**). For all doubly labelled oligonucleotides with an unmodified complement, the fluorescence dropped as the temperature was increased. To represent

these the clearest example with Cy5 is shown (figure 2.5.3). This behaviour could be explained by considering the conformations of the doubly labelled oligonucleotide at the two extremes: hybridised (low temp.) and single stranded (high temp.).

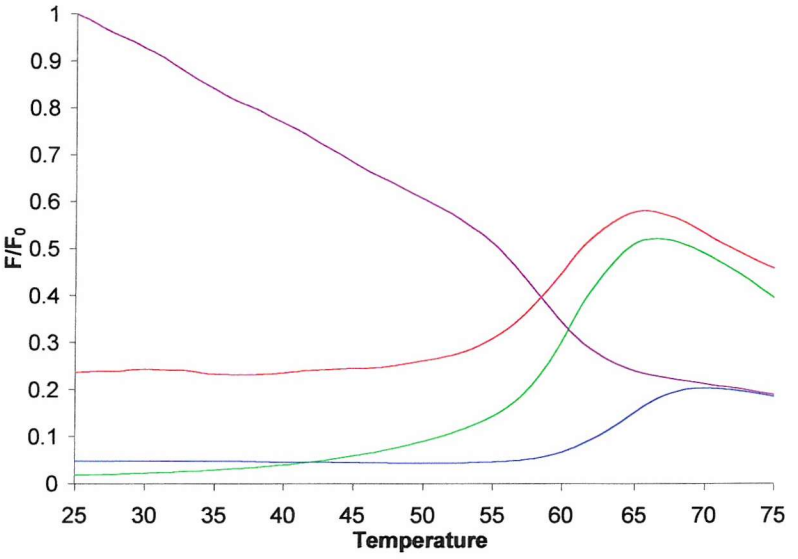


Figure 2.5.3: Fluorescence melting traces for Cy5/DB modified oligonucleotides. (a) — 3'-Cy5 (**ODN11**) + 5'-DB (**ODN14**), (b) — Doubly-labelled 5'-DB/3'-Cy5 (**ODN18**) + 5'-DB (**ODN14**), (c) — 3'-Cy5 (**ODN11**) + 5'-MR (**ODN19**), (d) — Doubly-labelled 5'-DB/3'-Cy5 (**ODN18**) + unmodified complement (**ODN19**). Fluorescence traces have been normalised i.e. F/Fo, where F= fluorecence intensity, Fo= maximum fluorescence intensity for each molecular beacon.

The oligonucleotide was 16 base pairs long so when hybridised to a complementary strand this equated to one and a half turns of the double helix. Consequently, not only would they be at opposite ends of the relatively rigid duplex, but they would orientate the fluorophore and quencher away from each other as well (figure 2.5.4).

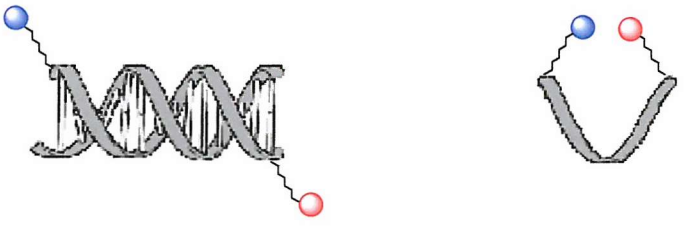


Figure 2.5.4: Diagrams showing the orientation of the fluorophore and quencher moieties for a rigid duplex 1.5 turns (left) and a single stranded oligonucleotide of the same length (right).

This increase in separation could reduce the level of quenching and so greater fluorescence would be observed. When the duplex is heated, the duplex structure dissociates and each oligonucleotide will become a highly disordered single strand. In this state it will be

possible for the fluorophore and quencher of the doubly modified oligonucleotide to come into close proximity more often and hence increase the amount of quenching.

When the doubly labelled oligonucleotides were studied with a quencher modified complementary strand similar results to the singly modified fluorescent melting were observed (figure 2.5.4). The duplex structure displayed low fluorescence, i.e. good quenching between the fluorophore in one strand and the quencher in the other at close proximity. At high temperature the doubly labelled oligonucleotide was a single strand structure and so quenching was apparent, but not as great as when in the duplex with a quencher modified complement.

These results demonstrate that the quencher worked best at close proximity to the fluorophore. Dipole orientations of the fluorophore and quencher moieties and possible intercalation into the duplex strand could also be possible reasons for reduced fluorescence quenching. It should be noted that the number of turns of the duplex is important when designing fluorescent probes and it is possible that an oligonucleotide probe could be designed in this format without the need for the hairpin structure of molecular beacons.

2.5.3 Study of DB molecular beacons

Molecular beacons have been synthesised and studied to assess the relative signal-to-noise ratios for beacons containing Cy5 (λ_{em}= 670nm) and FAM (λ_{em}= 520nm) dyes with methyl red (MR) and disperse blue monomer **16** (DB). The molecular beacon and target oligonucleotides were of general sequence:

Molecular beacon: **Q**-GCA GCC GTT TCC TCC ACT GTT GCG CTG C-**F**
Target (ODN24): GCA ACA GTG GAG GAA ACG

Oligonucleotide	Quencher, **Q**	Fluorophore, **F**
ODN20	DB	FAM
ODN21	MR	FAM
ODN22	DB	Cy5
ODN23	MR	Cy5

The fluorescent properties of these molecular beacons (**ODN20-23**) were studied with a fluorimeter over a range of temperature, with and without their target strand (**ODN24**), using a sodium phosphate buffer (0.1M, pH7, 0.1M NaCl, 1mM EDTA). In the presence of a target strand, a large increase in fluorescence was observed for all molecular beacons (figure 2.5.5).

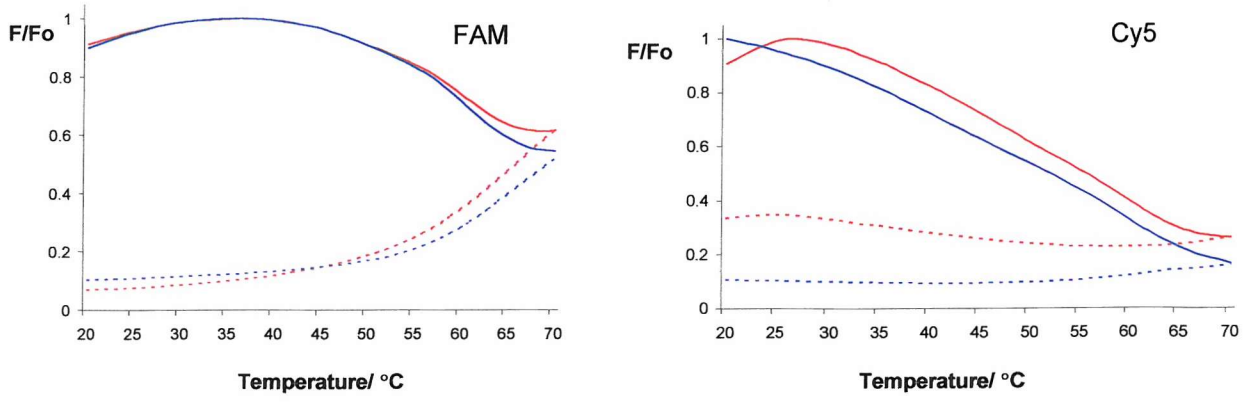


Figure 2.5.5: Fluorescence traces for the molecular beacons containing MR and DB. Key to traces: MR —; DB — . Dotted line = no target; solid line = with target. Fluorescence traces have been normalised i.e. F/Fo, where F= fluorecence intensity, Fo= maximum fluorescence intensity for each molecular beacon.

From these fluorescence traces a value for the signal-to-noise ratio was calculated for each molecular beacon (F_{max}/F_{min} = maximum fluorescence with target / minimum fluorescence without target). The signal-to-noise ratio was important because it allowed the assessment of different dye/quencher combinations relative to one another (figure 2.5.6).

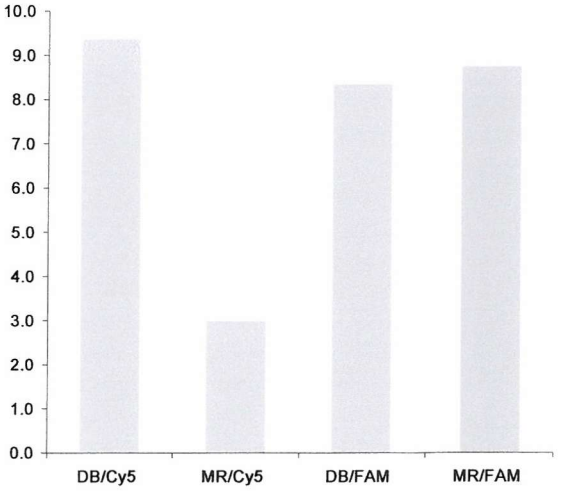


Figure 2.5.6: Signal-to-noise ratios for the molecular beacons studied.

The signal-to-noise ratios demonstrated that disperse blue (DB) was a significantly more effective quencher for the long wavelength dye Cy5. This was further evidence that methyl red was not a good quencher for long wavelength fluorescent dyes. Disperse blue and methyl red showed comparable values of signal-to-noise for the quenching of FAM with these molecular beacons.

2.5.4 TaqMan® PCR assays with DB oligonucleotides

TaqMan® probes were synthesised using the DB CPG resin **29** and tested in real-time PCR experiments based upon PCR amplification of the β-actin[31] site. All TaqMan® probes **ODN25-32** were of general formula:

TaqMan® probe: $^{5'}$ **F**-ATG CCC TCC CCC ATG CCA TCC TGC GTT-**Q** $^{3'}$

Oligonucleotide	Fluorophore, **F**	Quencher, **Q**
ODN25	TAMRA	DB
ODN26	TAMRA	MR
ODN27	ROX	DB
ODN28	ROX	MR
ODN29	JOE	DB
ODN30	JOE	MR
ODN31	FAM	DB
ODN32	FAM	MR

These oligonucleotide probes were used in a real-time PCR assay on an ABI 7700 machine and graphical readouts were obtained (figure 2.5.7). For further experimental detail see sections **4.5** and **4.6**.

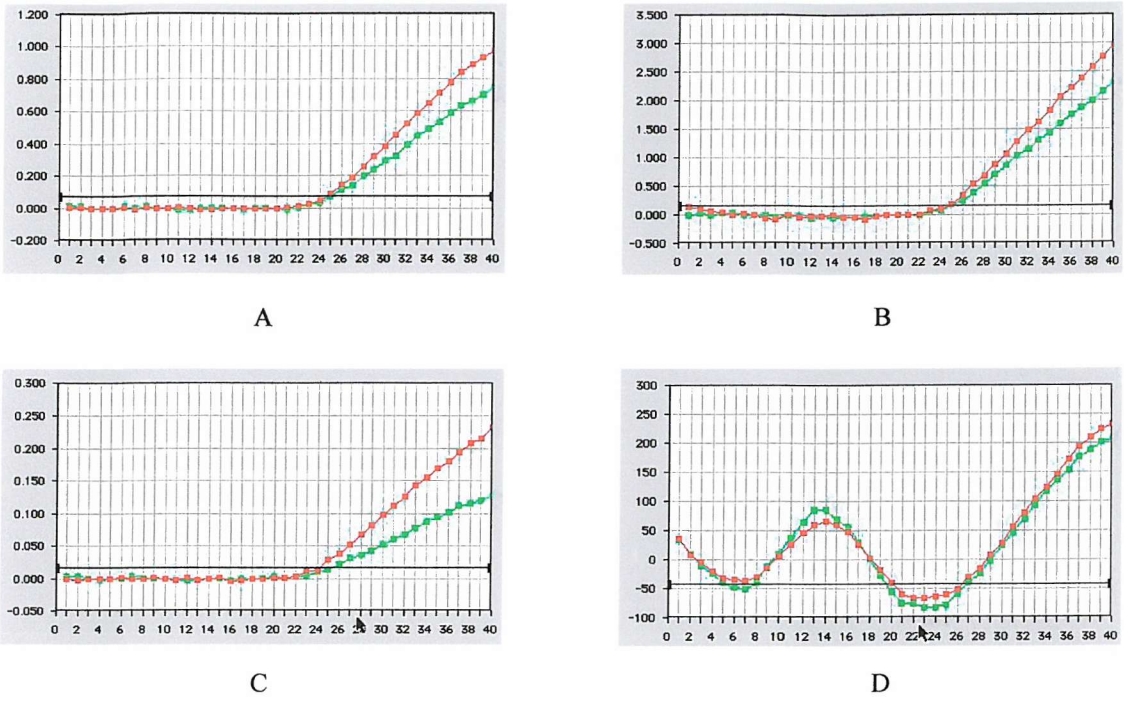


Figure 2.5.7: Comparison of disperse blue (—) relative to methyl red (—) with A) TAMRA, B) FAM, C) JOE and D) ROX as the fluorophore. X-axes: number of PCR cycles. Y-axes: relative fluorescence intensity.

In each PCR reaction the disperse blue probe showed a higher relative endpoint fluorescence than that for the methyl red probe. This was particularly pronounced for JOE where the endpoint was almost twice that of the conventional quencher and the C_t value was also improved (this is the cycle number at which the fluorescence rises above a threshold value). These results were very encouraging, indicating that disperse blue was a superior quencher to methyl red for all four dyes tested. The explanation for the greater endpoint fluorescence relative to methyl red in each case was that the disperse blue quencher was more efficient, creating a lower initial fluorescence intensity, leading to a greater range from initial to endpoint intensity.

2.5.5 Analysis of DB scorpion primers

Two "stem-loop" scorpion primers that used either methyl red (**ODN33**) or the disperse blue quencher (**ODN34**) and FAM as the fluorophore were synthesised and compared. The synthesis required that the quencher was positioned internally within the sequence, therefore the disperse blue quencher modified 2'-deoxyuridine phosphoramidite monomer **20** was used. Sequences were of the general format:

5' F-CCC GCG CCT TTC CTC CAC TGT TGC GCG CGG GQH ATG GTG TGT CTT GGG ATT CA 3'

where **F** = fluorophore: FAM; **Q** = quencher: MR (**ODN33**) or DB (**ODN34**); **H** = HEG PCR blocker.

The disperse blue quencher scorpion gave comparable real-time PCR results to the methyl red scorpion, both in terms of C_t value and fluorescent signal (figure 2.5.8). Scorpions were able to discriminate between homozygote (+/+) and heterozygote (+/-) for a cystic fibrosis mutation, where the two alleles present in the heterozygote differed by a single base in the region of the probe target.

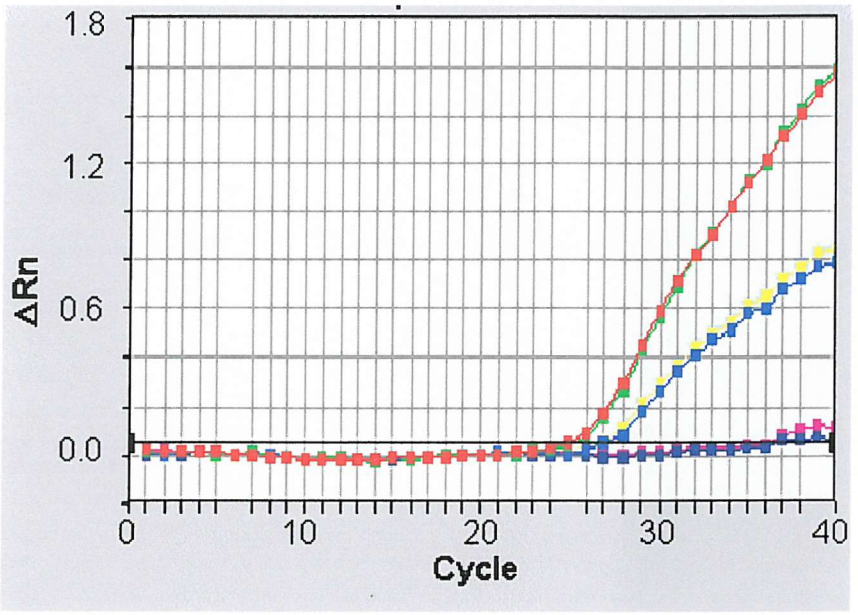


Figure 2.5.8: PCR traces for "stem-loop" scorpion primer. X-axis: relative fluorescence intensity. Y-axis: number of cycles. Disperse blue +/+; Disperse blue +/-; Disperse blue negative control; Methyl red +/+; Methyl red +/-; Methyl red negative control.

The "duplex" format scorpion primer was also tested. In this type of scorpion there is no stem-loop, the probe element has a fluorophore attached at its 5'-end and is annealed to a complementary oligonucleotide, bearing a quencher at the 3'-end. Hence, only a single fluorescent scorpion was required (**ODN35**) and two quencher oligonucleotides were synthesised with either methyl red (**ODN36**) or the disperse blue monomer **29** (**ODN37**) based on the sequences below:

Scorpion primer/probe:

5' F-CTT TCC TCC ACT GTT GCH ATGGTG TGT CTT GGG ATT CA 3'

Quencher oligonucleotide: 5' GCA ACA GTG GAG GAA AG-Q 3'

where **F** = fluorophore: FAM (**ODN35**); **H** = HEG PCR blocker; **Q** = quencher: MR (**ODN36**) or DB (**ODN37**).

Real-time fluorescence monitoring of PCR performed on the ABI Prism™ 7700 using the disperse blue quencher duplex scorpion improved upon that with methyl red, in terms of C_t and fluorescent signal (figure 2.5.9). Scorpions with both quenchers were able to discriminate between homozygote (+/+) and heterozygote (+/-) in a cystic fibrosis mutation (w1282x),[11] but the relative signal was always greatly enhanced using the disperse blue quencher.

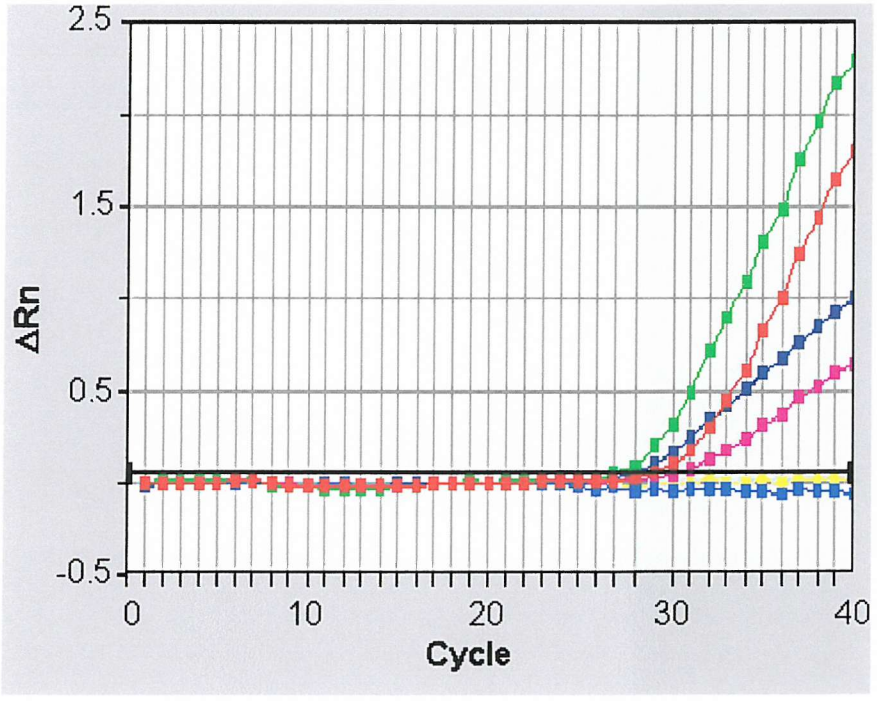


Figure 2.5.9: PCR traces for "duplex" scorpion primer. X-axis: relative fluorescence intensity. Y-axis: number of cycles. ■ Disperse blue +/+; ■ Disperse blue +/-; ■ Disperse blue negative control; ■ Methyl red +/+; ■ Methyl red +/-; ■ Methyl red negative control.

2.5.6 Distance dependence of DB quenching

Oligonucleotides were designed to assess the level of quenching over a range of distances. One of the three disperse blue monomers was used at different distances along a standard oligonucleotide of formula:

$^{5'}$ TCC AAG TTT TTT CTA $^{3'}$

The oligonucleotide was rich in thymidine nucleotides, because these could be readily replaced with the DB internal monomer **20**. Substitutions with monomer **20** were made at the thymidine positions coloured red (**ODN38-40**). Oligonucleotides were also made with a 3'-modification using monomer **29** (**ODN41**) and a 5'-modification using monomer **16** (**ODN42**), totalling 5 different positions of disperse blue. Two complementary oligonucleotides were synthesised, one with a FAM at the 3'-end (**ODN43**) and the other with FAM at the 5'-end (**ODN44**).

The fluorescent oligonucleotides (**ODN43/ODN44**, 0.1nmol) were then studied by fluorescence with and without the target quencher strand (**ODN38-42**, 5eq.), in sodium phosphate buffer (0.1M, pH7, 0.1M NaCl, 1mM EDTA). A value for the fluorescence intensity was calculated with and without target, for each possible pairing of oligonucleotides. Fluorescence with target divided by fluorescence without target gave values of the relative quenching. These values are displayed in a graphical form (figure 2.5.10), providing an indication of the relative quenching efficiency over a range of distance (in base pairs).

This method was only of limited accuracy because the disperse blue quencher monomers did not all have linkers of the same length. This gives the quencher a degree of flexibility, creating "errors" in the measurement of distance. However, a clear trend could be seen from zero separation to sixteen base pairs, as the relative fluorescence intensity increased, corresponding to decreasing quenching efficiency.

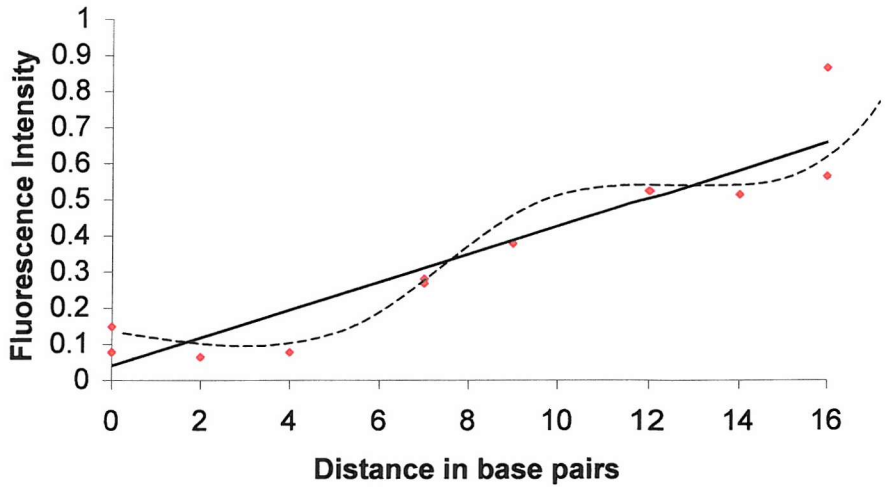


Figure 2.5.10: Distance dependence of disperse blue quenching. Quenching is greatest for lowest values of fluorescence intensity.

It also appeared that the data points formed a slightly undulating line (dotted line in figure 2.5.10) reaching a minimum at 5 and 15 base pairs (i.e. better quenching) and a maximum at 0 and 10 base pairs separation (i.e. worse quenching).

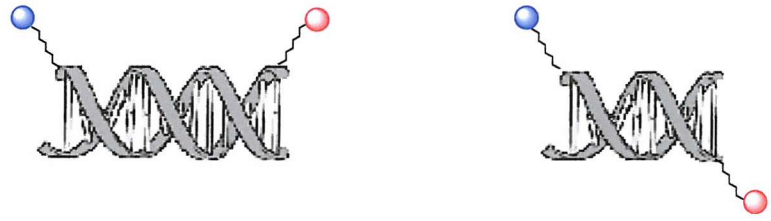


Figure 2.5.11: Diagrams showing the orientation of the fluorophore and quencher moieties for: Left: 1.5 turns (15bp); Right: 1 complete turn (10bp) of the double helix.

This could be related to the helical twist of the duplex formed (figure 2.5.11). If this was the case, the positions at 0 and 10 base pairs separation would be orientated away from each other, whereas at a distance of 5 and 15 base pairs separation the fluorophore and quencher would be on the same side of the duplex. Hence at separations of 5 and 15 base pairs, the fluorophore and quencher would be in slightly closer proximity to each other and therefore this could lead to improved quenching.

2.5.7 UV thermal melting with DB oligonucleotides

To investigate the effect of the disperse blue quencher on duplex stability, a series of oligonucleotides (**ODN38, 39, 40, 43, 45, 46, 47**) were synthesised. UV thermal melts were performed in triplicate, and the average melting temperature (T_m) value for each duplex was calculated (0.1M Sodium phosphate buffer, 0.1M NaCl, 1mM EDTA, pH7). The oligonucleotides (**ODN38-40**) contained the disperse blue quencher modified 2'-deoxyuridine **20** at three different positions. **ODN45** was a control oligonucleotide that was of the same sequence, but was unmodified. Three complements were prepared, all containing the sequence **ODN46** (blunt complement). **ODN43** differed by a 5'-FAM and **ODN47** had two additional bases (AT) at the 5'-end. The results of UV thermal melting (figure 2.5.12) demonstrated that in all cases incorporation of the disperse blue quencher had a stabilising effect on the duplex, causing an increase in T_m of up to 6.4°C.

Sequence	Oligo.	**ODN43** 5' FAM complement	ΔT_m*	**ODN46** blunt complement	ΔT_m*	**ODN47** long complement	ΔT_m*
TCCAAGTTTTTQCTA	**ODN38**	56.7	5.6	55.1	3.4	56.3	4.1
TCCAAGQTTTTTCTA	**ODN39**	54.2	3.1	55.0	3.3	55.4	3.2
TCCAAGTTTTTTCQA	**ODN40**	57.5	6.4	55.8	4.1	57.2	5.0
TCCAAGTTTTTTCTA (unmodified)	**ODN45**	51.1	-	51.7	-	52.2	-

Figure 2.5.12: UV-melting results for disperse blue oligonucleotides. *the difference in T_m between quencher modified duplexes and unmodified duplex. **Q** = Disperse blue 2'-deoxyuridine nucleotide (**20**).

The increase in T_m was greatest when the quencher was placed close to the end of the duplex (**ODN40**) and the effect was smallest when the quencher was in the middle of the duplex (**ODN39**). When the quencher was positioned in the middle of the duplex, changing the nature of the complementary oligonucleotide had very little effect on T_m. In addition, when the complementary sequence was labelled at the 5'-end with FAM there was significant stabilisation effect providing the quencher and the fluorophore were in close enough proximity. This could be due to stacking interactions between the two planar aromatic molecules.

There were two factors considered in order to explain the nature of this stabilisation effect. The disperse blue quencher nucleoside analogue **20** contained a stabilising propyne moiety at the 5-position of the heterocyclic base which was known to stabilise by 1-2°C.

However, stabilisation had also been observed with disperse blue quencher derivatives which did not contain the propyne moiety (data not shown), indicating that the stabilisation must be due to the disperse blue anthraquinone moiety. There were a number of possibilities that could cause this effect. Perhaps the most likely was that the anthraquinone was intercalating[172] (this family of structures is known to be potent DNA intercalators) into the DNA duplex due to its planar aromatic structure. Intercalation can bring about significant changes in duplex stability due to strong π-π interactions between the nucleotide bases and the planar aromatic intercalator. A second possibility was that the anthraquinone could be stacking at the 5' or 3'-end of the duplex and helping to reduce fraying. Reports of this kind of behaviour are described for cyanine dyes and related compounds.[173] Another possibility was that the molecule could be binding in the minor groove. Although the structure of the anthraquinone is not very similar to other minor groove binders, the linker contains amide bonds and points for hydrogen-bond contact.[174] The full nature of the sequence dependency of this stabilisation effect and its consequence on mismatch discrimination requires further investigation, as this could be used advantageously in the design of shorter, more specific probes.

2.6 Conclusions

Limitations with conventional quencher moieties in oligonucleotide probes (e.g. methyl red) have been observed when used with long wavelength fluorescent dyes (e.g. Cy5). Alternative chromophores were investigated for development of a novel quencher for oligonucleotide probes. Initially, diaminoanthraquinone was chosen because it had a long wavelength of absorption and primary amines for functionalisation and hence incorporation into oligonucleotides.

A diaminoanthraquinone monomer (**5**) for 5'-terminal labelling of oligonucleotide probes was synthesised and this monomer was incorporated into oligonucleotides. Appropriate deprotection conditions were ascertained and molecular beacons were studied using **5** as the quenching moiety. The quenching ability of this novel quencher was compared to methyl red, and this clearly demonstrated that **5** was a superior quencher for the long wavelength dye Cy5. A diaminoanthraquinone derivative (**13**) for incorporation at any point in an oligonucleotide backbone was also synthesised, but as with the synthesis of **5**, the initial alkylation reaction of the aromatic primary amine was low yielding. Consequently, an alternative starting material was sought for a cleaner, higher yielding synthetic route.

The alternative starting material chosen was disperse blue 3 (1-(1-hydroxyethyl)-4-(methylamino)-anthraquinone). This had a slightly longer wavelength of absorption and a hydroxyethyl moiety for derivatisation, but was only available 20% pure as a mixture. Following purification, three monomers for incorporation into oligonucleotides were synthesised. A 5'-terminal labelling monomer (**16**), an internal labelling monomer (**20**) and a 3'-labelling monomer (**29**). **16** was a simple phosphoramidite of disperse blue, **20** was a modified dU nucleoside with propyne linker for optimal stability in an oligonucleotide duplex and **29** was a functionalised dR CPG resin. Monomers **16**, **20** and **29** were incorporated into oligonucleotides and once suitable deprotection conditions were found, their fluorescence quenching characteristics were studied.

Initially the performance of monomer **16** was compared to methyl red quencher in a series of fluorescence melting experiments. Fluorescent dyes representing a region of the visible spectrum (500-700nm) were used and signal-to-noise ratios were calculated for each

quencher/dye pairing. The disperse blue monomer **16** was considerably better than methyl red at quenching the long wavelength dyes (Cy5 and Cy5.5) and slightly better for the mid range fluorophores (Cy3 and TAMRA). However, methyl red did show a very slight improvement over disperse blue in quenching the shortest wavelength dye studied (FAM). Fluorescence melting was also studied for doubly labelled oligonucleotides. These were labelled at 5'-end with monomer **16** and the 3'-end with a fluorescent dye. Complementary strands were either unmodified, or labelled with **16** at the 5'-end. These results revealed that quenching was distance dependent and greatest quenching was obtained for the most flexible single-stranded oligonucleotide structure.

Molecular beacons containing disperse blue monomer **16** were studied by fluorescence and comparison was made with methyl red. Two fluorescent dyes were used (FAM and Cy5), chosen because of the large difference in emission wavelengths (520 and 670nm respectively). The performance of the FAM containing molecular beacons (ODN20,21) were almost identical for both quenchers, but the Cy5 molecular beacons showed much better signal-to-noise ratios with **16** as a quencher relative to methyl red. These results indicate that monomer **16** is a highly efficient quencher, especially for the long wavelength fluorescent dyes. For shorter wavelength dyes the disperse blue moiety still works well, but not quite as efficiently as methyl red.

The disperse blue monomer **29** was studied in TaqMan® probes with a range of fluorescent dyes (FAM, TAMRA, JOE and ROX). Each TaqMan® probe was studied in real-time PCR by fluorescence. In all cases the probes containing disperse blue displayed a greater endpoint intensity than the methyl red containing probes. This was particularly the case for JOE where the fluorescence of the disperse blue probe was almost twice that for the equivalent methyl red probe. Monomer **29** has been shown to be a superior quencher for TaqMan® real-time PCR for all dyes studied.

Monomer **20**, designed for internal modifications to the oligonucleotide backbone, was required for the synthesis of stem-loop scorpion primer (**ODN34**). Study of this in real-time PCR showed almost identical fluorescence results to the scorpion containing methyl red (**ODN33**). As the fluorophore was FAM these results were expected, because FAM has a shorter than ideal emission wavelength for the disperse blue moiety. However, surprising

results were observed for the duplex scorpion studied (**ODN35**) with two different quencher oligonucleotides, identical except for the methyl red and disperse blue quencher moieties (**ODN36** and **ODN37**). Significant increases in fluorescence signal intensity were observed when disperse blue (**20**) was used as the quencher relative to methyl red. Improvements in C_t value and excellent homozygote/heterozygote discrimination were also observed for PCR with the disperse blue quencher oligonucleotide (**ODN37**).

All three disperse blue monomers (**16**, **20** and **29**) were studied in a series of 15mer oligonucleotides to indicate the degree of distance dependency in their level of quenching. Results were represented in a graph of fluorescence intensity versus distance in base pairs. A clear trend could be seen of decreasing quenching efficiency with increasing distance.

Finally, disperse blue monomer **20** was studied in a series of UV-melting experiments with oligonucleotides containing the modified nucleoside at different positions. Three complementary oligonucleotides were used, differing in length or modified with a FAM fluorescent dye. The quencher moiety increased the melting temperature in all cases by as much as 5°C with unmodified complements. An increase in T_m of up to 6.4°C was observed when hybridised to the FAM containing complementary strand. These results demonstrate that the disperse blue moiety is interacting with the oligonucleotide duplex in some way, but more work is needed to know what exactly is going on. These increases in T_m need to be taken into account when designing TaqMan® probes, molecular beacons and scorpions. The T_m increase might allow shorter, more specific probes to be used.

3. STABILISATION OF OLIGONUCLEOTIDE PROBES

3.1 Introduction to investigations into oligonucleotide stabilisation

By increasing duplex stability the required length of an oligonucleotide probe can be reduced and the destabilising effect of a single base pair mismatch enhanced. This has the desirable effect of increasing probe specificity. Therefore the stabilisation of oligonucleotide probes is an important goal. However, it is also important not to stabilise a probe in a manner that will encourage non-specific binding to occur. This may result if a large number of stabilising moieties are introduced into an oligonucleotide, particularly moieties that bind in a non-specific manner, e.g. protonated amines.

There are many ways of stabilising probe/target duplexes, including modifications to the base, sugar or backbone. It is also possible to incorporate intercalating or minor groove binding molecules into an oligonucleotide, imparting strong duplex-stabilising properties to the probe. Increasing the stability of the probe/target duplex is not the only requirement of an effective probe; for an oligonucleotide probe to be functional it must discriminate between a fully complementary strand and a mismatched strand to the level of a single base pair mismatch in twenty or more base pairs.

EPOCH Pharmaceuticals Inc. recently developed the Eclipse probe^{TM} system which incorporates a minor groove binding molecule at the 3'-end of the oligonucleotide. Large increases in duplex stability are observed with some targets [164] and as with other minor groove binding molecules it binds to A.T rather than G.C rich tracts. A 12mer Eclipse probe has the same T_m as an unmodified AT-rich 27mer probe, leading to discrimination at the level of a single base pair mismatch. An advantage of shorter probes is the closer proximity of fluorophore and quencher, leading to improved fluorescence quenching and hence better signal-to-noise ratios. Oligonucleotide probes have also been functionalised with spermine (poly-amine) residues to improve duplex stability by decreasing the repulsion between two oligonucleotide strands.[175] A large increase in stability was observed, but significant non-specific binding also occurred.

Our approach to duplex stabilisation was to find ways to precisely control the increase in stability by introducing several individual nucleotide modifications, rather than a single

DNA-binding moiety. In this chapter studies on the stabilisation properties of a number of modified nucleotides are presented.

3.2 Study of propyne-dU and diaminopurine

3.2.1 Introduction to p-dU /DAP study

C5-Propyne modified pyrimidine nucleotides (p-dU) and 2-aminoadenosine (DAP) have been previously studied in detail.[107] However, their effects with a mismatched target oligonucleotide have not been studied so thoroughly. In this study p-dU (**33**) and DAP (**37**) phosphoramidite monomers were synthesised by the literature methods[122,176] and incorporated into oligonucleotides to study the effect of modifications on duplex stability with mismatch target and fully complementary strands.

3.2.1 Synthesis of 5-propyne-dU phosphoramidite[176]

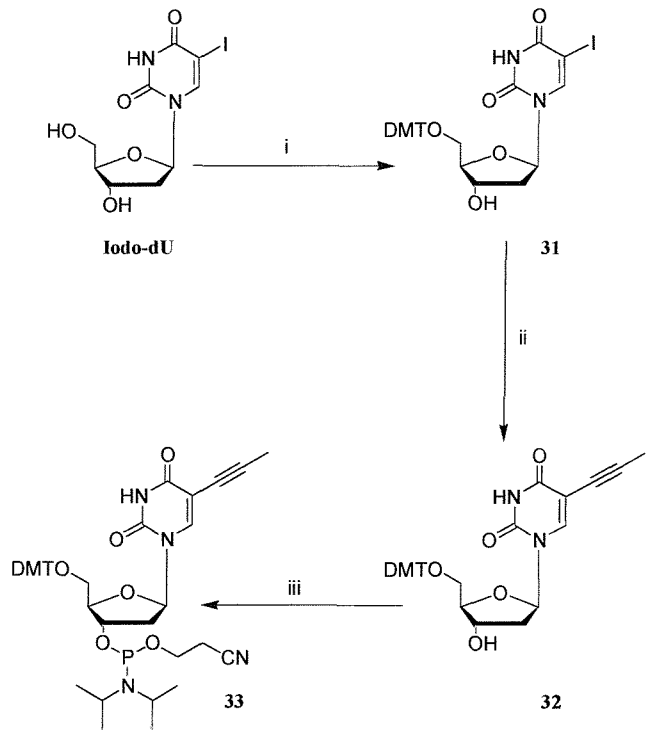


Figure 3.2.1: Regents and conditions: i) DMTCl (1.1eq), DMAP (0.5mol%), pyridine, 25°C, 14hrs. (70%); ii) Propyne (3eq), Cu(I)I (0.2eq), NEt_3 (4eq), $Pd(PPh_3)_4$ (0.1eq), DMF, 25°C, 15hrs. (12%); iii) 2-Cyanoethoxy-*N,N*-diisopropylamine chlorophosphine (1.1eq), DIPEA (4eq), THF, 25°C, 1.5hrs, under argon. (89%)

The propyne-dU phosphoramidite **33** was synthesised from 5-iodo-2'-deoxyuridine in a three step procedure (figure 3.1).[176] The first and third steps proceeded as described in the literature with yields of 70% and 89% respectively. The second step was a Sonogashira cross-coupling with Pd(0) catalyst, with modifications described by Hobbs.[177] In small-

scale reactions, adding all solid starting materials to the reaction flask and drying in a vacuum dessicator overnight worked particularly cleanly, but when performed on a large scale this was not so successful. The propyne (condensed by dry-ice/acetone bath into DMF) was in the liquid phase when transferred to the reaction vessel, but gradually evaporated into the atmosphere above the reaction mixture. The reaction was repeated with excess propyne and subsequent additions were also made. Difficulty in purification of the product by column chromatography was experienced and DCM/MeOH (0-3%) was found to be a much better eluant than that suggested in the literature[177] (EtOAc/Hexane, 1:1-3:1). Unfortunately the yield in this step was very low (~12%).

3.2.2 Synthesis of 2-amino-dA phosphoramidite[122]

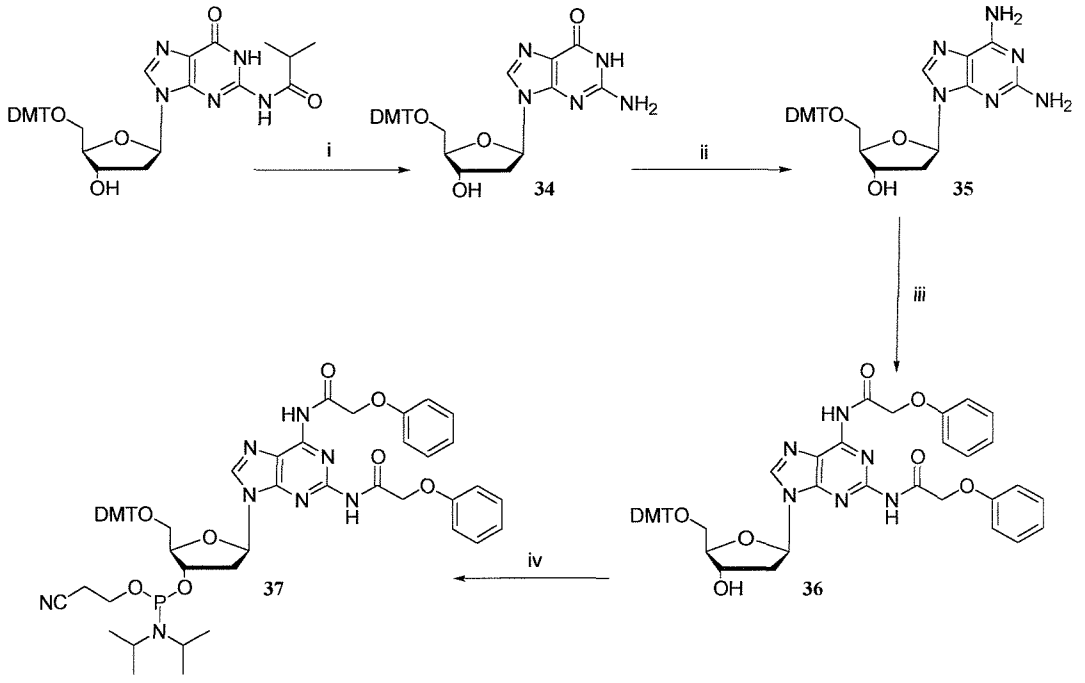


Figure 3.2.2: Reagents and conditions: i) Conc. ammonia/dioxane (1:1), 25°C, 120hrs, (79%); ii) TFA anhydride, pyridine, 0°C, 15mins, then, ammonia gas bubbled through for 2hrs (anhyd), pyridine, -20°C, 48hrs, (30%); iii) TMSCl (10eq), pyridine, 0°C, 30min, then, phenoxyacetic anhydride (5eq), pyridine, 25°C, 15hrs, (22%); iv) 2-Cyanoethoxy-*N,N*-diisopropylamine-chlorophosphine (1.1eq), DIPEA (4eq), THF, 25°C, 1.5hrs, (90%).

2-Amino-2'-deoxyadenosine phosphoramidite monomer **37** was synthesised from 5'-DMT-N^2-^{i}Bu-deoxyguanosine following a four stage synthetic procedure as described by Gryaznov and Schultz.[122] Following the initial deprotection, **34** was reacted in crude form to yield 2-amino-2'-deoxyadenosine (**35**) (2 steps: 24%). The amines were protected with

phenoxyacetyl, but problems were encountered seperating **36** from the two mono-protected by-products by silica gel column chromatography. Elution with DCM/acetone (9:1-7:3) gave **36** in modest yield (22%). Finally the phosphoramidite moiety was introduced to the 3'-hydroxyl group following a standard procedure[6] and the product (**37**) was isolated using a short silica column (EtOAc), under argon in 90% yield.

3.2.3 Study of p-dU (33) and DAP (37) fidelity

The 5-propyne-2'-deoxyuridine (**33**) and 2-amino-2'-deoxyadenosine (**37**) nucleosides were incorporated into oligonucleotides in place of thymidine and adenosine respectively. Early experiments had shown that 8mer oligonucleotides were too short (T_m too low). Instead 10mer oligonucleotides (**ODN48-67**) were synthesised, which were expected to form more stable duplexes, thus simplifying the study. These oligonucleotides were of the general sequence:

$^{5'}$ GCG TAA TGC G $^{3'}$
$^{3'}$ CGC ATT ACG C $^{5'}$

and were studied by UV-melting (0.1M sodium phosphate buffer, pH7.0, 1M NaCl, 1mM EDTA) to determine their melting temperatures (T_m). In addition to the complementary oligonucleotide pairings, a number of single nucleotide mismatch oligonucleotides were synthesised. In each case the mismatch was opposite the modified base. A full table of results is included in the appendix, but a summary is shown below (figure 3.2.3). This table shows the oligonucleotide sequences in the left-hand column with the base pair studied marked as **X** and **Y**. The identities of **X** and **Y** are given in the second column and for each combination the average melting temperature is shown. Also indicated is the nature of the base pair; i.e. Watson-Crick (wc), or mismatch (m). The oligonucleotide code numbers are shown for reference with section 4.6.

Oligonucleotide sequences	Base pair **X.Y**	Oligonucleotide numbers	Average T_m (°C)
$^{5'}$CGCA**X**TACGC$^{3'}$ $^{3'}$GCGT**Y**ATGCG$^{5'}$	**p-dU.A** (wc)	**ODN48/49**	54.3
	T.A (wc)	**ODN52/49**	53.0
	p-dU.G (m)	**ODN48/50**	38.9
	T.G (m)	**ODN52/50**	40.8
	p-dU.C (m)	**ODN48/51**	34.9
	T.C (m)	**ODN52/51**	34.7
$^{5'}$GCGTAA**X**GCG$^{3'}$ $^{3'}$CGCATT**Y**CGC$^{5'}$	**p-dU.A** (wc)	**ODN53/52**	55.2
	T.A (wc)	**ODN49/52**	53.4
	p-dU.G (m)	**ODN53/54**	38.1
	T.G (m)	**ODN49/54**	39.0
	p-dU.C (m)	**ODN53/55**	33.9
	T.C (m)	**ODN49/55**	31.2
$^{5'}$CGC**X**TTACGC$^{3'}$ $^{3'}$GCG**Y**AATGCG$^{5'}$	**DAP.T** (wc)	**ODN56/49**	56.1
	A.T (wc)	**ODN52/49**	53.4
	DAP.G (m)	**ODN56/57**	38.2
	A.G (m)	**ODN52/57**	42.7
	DAP.C (m)	**ODN56/58**	26.1
	A.C (m)	**ODN52/58**	26.2
$^{5'}$GCGT**X**ATGCG$^{3'}$ $^{3'}$CGCA**Y**TACGC$^{5'}$	**DAP.T** (wc)	**ODN59/52**	54.9
	A.T (wc)	**ODN49/52**	53.5
	DAP.G (m)	**ODN59/60**	37.5
	A.G (m)	**ODN49/60**	39.4
	DAP.C (m)	**ODN59/61**	37.9
	A.C (m)	**ODN49/61**	31.0
$^{5'}$GCG**X**ATTGCG$^{3'}$ $^{3'}$CGC**Y**TAACGC$^{5'}$	**DAP.T** (wc)	**ODN62/63**	58.8
	A.T (wc)	**ODN64/63**	57.3
	DAP.G (m)	**ODN62/65**	35.7
	A.G (m)	**ODN64/65**	38.5
	DAP.C (m)	**ODN62/66**	42.3
	A.C (m)	**ODN64/66**	39.6
$^{5'}$CGC**X**TT**X**CGC$^{3'}$ $^{3'}$GCG**Y**AA**Y**GCG$^{5'}$	**DAP.T** (wc)	**ODN 67/49**	58.8

Figure 3.2.3: A summary of the UV-melting results collated for both propyne-dU and 2-amino-dA modified oligonucleotides. (wc) = Watson-Crick base pair, (m) = mismatch base pair.

Both base modifications lead to an increase in stability of the pseudo-Watson-Crick base pair, in agreement with the literature (refer to first two rows in each section of figure 3.2.3).[107,108] For p-dU (**ODN48,53**) this was ~1.5°C, whilst for DAP (**ODN56,59,62**) it varied from 1.4-2.7°C for a single modification. These modifications also destabilised some mismatch base pairs, particularly G.T and G.A, which can otherwise be relatively

stable. However, for C-containing mismatches there was often a small stabilisation. This is not important because the C.T and C.A mismatches are intrinsically much less stable than an A.T match (>10°C). The nature of the stabilisation of C-containing mismatches is not known at the present time, but warrants further investigation.

A slight variation in the stability of different sequences with a single base modification was observed. This indicates that the degree of stabilisation for a particular modification is dependent on oligonucleotide sequence/base stacking. The doubly modified DAP oligonucleotide (**ODN67**) displayed a greater increase in stability (2.7°C), than the singly modified oligonucleotide (**ODN56**).

In conclusion, the results suggest that these modifications could be employed to advantage in probes such as molecular beacons for stabilising stem structures, shortening probe regions (hence increasing specificity), and destabilising mismatches in probe/target duplexes.

3.3 Study of stabilisation with amino moieties

3.3.1 Introduction

Studies with DAP (**37**) and p-dU (**33**) revealed increases in duplex stability by ~2°C per modification. However, the literature[107,112,122] states that the additional stabilisation caused by each subsequent addition is progessively smaller. Hence, if these nucleosides were incorporated into oligonucleotide probes, only a limited stabilisation would be possible. There would come a point where the addition of another modified nucleoside would increase the stability by such a small amount that it would not be worthwhile. Therefore alternative methods of duplex stabilisation are worth pursuing. This section describes studies to introduce amino groups onto nucleosides and their effect on duplex stability. In particular the propargyl amino moiety was studied. This structure incorporates the propyne and amino functionalities. Some initial investigations have been made by Heystek *et al.*[113] who reported large stabilisation with high levels of specific binding. It was also decided to investigate modification of the deoxyribose sugar to incorporate amino groups and the consequence of such modifications on duplex stabilisation. Recently 2'-aminoethoxy modified nucleosides have been developed for stabilisation of triplexes,[135] but their hybridisation properties in oligodeoxynucleotide duplexes have not been described.

Propargylamino deoxyuridine was synthesised[176] and compared with p-dU and 2'-aminoethoxy-dU[135] [φ] for singly, doubly and triply modified short oligodeoxynucleotide duplexes. It was envisaged that by studying these compounds, the best modifications for optimum stability would be discovered.

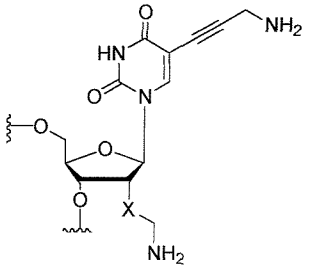


Figure 3.3.1: Doubly modified monomer for increased duplex stability (where X = OCH_2, CH_2, or something else)

[φ] This phosphoramidite monomer was provided by Matthieu Sollogoub in TB research group.

Study of these C5-pyrimidine and 2'-ribose modifications were carried out with a view to incorporating both modifications into a single nucleoside (figure 3.3.1). This would hopefully lead to a highly stabilising nucleoside monomer. Hence fewer modified nucleosides would be needed for maximum effect.

3.3.2 Synthesis of 5-propargylamino-dU phosphoramidite [176]

5-Iodo-5'-*O*-(4,4'-dimethoxytrityl)-2'-deoxyuridine (**31**) was subjected to a Sonogashira Pd(0) coupling (figure 3.3.2), with propargyltrifluoroacetamide to yield nucleoside **38** (NB. The trifluoroacetate protecting group can be easily removed under conventional oligonucleotide deprotection). The 3'-hydroxyl group was functionalised as a phosphoramidite by the usual method[6] to give monomer **39** in good yield (73%).

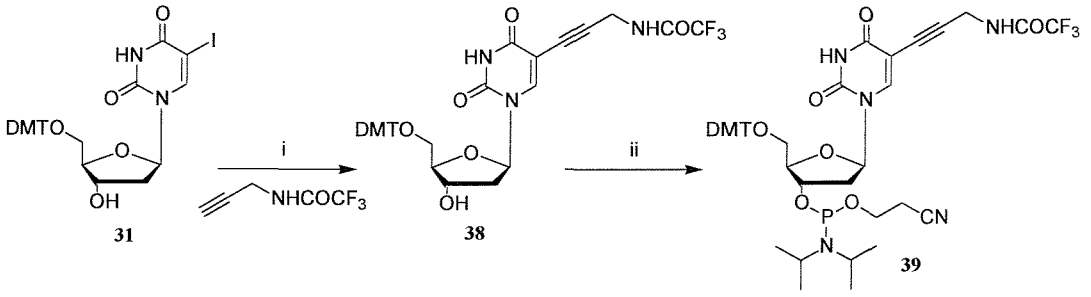


Figure 3.3.2: Reagents and conditions: i) $Pd(PPh_3)_4$ (0.1eq), CuI (0.2eq), NEt_3 (5eq), DMF, rt, 4hrs (69%); ii) 2-Cyanoethoxy-*N,N*-diisopropylamine chlorophosphine (1.1eq), DIPEA (2.2eq), THF, rt, 2hrs (73%).

3.3.3 Determination of amino moiety significance by UV melting

Three sets of 10mer oligonucleotides were synthesised for the pa-dU monomer **39** **(ODN53,68,69)**, 2'-aeo-dU[135] (**ODN70,71,72**) and p-dU monomer **33** (**ODN73,74,75**). For each modification a singly, doubly and triply modified oligonucleotide was synthesised. The relative duplex stabilisation effects of these oligonucleotides with their unmodified complementary strands (**ODN52** and **ODN77**) were studied. Comparison was made with unmodified control oligonucleotides (**ODN49** and **ODN76**).

unmodified control 1	1 modification	2 modifications
5'GCGTAATGCG3'	5'GCGTAA**X**GCG3'	5'GCG**X**AA**X**GCG3'
3'CGCATTACGC5'	3'CGCATTACGC5'	3'CGCATTACGC5'

unmodified control 2	3 modifications	
5'GCGTAATGTG3'	5'GCG**X**AA**X**G**X**G3'	**X** = modified nucleoside
3'CGCATTACAC5'	3'CGCATTACAC5'	(p-dU, pa-dU, 2'-aeo-dU)

	p-dU		pa-dU		2'-aeo-dU	
1 modification	2.5	**ODN53/52**	2.5	**ODN70/52**	-2.7	**ODN73/52**
2 modifications	6.1	**ODN68/52**	6.7	**ODN71/52**	-5.4	**ODN74/52**
3 modifications	4.2	**ODN69/77**	6.5	**ODN72/77**	-1.3	**ODN75/77**

Figure 3.3.3: T_m data for the three different modified nucleosides. All values are in °C at pH 7.0 and show relative stabilisation to the respective unmodified duplex (see text) with 0.1M sodium phosphate buffer (1M NaCl, 1mM EDTA). See Appendix I for full T_m data.

In this experiment the p-dU oligonucleotides (**ODN53,68,69**) were used as references, because of the previous studies and a number of publications relating to oligonucleotides containing this compound.[107,112] Pa-dU oligonucleotides (**ODN70,71,72**) were seen to stabilise by a similar amount to p-dU (**ODN53,68,69**), but as additional numbers of modifications were introduced, the stabilisation increased significantly more for pa-dU than p-dU oligonucleotides (figure 3.3.3). A possible reason for this is that the amino functionalities are helping to neutralise the repulsion of the two negatively charged phosphate backbones. The effect of singly modified oligonucleotides appears to be the same for p-dU (**ODN53**) and pa-dU (**ODN70**), but when oligonucleotides with three modifications are studied pa-dU (**ODN69**) stabilises by 2.3°C more than p-dU (**ODN72**).

These initial observations suggest that a positive effect from amino groups becomes particularly significant when multiple modifications are present. If amino modifications work best in higher abundance, then a doubly modified nucleoside (figure 3.3.1) could be an effective way of achieving extra modification efficiently.

Oligonucleotides containing 2'-aeo-dU (**ODN73,74,75**) significantly destabilised all duplexes. The magnitude of this destabilisation appears to be site/sequence specific. A likely cause for this is that the 2'-aeo-dU monomer adopts a C3'-endo conformation (c.f. RNA, figure 3.3.4), which may destabilise the duplex with a DNA strand (C2'-endo conformation). There is evidence for this C3'-endo conformation of 2'-aeo-dU containing oligonucleotides in the literature[135] and sugar conformation is thought to significantly affect duplex stability.

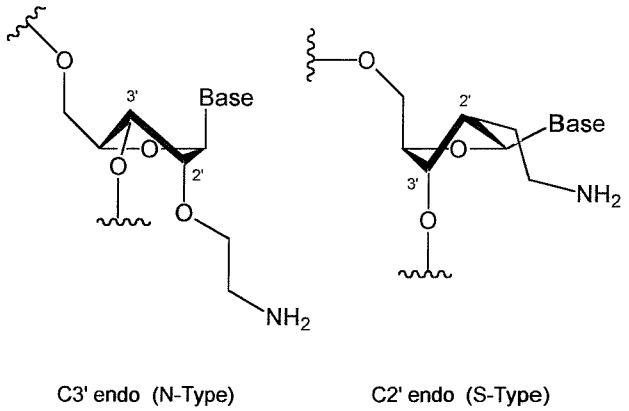


Figure 3.3.4: Proposed conformations of the furanose ring for different 2'-modifications. Left: with a heteroatom at the 2'-position. Right: with no heteroatom at the 2'-position.

These results look very promising and development of a suitably doubly labelled nucleoside monomer was now particularly attractive. To achieve this, further development of the 2'-modification was needed. This prompted investigation into the synthesis of 2'-aminoethyl-dU, with no electronegative heteroatom at the 2'-position. This modification should allow the nucleoside to adopt a C2'-endo configuration more readily (figure 3.3.4) thus avoiding destabilisation of the B-type duplex.

3.4 Investigation into synthesis of 2'-aminoethyl nucleosides

3.4.1 Consideration of synthetic routes available

To introduce the 2'-aminoethyl functionality, a number of routes were considered:

1. Build up the furanose sugar with the 2'-functionality and then glycosylate the base (figure3.4.1).[178]

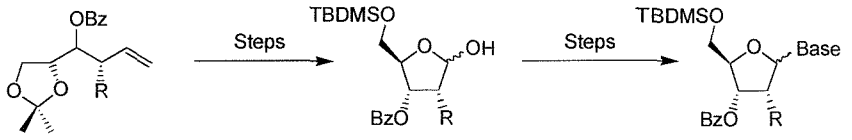


Figure 3.4.1: Synthesis of a 2'-*C*-alkyl-2'deoxynucleoside from an acyclic starting material.

This synthesis is based upon a published route following the methods by Schmidt *et al.* The literature describes routes for methyl and phenyl "R groups",[178] so some changes would be required to introduce the desired 2'-aminoethyl group. This would be a long synthetic route with the possibility of a low yield for the glycosylation step due to the formation of α and β anomers. However, it might be a general route to a number of different nucleoside derivatives as the modified sugar could be used to glycosylate any chosen nucleoside base.

2. Synthesise the 2,2'-cyclouridine molecule and then alkylate with a carbon nucleophile at the 2'-position (figure 3.4.2).

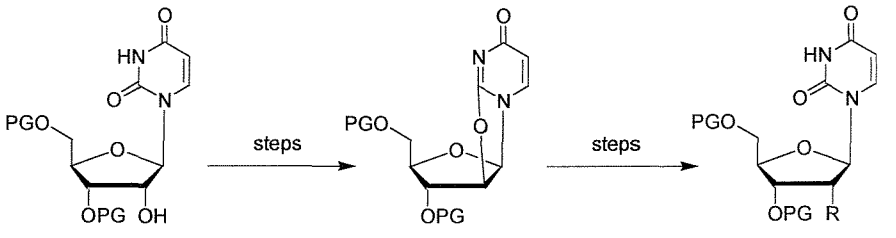


Figure 3.4.2: Synthesis of 2'-*C*-alkyl-2'-deoxynucleoside *via* 2'-anhydro derivative.

Cyclisation of 5'-*O*-trityluridine by basic diphenylcarbonate is reported to proceed in good yield.[179] There are also methods described for the alkylation of the 3'-position *via* a 2,3'-

cyclothymidine compound, but yields with carbon nucleophiles are relatively low.[180] This route looks interesting, but the alkylation step may not be reliable.

3. Functionalise the 2'-hydroxyl of a ribose nucleoside with a phenoxysulfanone group and create a radical for alkylation by an allyltributyltin (figure 3.4.3).[181,182]

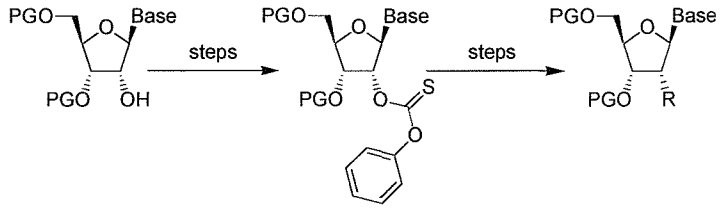


Figure 3.4.3: Synthesis of 2'-*C*-alkyl derivative *via* radical alkylation.

Most steps in this route have been described recently in reputable journals, quoting good yields, so it should therefore be reliable.[181] The phenoxysulfanone group is replaced with an allyl substituent which can then be oxidised to the 2'-hydroxyethyl derivative.[182] Functional group conversion of this alcohol to an amine should then be relatively simple to yield the desired compound. One minor drawback for this route is that a ribonucleoside is required as a starting material so uridine will have to be used, unless further steps are introduced to synthesise ribothymidine for use as a starting material.

4. Use a 2'-deoxyribose nucleoside and perform an intramolecular C-H insertion reaction at the 2'-position (figure 3.4.4).[179]

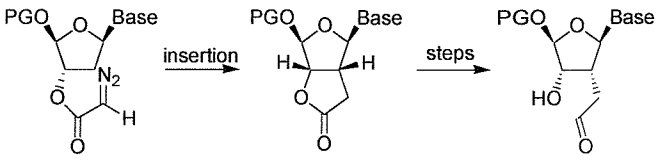


Figure 3.4.4: Synthesis of 2'-*C*-alkyl nucleoside *via* an intramolecular C-H insertion.

This C-H insertion has been described in a recent communication and is reported to proceed in good yield.[179] Procedures are described for the reduction of the intermediate butyrolactone to the aldehyde, which can be further reduced to the 2'-hydroxyethyl compound.[183,184] An advantage of this synthesis is that the insertion has been described for

all natural deoxynucleosides, so starting materials should be readily available and relatively cheap.

Of these synthetic routes, those outlined in 3 and 4 had more literature precedent. The C-H insertion method would allow use of a 2'-deoxyribose starting material, hence 2'-deoxy thymidine could be used. This is a natural, relatively cheap, starting material, which only requires a single protective group on the 5'-hydroxyl. Thymidine also shows a slight duplex stabilisation relative to uridine.[107]

3.4.2 Discussion on C-H insertions

Intramolecular [1,5] C-H insertion reactions of α-diazocarbonyl compounds with dirhodium(II) catalysts have been used extensively for the construction of functionalised five membered rings. Dirhodium(II) tetraacetate has been used to cyclise substrates *via* C-H insertion, however there is only a single report of it being used to functionalise the furanose ring of nucleosides.[179] Lim *et al.* describe intramolecular C-H insertion reactions on a nucleoside substrate, with a range of 2'-deoxy-3'-diazocarbonyl derivatives (diazoester, diazo-β-ketoester, diazomethoxy-β-ketoester and diazoethoxy-β-ketoester). All isolated yields quoted are 60% or higher, after a short period of reflux (1-3hrs) in dichloromethane with dirhodium(II) tetraacetate catalyst. It is unusual for a C-H insertion of this type to require heating to reflux temperature, but Lim *et al.* found only trace amounts of insertion product were recovered otherwise.

The rhodium catalyst used exists as a binuclear structure with two vacant co-ordination sites (figure 3.4.5).

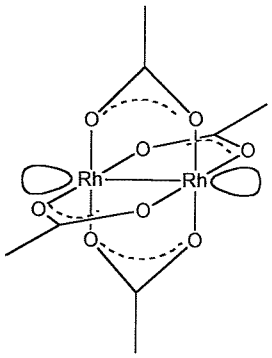


Figure 3.4.5: Structure of rhodium acetate catalyst displaying vacant co-ordination sites.

These vacant acceptor sites bind Lewis basic donor ligands. The mechanism of the rhodium(II) mediated C-H insertion reaction of diazocarbonyl compounds is thought to proceed *via* the proposed mechanism (figure 3.4.6).

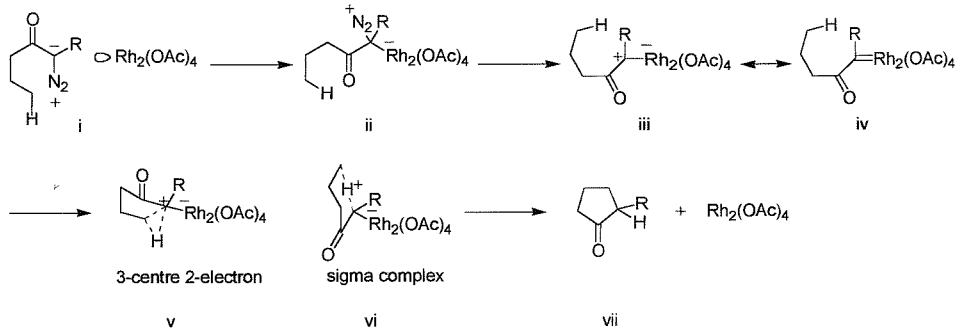


Figure 3.4.6: Proposed mechanism for intramolecular C-H insertion of diazoester with rhodium acetate catalyst.

It has been suggested[185] that an intermediate metal carbene is generated (figure 3.4.6) by electron donation from the electron rich alpha carbon of diazoketone with one of the vacant rhodium co-ordination sites (ii). The nitrogen is expelled to yield the metal carbene (iii). This carbenoid can be perceived as a ylide-type structure, possessing an electrophilic p-orbital capable of interacting with a neighbouring sigma C-H bond. Insertion proceeds *via* either a three-centre two-electron system (v), or a sigma complex (vi). Finally, the complex collapses to produce the cyclisation product and regenerated catalyst (vii) (figure 3.4.6).

Although the intramolecular C-H insertion reaction is favoured kinetically, it is not exclusive. In fact there are three major reaction pathways for metallocarbenes: olefin cyclopropanation, ylide formation and X-H insertion (where X= C, Si, O, N). Therefore for a given substrate several reaction pathways are sometimes possible. The chemoselectivity is often dependent on both the substrate and the catalyst. Generally for C-H insertions, formation of a five-membered ring is favoured, but isomers can be observed for equidistant C-H bonds. These insertions also proceed with a high retention of stereochemistry. Site selectivity and stereoselectivity can be controlled using steric, electronic and conformational factors. C-H insertions proceed with a clear order of reactivity from methine to methylene to methyl, due to their decreasingly electron-rich nature. The selectivity for methine has been enhanced, using a more electron rich catalyst, or by introducing heteroatoms capable of donating electron density to an adjacent carbon-hydrogen bond. Electron withdrawing groups α to C-H hinder insertion. In some cases the

electronic preference for methine is so strong that a four membered spiro-ring is formed instead of the usual five membered ring, as studied by Ikegami *et al.* (figure 3.4.7).

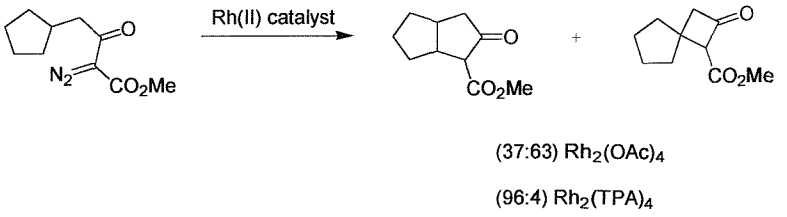


Figure 3.4.7: Control of the C-H insertion by increasing the steric bulk of the catalyst.

Ikegami *et al.*[186] controlled this problem by preparing a bulky catalyst: rhodium(II) tetra(triphenylacetate) ($Rh_2(TPA)_4$). When cyclisation was carried out with the more sterically crowded catalyst the [1,5] insertion product was formed despite the electronic preference for methine (figure 3.4.7).

3.4.3 Attempted synthesis of 2'-aminoethyl-dT

A synthesis was devised (figure 3.4.8), initially with 4,4'-dimethoxytrityl protection of the 5'-hydroxyl (convenient for oligonucleotide synthesis). The acyl chloride **41** was prepared by the method of House & Blankley[187,188] *via* the acid **40**. The 3'-diazoester derivative of thymidine (**45**) was then obtained from reaction of **41** with DMT-dT (**42**).[189]

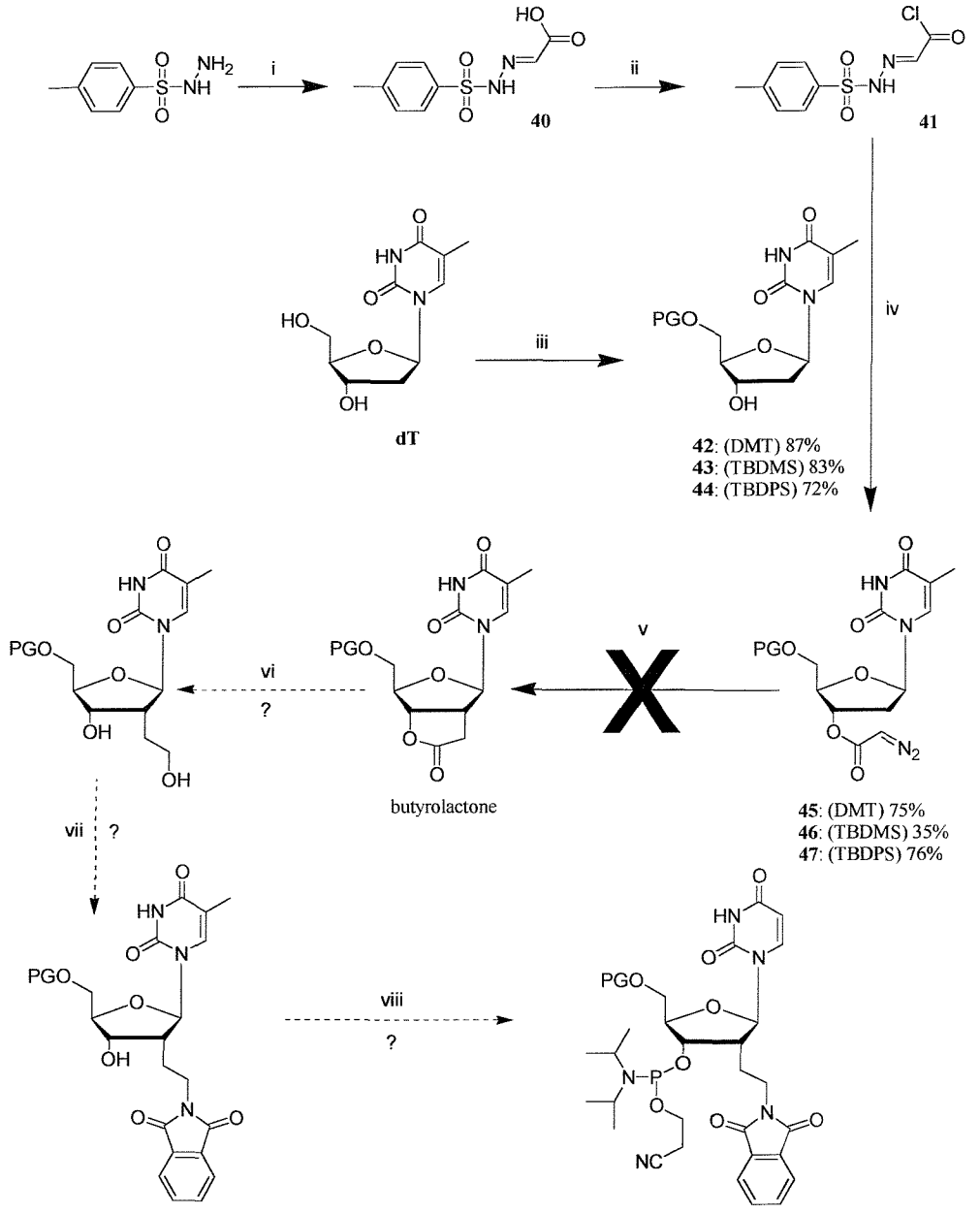
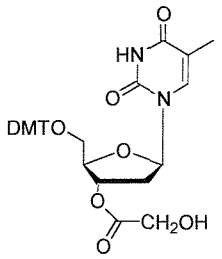


Figure 3.4.8: Reagents and conditions: i) Glyoxylic acid (1eq), HCl, H_20, 60°C, 1hr; ii) $SOCl_2$ (2.2eq), toluene, reflux, 2hrs iii) Protecting group chloride (i.e. DMTCl 3hrs, TBDMSCl 5hrs, TBDPSCl 15hrs) (1.1eq), pyr, rt; iv) Dimethylaniline (1.9eq), CH_2Cl_2, 0°C, 15min, then Et_3N (5eq), 0°C, 10min, then rt, 15min; v) $Rh_2(OAc)_4$ (10mol%), CH_2Cl_2, reflux; vi) DIBAL, THF, -78°C - rt; vii) Phthalimide, DEAD, PPh_3, THF; viii) 2-Cyanoethoxy-*N,N*-diisopropylamine chlorophosphine, DIPEA, THF, rt;. X = failed reaction. ? = not yet attempted. PG = protecting group (TBDPS, TBDMS or DMT).

The C-H insertion with rhodium catalyst,[179] should then have been used to afford the γ-butyrolactone, but this reaction failed to yield any significant amount of the desired product. In an attempt to rectify this problem, the silyl protecting groups *tert*-butyldimethylsilyl and *tert*-butyldiphenylsilyl[179] were each used instead, but no

improvement in yield of the required product was observed. The reaction was also attempted at low temperature (0°C), but only **48** was recovered (figure 3.4.9). Presumably the carbenoid formed was inserting into an HO-H bond rather than the desired C-H bond, despite the precautions of dry apparatus and solvents. This suggested that the carbenoid was forming, but that it was reluctant to insert into the desired C-H bond. This was particularly surprising because carbenes are normally so reactive they react intramolecularly before any intermolecular reaction can take place.[190]



48

Figure 3.4.9: Product of carbene insertion reaction at 0°C, compound **48**.

It was decided to study the C-H insertion further with an alternative substrate made by a different method. If a major insertion product was found then the conditions could be optimised and the reaction attempted with the other diazoesters (**45**, **46**, **47**). Routes to the 3'-*O*-β-ketodiazoester nucleosides (**52**, **53**) were pursued (figure 3.8.4). The β-ketodiazoester is a widely used substrate for carbenoid formation[191] and its reaction with the 2'-CH_2 of a nucleoside is described by Lim *et al.*[179] However, an alternative route to the β-ketoester was followed. This route utilised the 2,2,6-trimethyl-4*H*-1,3-dioxin-4-one reagent in xylenes, which on heating rearranges to form a ketene (figure 3.4.9). [192,193]

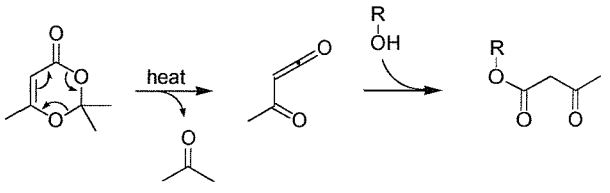


Figure 3.4.9: β-Ketoester formation from 2,2,6-trimethyl-4*H*-1,3-dioxin-4-one.

This highly reactive intermediate is trapped by the hydroxyl group of the nucleoside to yield the β-ketoester. Diazo transfer with *p*-carboxybenzenesulfonyl azide then furnished the desired diazoester in good yield (figure 3.4.10).

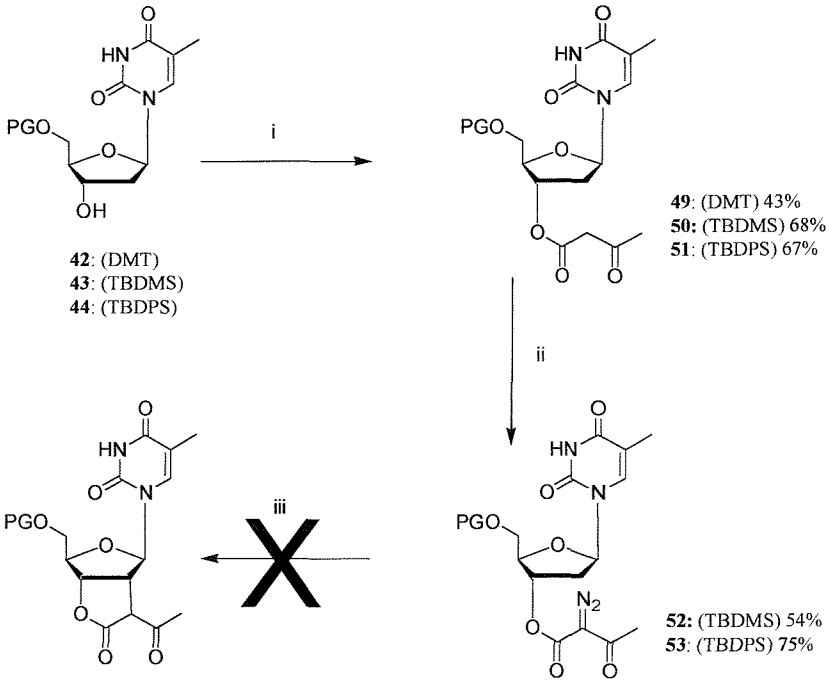


Figure 3.4.10: Reagents and conditions: i) 2,2,6-trimethyl-4*H*-1,3-dioxin-4-one (1.1eq), xylenes, 200°C, 30min; ii) *p*-carboxybenzenesulfonyl azide (1.3eq), DIPEA (2.3eq), MeCN, rt, 15hr; iii) $Rh_2(OAc)_4$ (10mol%), CH_2Cl_2, reflux; X = failed reaction. PG = protecting group (TBDPS, TBDMS or DMT).

The diazo-β-ketoester was synthesised with a number of protection groups of differing steric bulk. Unfortunately in each case reaction with the dirhodium(II) tetraacetate catalyst gave a mixture of inseparable products, amongst which the desired γ-butyrolactone was not found.

It was decided that perhaps changing the catalyst may improve the carbene specificity in favour of the desired C2' methylene 1,5-CH insertion. As discussed earlier, Ikegami *et al.* managed to promote five-membered ring formation at a methylene C-H bond by using a bulky catalyst. To this end, dirhodium(II) tetra(triphenylacetate) was synthesised by heating rhodium acetate in triphenylacetate.[186] This catalyst was used with diazoesters **45**, **46**, **47** as before, but still no improvement in selectivity or yield was observed (figure 3.4.11).

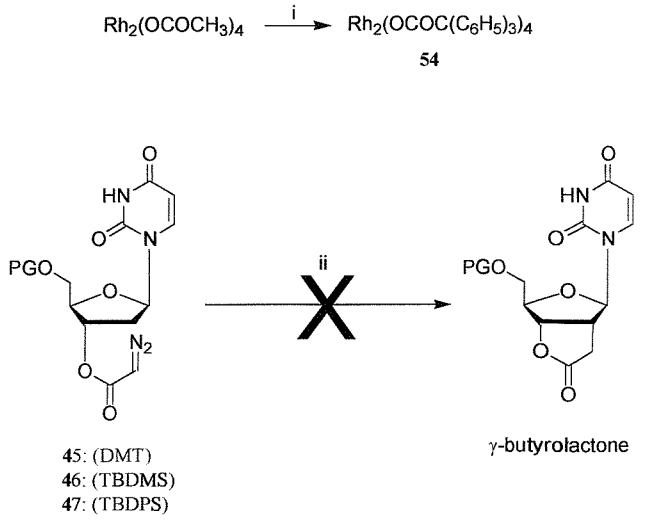


Figure 3.4.11: Reagents and conditions: i) Triphenylacetic acid (8eq), chlorobenzene, reflux, 3hrs (85%); ii) **54** (10mol%), CH_2Cl_2, reflux, 2hrs.

Since it seemed that this methodology was unlikely to yield the required γ-butyrolactone, it was decided to investigate the alternative synthesis, using a radical alkylation with alkyl tin to introduce the desired 2'-aminoethyl moiety (figure 3.4.3).

3.5 Redesign of synthesis for 2'-aminoethyl nucleoside

3.5.1 Discussion of radical method

Functionalisation of the 2'- or 3'-hydroxyls with a phenoxythioester followed by radical displacement has been described as a relatively mild method for the alkylation of nucleoside analogues.[194] Formation of a phenoxythioester, first described by Barton,[195] has been shown to provide an efficient route to the desired nucleoside radical intermediate.[181] This radical can then be reacted with a tin derivative to introduce the desired modification. Tributyltin hydride has often been used for the transformation of a ribonucleoside to a deoxynucleoside. In the same way, tributylallyltin reacts with the nucleoside radical to yield the allyl derivative in good yield.[196] Lawrence *et al.* have then described a reliable method from this to the 2'-hydroxyethyl nucleoside. Oxidation of the allyl derivative to cleave the alkene double bond furnishes an aldehyde and subsequent reduction to the alcohol proceeds in good yield.[182]

3.5.2 Synthesis of 2'-aminoethyl-dU phosphoramidite

The redeveloped synthesis started with a ribonucleoside, so thymidine could not be used (no 2'-hydroxyl functionality). Ribothymidine is an unnatural nucleoside, which was too expensive for a starting material here. Instead uridine was used, which although not stabilising in duplex DNA, would serve as a suitable material for initial investigations into the effect of the 2'-modification.

Initial protection was with TIPS chloride, which furnished the nucleoside **55** in good yield (figure 3.5.1). The phenoxythioester was introduced at the 2'-position (**56**) and a radical reaction with AIBN and tributyl-allyl-tin was used to alkylate the nucleoside yielding the allyl derivative **57**. The alkene was then oxidised in a two step proceedure with sodium periodate and osmium tetroxide to yield aldehyde **58**. The aldehyde was reduced with DIBAL to give the 2'-hydroxyethyl-2'-deoxyuridine nucleoside (**59**), in good yield. A Mitsunobu reaction was then used to substitute the hydroxyl with a phthaloyl derivative, hence introducing a protected amino moiety (**60**). Removal of the disiloxane with TBAF presented the deprotected nucleoside (**61**) and the 5'-hydroxyl was reprotected with dimethoxytrityl for oligonucleotide synthesis (**62**). Finally the phosphoramidite moiety was added to the 3'-position to yield the final monomer **63**, as a white foam.

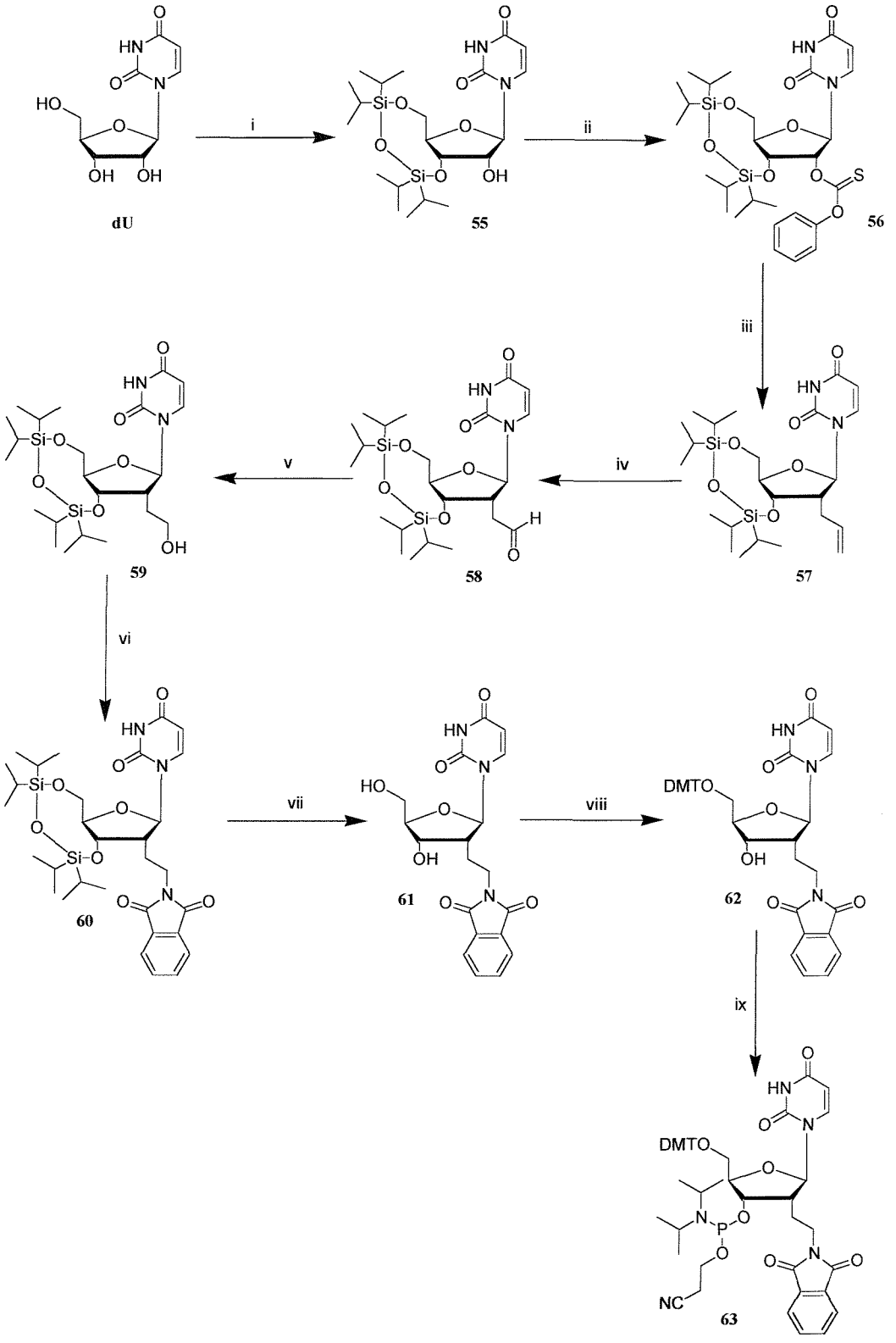


Figure 3.5.1: Reagents and conditions: i) TIPSCl (1.1eq), pyr, rt, 2hrs; ii) PhOCSCl (1.1eq), DMAP (1.1eq), CH_2Cl_2, rt, 18hrs; iii) $AllylSnBu_3$ (5eq), AIBN (0.5eq), PhH, *hv*, rt, 5hrs; iv) OsO_4 1% (w/v) aqueous solution, *N*-methylmorpholine *N*-oxide (1.1eq), acetone:water (1:1), rt, 1.5hrs, then $NaIO_4$, dioxane:water (3:1), rt, 45mins; v) $NaBH_4$, MeOH, 0°C, 1hr; vi) Phthalimide (1.03eq), PPh_3 (1.03eq), DEAD (1.03eq), THF, rt, 18hrs; vii) TBAF (1M) in THF (6eq), rt, 1hr; viii) DMTCl (1.1eq), pyr, rt, 18hrs; ix) 2-Cyanoethoxy-*N,N*-diisopropylamine chlorophosphine (1.1eq), DIPEA (4eq), THF, rt, 5hrs.

3.6 Incorporation of 2'-aminoethyl-dU into oligonucleotides

Two oligonucleotides were designed to assess the properties and suitability of this novel nucleoside as a monomer for oligonucleotide synthesis. These oligonucleotides were based upon sequences used in previous studies so that the melting temperatures could be compared.

ODN78	GCG TAA **X**GC G	**X** = 2'-ae-dU monomer **63**
ODN79	GCG **X**AA **X**GC G	

Unfortunately, coupling of the 2'-ae-dU monomer during oligonucleotide synthesis did not proceed in high yield (figure 3.6.1).

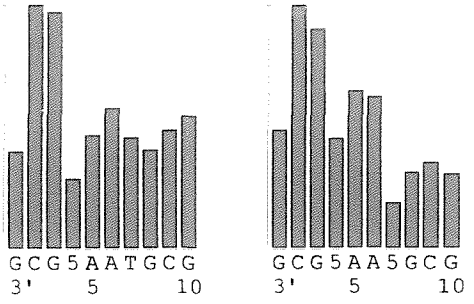


Figure 3.6.1: Oligonucleotide synthesis histogram readouts from ABI Expedite™ synthesiser. **ODN78** (left), **ODN79** (right). Bars indicate trityl yield for each addition. 5 = 2'-ae-dU monomer addition (**63**).

The reason for this drop in yield is not obvious, and if time allowed, greater quantities of monomer **63** could have been synthesised to investigate this further. However, the oligonucleotide yields were sufficiently good for purification by RP-HPLC and in each case relatively clean chromatographs were observed (figure 3.6.2).

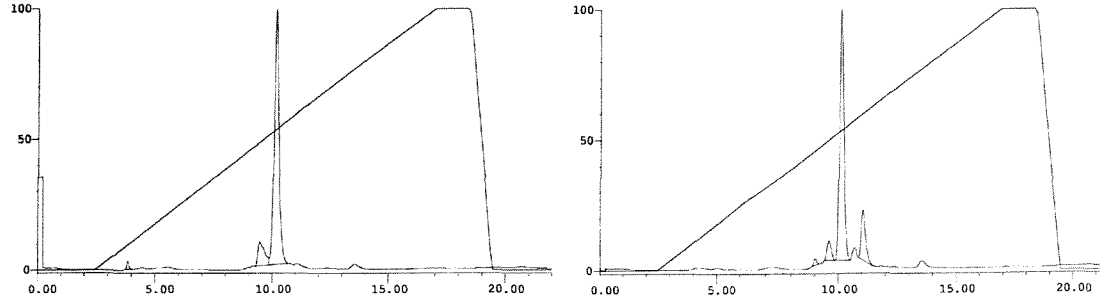


Figure 3.6.2: HPLC chromatograms of **ODN78** (left) and **ODN79** (right).

ODN78 and **ODN79** were then analysed by MALDI-TOF mass spectrometry. In each case peaks relating to the calculated mass were found (figure 3.6.3).

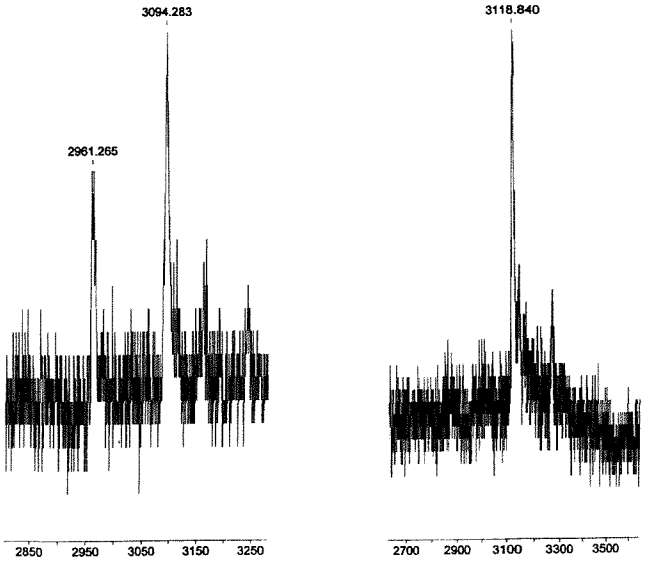


Figure 3.6.3: MALDI-TOF mass spectrometry for **ODN78** (expected: 3097, found: 3094) and **ODN79** (expected: 3126, found: 3119).

It appears that this modification is suitable for incorporation into oligonucleotides, although problems with coupling of this monomer during oligonucleotide synthesis need further investigation. Once this is achieved the effect of this 2'-modification can be studied in oligonucleotides and if successful, development of a doubly modified nucleoside monomer can be investigated.

3.7 Conclusions

5-Propyne-2'-deoxyuridine and 2-amino-2'-deoxyadenosine phosphoramidite monomers (**33** and **37** respectively) have been synthesised and incorporated into oligonucleotides for studies by UV-melting (**ODN48-67**). Increases in duplex stability were observed (~1.5°C per modification) for incorporation of either monomer, as reported in the literature.[107,122] In addition, studies with mismatched complementary strands showed that these modifications do discriminate against mismatch targets successfully. This is particularly the case for G containing mismatches, which are generally the most stable mismatched structures. These results show promise for the introduction of stabilising modifications into oligonucleotide probes such as molecular beacons.

5-Propargylamino-2'-deoxyuridine phosphoramidite (**39**) was synthesised and incorporated into oligonucleotides. Oligonucleotides were also synthesised containing 5-propyne-2'-deoxyuridine (**33**) and 2'-aminoethoxy-2'-deoxyuridine with one, two or three additions of each modified monomer. UV-melting results indicated that the presence of monomer **39** in an oligonucleotide stabilises the duplex by a similar amount to monomer **33**, but as more modifications were introduced, the stabilisation increased significantly more for oligonucleotides containing **39** than for **33**. Hence it was concluded that the amino group was having a positive effect on the duplex stabilisation particularly when several modifications were present. The 2'-aminoethoxy modification destabilised all duplexes. It is proposed that this destabilisation is due to this modification preferring a C3'-endo ring pucker which would not be compatible with a B-type DNA duplex.

Synthesis of a 2'-aminoethyl nucleoside was attempted *via* an intramolecular C-H insertion with dirhodium(II) tetraacetate catalyst.[179] However, this was unsuccessful despite varying conditions and substrates. The more bulky dirhodium(II) tetra(triphenylacetate) catalyst was synthesised and used for attempted C-H insertion without success. Reasons for the failure of this C-H insertion to work are not known.

However, 2'-aminoethyl-2'-deoxyuridine (**63**) was synthesised *via* 2'-allyl-2'-deoxyuridine using radical chemistry to introduce the 2'-*C*-substituent. Monomer **63** was

incorporated into oligonucleotides, but coupling yields were low. Consequently, further study on this monomer was not possible. This work will be continued in the future.

4. EXPERIMENTAL

4.1 Solvents and reagents

Dichloromethane (41°C), pyridine (115°C), toluene (110°C) and triethylamine (89°C) were distilled from CaH_2. Tetrahydrofuran (66°C) was freshly distilled from sodium wire and benzophenone. Methanol (65°C) was distilled from magnesium and iodine. Acetone (56°C) was freshly distilled from molecular sieves. Anhydrous acetonitrile was purchased from Applied Biosystems Limited (ABI). Anhydrous benzene, *N,N*-diisopropylethylamine and dimethylformamide were purchased from Aldrich. Other reagents were purchased from Sigma/Aldrich or Lancaster. Osmium tetroxide was purchased from Strem. Dirhodium(II) tetraacetate was purchased from Aldrich. 2-Cyanoethyl-*N,N*-diisopropyl chlorophosphoramidite and 4,4-dimethoxytrityl chloride were purchased from Link Technologies Ltd. 5-(*N*-(2-propynyl)-6-amino-hexanamide)-5'-*O*-(4,4'-dimethoxytrityl)-2'-deoxyuridine was provided by Oswel Research Products Ltd.

4.2 Analytical techniques

4.2.1 Chromatography

Flash column chromatography was carried out using 60 mesh silica gel (Merck or Apollo). Thin layer chromatography (TLC) was carried out on silica gel 60, F254, 0.2mm layer aluminium sheets (Merck) using the designated solvent system. TLC systems:

A	CH_2Cl_2:MeOH (9:1)
B	EtOAc (100%)
C	Toluene:EtOAc (8:2)
D	EtOAc:MeOH:NEt_3 (5:1:1)
E	Hexane:EtOAc (2:1)
F	Hexane:CH_2Cl_2 (2:1)
G	CH_2Cl_2:EtOAc (9:1)
H	Acetone:CH_2Cl_2 (1:2)
I	Hexane:EtOAc (1:2)
J	Hexane:EtOAc (1:1)
K	CH_2Cl_2:EtOAc:MeOH (5:4.5:0.5)
L	CH_2Cl_2:MeOH (9.5:0.5)

Products were visualised on TLC using the following techniques:

(i) UV absorption at 264nm for nucleosides.

(ii) Heating caused compounds containing a dimethoxytrityl group to produce a strong orange colour.

(iii) Spraying with a solution of 4-methoxybenzaldehyde: glacial acetic acid: conc. sulphuric acid: ethanol (5:1:1:50, v:v:v:v). Compounds with a protected or unprotected 1,2-diol function gave a dark blue colour on strong heating.

(iv) Spraying with a solution of ninhydrin:ethanol (1:100, wt:vol). Compounds with primary-amino groups gave a dark brown colour on heating.

(v) Spraying with polymolybdic acid:ethanol (1:10, v:v). Compounds were stained a dark purple colour on strong heating.

4.2.2 NMR spectroscopy

^{1}H NMR spectra were recorded on a Bruker WP-300 spectrometer (300 MHz) or a Bruker WP-400 spectrometer (400MHz). ^{13}C NMR spectra (75.5 MHz) and ^{31}P spectra (90 MHz) were recorded on a Bruker WP-300 machine. Multiplicities of carbon–13 signals were determined using distortionless enhancement by phase transfer, DEPT spectral editing technique. All spectra were referenced to $CDCl_3$, CD_3OD or d_6-DMSO.

4.2.3 Mass spectrometry

Electrospray data was obtained on a VG Organic Quattro II machine, with quadrupole analyser and photomultiplier ion detection, using the positive ion and oligo programs. Oligos were run in isopropanol/water 1:1 + 0.002% TPA, whilst all other compounds were run in 100% acetonitrile. MALDI-TOF data was obtained on a ThermoBioAnalysis Dynamo machine using a 3-hydroxypicolinic acid / picolinic acid (4:1) matrix with 50% aqueous acetonitrile solvent.

4.2.4 UV/vis spectroscopy

UV-vis data was recorded on a Perkin Elmer Lambda 2 spectrometer, using 1ml cuvettes at 25°C. All extinction coefficients are quoted in brackets (cm^2 mol^{-1}).

4.2.5 IR spectroscopy

Infrared spectra were taken on a BIORAD FTIR machine, using a golden gate adapter and BIORAD WIN-IR software. Absorptions are described as strong (s), medium (m), or weak (w).

4.2.6 Elemental analysis

C, H, N elemental analysis was undertaken by MEDAC Ltd. Calculated and observed values are shown.

4.2.7 Melting point analysis

Melting points were determined using a Gallenkamp electrothermal melting point apparatus and values are quoted as a range of temperature (°C).

4.3 List of compounds

1. **1-((6-Hydroxyhexyl)amino)-4-amino-anthraquinone**
2. **1,4-Di((6-hydroxyhexyl)amino)-anthraquinone**
3. **1-((Acetoxyhexyl)amino)-4-(acetylamino)-anthraquinone**
4. **1-((6-Hydroxyhexyl)amino)-4-(acetylamino)-anthraquinone**
5. **1-(2-Cyanoethyl-*N,N*-diisopropylphosphoramidyl)hexyl)amino)-4-(acetylamino)-anthraquinone**
6. **4,4'-Dimethoxytrityl 1-bromohexyl ether**

7. **Racemic 5,6-isopropylidenedioxyhexan-1-ol**
8. **Racemic 5,6-isopropylidenedioxy-1-bromohexane**
9. **1-((4-(2,2-Dimethyl-1,3-dioxolan-4-yl)butyl)amino)-4-amino-anthraquinone**
10. **1-((4-(2,2-Dimethyl-1,3-dioxolan-4-yl)butyl)amino)-4-(acetylamino)-anthraquinone**
11. **1-((5,6-Dihydroxyhexyl)amino)-4-(acetylamino)-anthraquinone**
12. **1-((6-(4,4'-Dimethoxytrityloxy)-5-hydroxyhexyl)amino)-4-(acetylamino)-anthraquinone**
13. **1-((6-(4,4'-Dimethoxytrityloxy)-5-(2-cyanoethyl-*N,N*-diisopropylphosphoramidyl)hexyl)amino)-4-(acetylamino)-anthraquinone**
14. **Racemic *N*-phthaloyl-5,6-isopropylidenedioxyhexylamine**
15. **Racemic 5,6-isopropylidenedioxyhexylamine**

16. **1-(((2-Cyanoethyl-*N,N*-diisopropylphosphoramidyl)ethyl)amino)-4-(methylamino)-anthraquinone**
17. **1-((2-Aminoethyl)amino)-4-(methylamino)-anthraquinone**
18. **1-(2-Succinamido-ethylamine)-4-(methylamino)-anthraquinone**
19. **5'-*O*-(4,4'-Dimethoxytrityl)-5-(3-(6-*N1*-(2-((4-(methylamino)-anthraquinone)amino)ethyl)succinamidohexamide)propynyl)-2'-deoxyuridine**
20. **5'-*O*-(4,4'-Dimethoxytrityl)-5-(3-(6-*N1*-(2-((4-(methylamino)-anthraquinone)amino)ethyl)succinamidohexamide)propynyl) 3'-*O*-(2-cyanoethyl-*N,N*-diisopropylphosphoramidyl)-2'-deoxyuridine**
21. **3',5'-*O*-Ditoluoyl-1'-methoxy-2'-deoxy-D-ribose**

22. 3',5'-*O*-Ditoluoyl-1'-chloro-2'-deoxy-D-ribose

23. 3',5'-*O*-Ditoluoyl-1'-*O*-(6-trifluoroacetylamidohexyl)-2'-deoxy-D-ribose

24. 1'-*O*-(6-Trifluoroacetylamidohexyl)-2'-deoxy-D-ribose

25. 5'-*O*-(4,4'-Dimethoxytrityl)-1'-*O*-(6-trifluoroacetylamidohexyl)-2'-deoxy-D-ribose

26. 5'-*O*-(4,4'-Dimethoxytrityl)-1'-*O*-(6-aminohexyl)-2'-deoxy-D-ribose

27. 5'-*O*-(4,4'-Dimethoxytrityl)-1'-*O*-(6-*N1*-(2-((4-(methylamino)-anthraquinone)amino)ethyl)succinamidohexyl)-2'-deoxy-D-ribose

28. 5'-*O*-(4,4'-Dimethoxytrityl)-1-*O*-(6-*N1*-(2-((4-(methylamino)-anthraquinone)amino)ethyl)succinamidohexyl)-3'-*O*-oxobutanoic acid-2'-deoxy-D-ribose

29. 5'-*O*-(4,4'-Dimethoxytrityl)-1-*O*-(6-*N1*-(2-((4-(methylamino)-anthraquinone)amino)ethyl)succinamidohexyl)-3'-*O*-oxobutanoic acid-2'-deoxy-D-ribose LCAA-CPG

30. 6-Trifluoroacetamidohexan-1-ol

31. 5'-*O*-(4,4'-Dimethoxytrityl)-5-iodo-2'-deoxyuridine

32. 5'-*O*-(4,4'-Dimethoxytrityl)-5-(1-propynyl)-2'-deoxyuridine

33. 5'-*O*-(4,4'-Dimethoxytrityl)-5-(1-propynyl)-3'-*O*-(2-cyanoethyl-*N,N*-diisopropylphosphoramidyl)-2'-deoxyuridine

34. 5'-*O*-(4,4'-Dimethoxytrityl)-2'-deoxyguanosine

35. 5'-*O*-(4,4'-Dimethoxytrityl)-2-amino-2'-deoxyadenosine

36. 5'-*O*-(4,4'-Dimethoxytrityl)-N^2,N^6-bisphenoxyacetyl-2-amino-2'-deoxyadenosine

37. 5'-*O*-(4,4'-Dimethoxytrityl)-N^2,N^6-bisphenoxyacetyl-2-amino-3'-*O*-(2-cyanoethyl-*N,N*-diisopropylphosphoramidyl)-2'-deoxyadenosine

38. 5'-*O*-(4,4'-Dimethoxytrityl)-5-(3-*N*-(2,2,2-trifluoroacetyl)amino-1-propynyl)-2'-deoxyuridine

39. 5'-*O*-(4,4'-Dimethoxytrityl)-5-(3-*N*-(2,2,2-trifluoroacetyl)amino-1-propynyl)-3'-*O*-(2-cyanoethyl-*N,N*-diisopropylphosphoramidyl)-2'-deoxyuridine

40. 2-((4-Methylphenyl)sulfonyl)hydrazonoacetic acid

41. 2-((4-Methylphenyl)sulfonyl)hydrazonoethanoyl chloride

42. 5'-*O*-(4,4'-Dimethoxytrityl)-2'-deoxythymidine

43. 5'-*O*-(*tert*-Butyldimethylsilyl)-2'-deoxythymidine

44. 5'-*O*-(*tert*-Butyldiphenylsilyl)-2'-deoxythymidine

45. 5'-*O*-(4,4'-Dimethoxytrityl)-3'-(α-diazoacetyl)-2'-deoxythymidine

46. 5'-*O*-(*tert*-Butyldimethylsilyl)-3'-(α-diazoacetyl)-2'-deoxythymidine

47. 5'-*O*-(*tert*-Butyldiphenylsilyl)-3'-(α-diazoacetyl)-2'-deoxythymidine

48. 5'-*O*-(4,4'-Dimethoxytrityl)-3'-(2-hydroxyacetyl)-2'-deoxythymidine

49. 5'-*O*-(4,4'-Dimethoxytrityl)-3'-*O*-(β-ketoester)-2'-deoxythymidine

50. 5'-*O*-(*tert*-Butyldimethylsilyl)-3'-*O*-(β-ketoester)-2'-deoxythymidine

51. 5'-*O*-(*tert*-Butyldiphenylsilyl)-3'-*O*-(β-ketoester)-2'-deoxythymidine

52. 5'-*O*-(*tert*-Butyldimethylsilyl)-3'-*O*-(β-ketodiazoester)-2'-deoxythymidine

53. 5'-*O*-(*tert*-Butyldiphenylsilyl)-3'-*O*-(β-ketodiazoester)-2'-deoxythymidine

54. Dirhodium(II) tetra(triphenylacetate)

55. 3',5'-*O*-(1,1,3,3-Tetraisopropyldisiloxy)uridine

56. 3',5'-*O*-(1,1,3,3-Tetraisopropyldisiloxy)-2'-deoxy-2'-α-*C*-(phenoxythioester)uridine

57. 3',5'-*O*-(1,1,3,3-Tetraisopropyldisiloxy)-2'-deoxy-2'-α-*C*-(allyl)uridine

58. 3',5'-*O*-(1,1,3,3-Tetraisopropyldisiloxy)-2'-deoxy-2'-α-*C*-(2-oxyethyl)uridine

59. 3',5'-*O*-(1,1,3,3-Tetraisopropyldisiloxy)-2'-deoxy-2'-α-*C*-(2-hydroxyethyl)uridine

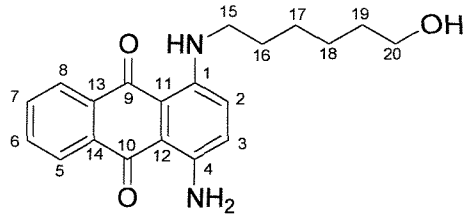
60. 3',5'-*O*-(1,1,3,3-Tetraisopropyldisiloxy)-2'-deoxy-2'-α-*C*-(2-*N*-phthaloylethyl)uridine

61. 2'-Deoxy-2'-α-*C*-(2-*N*-phthaloylethyl)uridine

62. 5'-*O*-(4,4'-Dimethoxytrityl)-2'-deoxy-2'-α-*C*-(2-*N*-phthaloylethyl)uridine

63. 5'-*O*-(4,4'dimethoxytrityl)-3'-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidyl)-2'-deoxy-2'-α-*C*-(2-*N*-phthaloylethyl)uridine

4.4 Chemical synthesis



1 $C_{20}H_{22}N_2O_3$

1-((6-Hydroxyhexyl)amino)-4-amino-anthraquinone (1).

Alkylation was carried out by the method described by Frier *et al.*[166]

1,4-Diaminoanthraquinone (3.0g, 12.6mmol) was dissolved in anhydrous DMF (50ml) and to this was added 6-bromohexanol (5.0g, 3.6ml, 27.6mmol). The mixture was stirred at 95°C for 48hrs, and followed by TLC (system A). When no further reaction was observed, the solvent was removed and the residue dissolved in dichloromethane (200ml). This was washed with 10% w/v sodium bicarbonate (2x 200ml), dried with anhydrous sodium sulfate and evaporated to dryness. The mixture was purified by silica gel chromatography, eluting with CH_2Cl_2/MeOH (0-5%). Two major solid products, monoalkylated (850mg, 20%, **1**) and bisalkylated (50mg, 1%, **2**) were obtained, in addition to recovered starting material 1.8g (60%).

1-((6-Hydroxyhexyl)amino)-4-amino-anthraquinone (1).

R_f (system A) 0.4.

^{1}H NMR (DMSO-d_6) δ: 10.90 (br, 1H, N**H**), 8.45 (br, 2H, N**H**$_2$), 8.32-8.24 (m, 2H, Ar**H**^{5,8}), 7.85-7.78 (m, 2H, Ar**H**^{6,7}), 7.42 (d, 2H, J = 9.5 Hz, Ar**H**^{3}), 7.31 (d, 2H, J = 9.5 Hz, Ar**H**^{2}), 4.39 (t, 1H, J = 5.0 Hz, O**H**), 3.52-3.43 (m, 4H, C**H**$_2^{15}$, C**H**$_2^{20}$), 1.71-1.64 (m, 2H, C**H**$_2^{16}$), 1.48-1.32 (m, 6H, C**H**$_2^{17,18,19}$).

^{13}C NMR (DMSO-d_6) δ: 181.65 (**C**^{10}), 180.78 (**C**^{9}), 147.25 (**C**^{14}), 146.54 (**C**^{13}), 134.69 (**C**^{4}), 134.23 (**C**^{1}), 132.77 (**C**H^6), 132.55 (**C**H^7), 130.21 (**C**H^{5,8}), 126.16 (**C**H^3), 124.26 (**C**H^2), 108.38 (**C**^{12}), 108.22 (**C**^{11}), 60.10 (**C**H$_2^{20}$), 42.47 (**C**H$_2^{15}$), 32.93 (**C**H$_2^{19}$), 29.74 (**C**H$_2^{16}$), 26.89 (**C**H$_2^{17}$), 25.70 (**C**H$_2^{18}$).

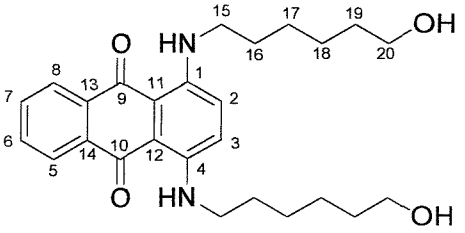
IR ν_{max}/cm^{-1}: 3351 (s), 2923 (m), 2852 (m), 1568 (s), 1530 (s).

UV/vis (CH_2Cl_2): sh 538nm ($9.6x10^6 cm^2mol^{-1}$), 573nm ($16.4x10^6 cm^2mol^{-1}$), 616nm ($16.6x10^6 cm^2mol^{-1}$).

ES^+/MS: 339.4 $(M+H)^+$.

HRMS (ES^+) for $C_{20}H_{22}N_2O_3$ $(M+Na)^+$: calculated 361.1522, found 361.1529.

Mpt. 160-165°C.



2 $C_{26}H_{34}N_2O_4$

1,4-Di((6-hydroxyhexyl)amino)-anthraquinone (2).

R_f (system A) 0.3.

^1H NMR ($CDCl_3$) δ: 10.73 (br s, 2H, N**H**), 8.27-8.18 (m, 2H, Ar**H**^{5,8}), 7.62-7.54 (m, 2H, Ar**H**^{6,7}), 7.05 (s, 2H, Ar**H**^{2,3}), 3.61 (t, 4H, J = 5.9 Hz, C**H**$_2^{20}$), 3.30 (t, 4H, J = 6.6 Hz, C**H**$_2^{15}$), 1.80-1.30 (m, 16H, C**H**$_2^{16,17,18,19}$).

^{13}C NMR (DMSO-d_6) δ: 180.19 (**C**^{9,10}), 145.73 (**C**^{13,14}), 133.57 (**C**^{1,4}), 131.84 (**C**H^{6,7}), 125.36 (**C**H^{5,8}), 124.25 (**C**H^{2,3}), 107.89 (**C**^{11,12}), 60.36 (**C**H$_2^{20}$), 41.74 (**C**H$_2^{15}$), 32.19 (**C**H$_2^{19}$), 28.98 (**C**H$_2^{16}$), 26.16 (**C**H$_2^{17}$), 24.97 (**C**H$_2^{18}$).

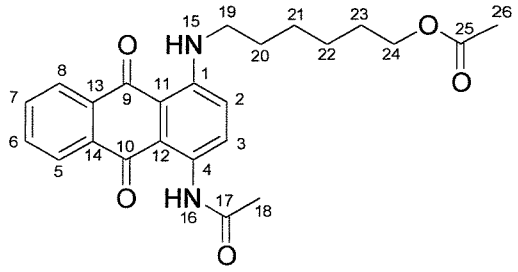
IR ν_{max}/cm^{-1}: 3309 (s), 2923 (m), 2852 (m), 1572 (s).

UV/vis (CH_2Cl_2): sh 563nm ($1.8x10^6 cm^2mol^{-1}$), 599nm ($2.4x10^6 cm^2mol^{-1}$), 646nm ($2.1x10^6 cm^2mol^{-1}$).

ES^+/MS: 439.3 $(M+H)^+$.

HRMS (ES^+) for $C_{26}H_{34}N_2O_4$ $(M+Na)^+$: calculated 461.2411, found 461.2409.

Mpt. 118-120°C.



3 $C_{24}H_{26}N_2O_5$

1-((Acetoxyhexyl)amino)-4-(acetylamino)-anthraquinone (3).

Compound **1** (400mg, 1.18mmol) was dissolved in pyridine (50ml) and acetic anhydride (25 ml, d= 1.082g/ml, 265mmol) was added. The mixture was stirred at room temperature for 12hrs. TLC (system A) showed starting material still present, so the reaction was heated at 50°C for 5hrs to drive the reaction to completion. The mixture was evaporated to dryness, then dissolved in CH_2Cl_2 (100ml), washed with water (200ml), sodium bicarbonate (200ml) and brine (200ml), followed by drying with sodium sulfate. Following filtration the solution was evaporated to dryness to yield solid acetylated product **3** (0.46g, 92%).

R_f (system A) 0.75.

^{1}H NMR ($CDCl_3$) δ: 12.40 (s, 1H, N**H**^{16}), 10.20 (s, 1H, N**H**^{15}), 8.90 (d, 1H, J = 9.5 Hz, Ar**H**^{3}), 8.16 (d, 1H, J = 7.5 Hz, Ar**H**^{5}), 8.11 (d, 1H, J = 7.5 Hz, Ar**H**^{8}), 7.67 (t, 1H, J = 7.5 Hz, Ar**H**^{6}), 7.61 (t, 1H, J = 7.5 Hz, Ar**H**^{7}), 7.00 (d, 1H, J = 9.5 Hz, Ar**H**^{2}), 4.01 (t, 2H, J = 6.5 Hz, C**H**$_2^{24}$), 3.21 (dt, 2H, J = 12.5 Hz, J = 7.0 Hz, C**H**$_2^{19}$), 2.20 (s, 3H, C**H**$_3^{18}$), 2.00 (s, 3H, C**H**$_3^{26}$), 1.80-1.20 (m, 8H, C**H**$_2^{20,21,22,23}$).

^{13}C NMR ($CDCl_3$) δ: 186.02 (**C**^{10}), 182.52 (**C**^{9}), 170.15 (**C**^{25}), 168.75 (**C**^{17}), 147.84 (**C**^{14}), 133.40 (**C**^{13}), 132.91 (**CH**^{6}), 132.70 (**C**^{4}), 132.18 (**C**^{1}), 131.64 (**CH**^{7}), 128.76 (**CH**^{5}), 125.62 (**CH**^{8}), 125.28 (**CH**^{3}), 120.23 (**CH**^{2}), 115.67 (**C**^{12}), 109.02 (**C**^{11}), 63.37 (**CH**$_2^{24}$), 43.24 (**CH**$_2^{19}$), 41.79 (**CH**$_2^{23}$), 28.18 (**CH**$_2^{20}$), 27.52 (**CH**$_2^{21}$), 24.74 (**CH**$_3^{18}$), 24.71 (**CH**$_2^{22}$), 19.98 (**CH**$_3^{26}$).

IR ν_{max}/cm^{-1}: 2914 (m), 1732 (s), 1688 (s), 1564 (s), 1512 (m).

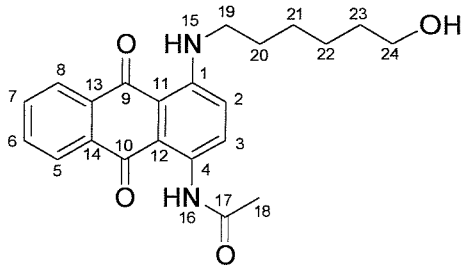
UV/vis (CH_2Cl_2): 565nm ($9.5x10^6 cm^2mol^{-1}$), 596nm ($8.4x10^6 cm^2mol^{-1}$).

ES$^+$/MS: 423.2 $(M+H)^+$.

HRMS (ES^+) for $C_{24}H_{26}N_2O_5$ $(M+Na)^+$: calculated 445.1734, found 445.1714.

Mpt. 110-115°C.

Elem. Anal. ($C_{24}H_{26}N_2O_5$): Calculated C: 68.23, H: 6.20, N: 6.63; found C: 67.84, H: 6.22, N: 6.50.



4 $C_{22}H_{24}N_2O_4$

1-((6-Hydroxyhexyl)amino)-4-(acetylamino)-anthraquinone (4).

Compound **3** (0.44g, 1.04mmol) was dissolved in methanol (40ml) and to this was added NaOMe (0.085g, 1.5mmol). The mixture was stirred at room temperature for 5hrs and when the TLC (system A) showed consumption of the starting material, the reaction was worked up with DOWEX, H^+. DOWEX was washed with water several times and then methanol before use. The beads were then added to the stirred reaction vessel and the mixture filtered. The filtrate was evaporated to dryness to yield crude product. The crude material was purified by silica gel column chromatography eluting with CH_2Cl_2/MeOH (0-2%) to yield compound **4** 0.16g (41%), as a coloured solid.

R_f (system A) 0.45.

^{1}H NMR ($CDCl_3$) δ: 12.30 (s, 1H, N**H**^{16}), 10.20 (br s, 1H, N**H**^{15}), 8.85 (d, 1H, J = 9.5 Hz, Ar**H**^{3}), 8.20 (d, 1H, J = 7.5 Hz, Ar**H**^{5}), 8.12 (d, 1H, J = 7.5 Hz, Ar**H**^{8}), 7.69 (t, 1H, J = 7.5 Hz, Ar**H**^{6}), 7.60 (t, 1H, J = 7.5 Hz, Ar**H**^{7}), 7.05 (d, 1H, J = 9.5 Hz, Ar**H**^{2}), 3.60 (t, 2H, J = 6.5 Hz, C**H**$_2^{24}$), 3.30 (dt, 2H, J = 12.5 Hz, J = 7.0 Hz, C**H**$_2^{19}$), 2.20 (s, 3H, C**H**$_3^{18}$), 1.70-1.30 (m, 8H, C**H**$_2^{20,21,22,23}$).

^{13}C NMR ($CDCl_3$) δ: 187.49 (**C**^{10}), 183.97 (**C**^{9}), 170.25 (**C**^{17}), 149.36 (**C**^{14}), 134.85 (**C**^{13}), 134.36 (**CH**^{6}), 134.11 (**C**^{4}), 133.62 (**C**^{1}), 133.08 (**CH**^{7}), 130.24 (**CH**^{5}), 127.06 (**CH**^{8}), 126.72 (**CH**^{3}), 121.71 (**CH**^{2}), 117.14 (**C**^{12}), 110.43 (**C**^{11}), 63.12 (**CH**$_2^{24}$), 43.24(**CH**$_2^{19}$), 33.01 (**CH**$_2^{23}$), 29.62 (**CH**$_2^{20}$), 27.33 (**CH**$_2^{21}$), 26.14 (**CH**$_3^{18}$), 25.91 (**CH**$_2^{22}$).

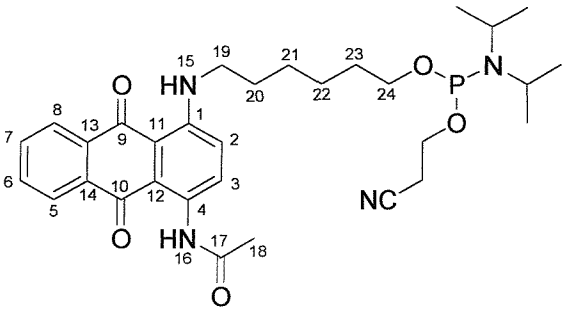
IR ν_{max}/cm^{-1}: 3471 (s), 2933 (m), 2856 (m), 1687 (s), 1568 (s), 1556 (m).

UV/vis (CH_2Cl_2): 566nm ($12.4x10^6 cm^2mol^{-1}$), 597nm ($10.7x10^6 cm^2mol^{-1}$).

ES$^+$/MS: 381.3 $(M+H)^+$.

HRMS (ES^+) for $C_{22}H_{24}N_2O_4$ $(M+Na)^+$: calculated 403.1628, found 403.1634.

Mpt. 145-148°C.



5 $C_{31}H_{41}N_4O_5P$

1-(2-Cyanoethyl-*N,N*-diisopropylphosphoramidyl)oxyhexyl)amino)-4-(acetylamino)-anthraquinone (5).

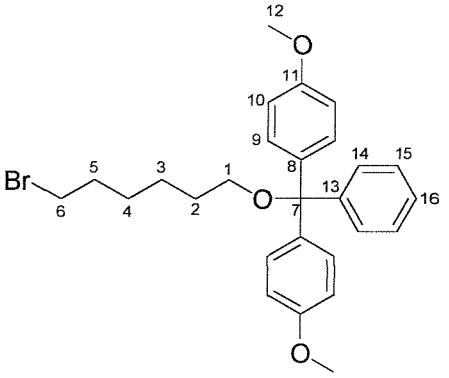
Phosphitylation was carried out by the method described by Caruthers *et al.* [6]

Compound **4** (0.14g, 0.37mmol) was dissolved in dry THF (20ml), to which DIPEA (0.095g, 0.74mmol) and 2-cyanoethoxy-*N,N*-diisopropylamine chlorophosphine (0.097g, 0.44mmol) were added under argon. The mixture was stirred for 1.5hrs and observed with TLC (system B), showing only partial consumption, so a further 0.1 equivalents of 2-cyanoethoxy-*N,N*-diisopropylamine chlorophosphine were added to drive the reaction to completion. The mixture was diluted with EtOAc (50ml), washed with sat. KCl (50ml), dried (anhydrous sodium sulfate), filtered and evaporated to yield crude product (0.29g) as a blue/purple solid. This was purified with silica column chromatography under argon, eluting with EtOAc (100%). The solid product was very sensitive to air and decomposed on TLC rapidly. A resultant yield of 0.19g (89%) was obtained. This product was dissolved in acetonitrile (anhyd. 5ml), filtered and evaporated to confirm solubility for use as an automated-synthesis monomer.

R_f (system B) 0.8.

^{31}P NMR ($CDCl_3$) δ: 147.87.

ES^+/MS: 581.3 $(M+H)^+$.



6 $C_{27}H_{31}O_3Br$

4,4'-Dimethoxytrityl 1-bromohexyl ether (6).

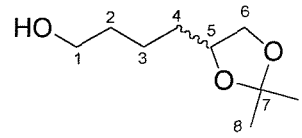
6-Bromohexan-1-ol (1.0g, 5.5mmol) was added to a solution of 4,4'-dimethoxytrityl chloride (1.9g, 5.6mmol) in dry pyridine (15 ml) and it was left stirring for 15hrs. The solution decolourised and a white precipitate formed. The reaction was quenched with methanol before evaporating to dryness, then redissolving in dichloromethane (200ml). The mixture was washed with sat. KCl (200ml) and dried with anhydrous Na_2SO_4. The product was purified by silica gel column chromatography, eluting with hexane:CH_2Cl_2 (1:1) with 0.1% of NEt_3 to cap acidic silica sites. Fractions were combined to yield tritylated product **6**, 2.1g (79%) as a colourless oil.

R_f (system F) 0.5.

^{1}H NMR ($CDCl_3$) δ: 7.35 (d, 2H, J = 7.4 Hz, Ar**H**^{14}), 7.28 (d, 4H, J = 8.8 Hz, Ar**H**^{10}), 7.25-7.10 (m, 3H, Ar**H**^{15,16}), 6.72 (d, 4H, J = 8.8 Hz, Ar**H**^{9}), 3.66 (s, 6H, C**H**$_3^{12}$), 3.43 (t, 2H, J = 6.6 Hz, C**H**$_2^{1}$), 2.96 (t, 2H, J = 4.4 Hz, C**H**$_2^{6}$), 1.70-1.25 (m, 8H, C**H**$_2^{2,3,4,5}$).

^{13}C NMR ($CDCl_3$) δ: 158.33 (**C**^{11}), 145.44 (**C**^{13}), 136.69 (**C**^{8}), 130.12 (**CH**^{9}), 128.21 (**CH**^{14}), 127.75 (**CH**^{15}), 126.63 (**CH**^{16}), 112.99 (**CH**^{10}), 85.69 (**C**^{7}), 63.21 (**C**H$_2^{1}$), 55.23 (**C**H$_3^{12}$), 45.15 (**C**H$_2^{6}$), 32.81 (**C**H$_2^{2}$), 29.95 (**C**H$_2^{5}$), 26.81 (**C**H$_2^{3}$), 25.68 (**C**H$_2^{4}$).

ES$^+$/MS: 484.8 $(M+H)^+$.



7 $C_9H_{18}O_3$

Racemic 5,6-isopropylidenedioxyhexan-1-ol (7).[167]

Racemic 1,2,6-hexanetriol (24.0g, 1.105g/ml, 21.8ml, 88mmol) was dissolved in acetone (anhyd. 250ml). Anhydrous Na_2SO_4 (50.0g) and molecular sieves (20.0g, powder, flame dried) were added followed by conc. $HCl_{(aq)}$ (3.5ml). The reaction was performed under argon and stirred at room temperature for 20hrs. A further addition of Na_2CO_3 (anhyd. 70.0g) was added and the reaction was stirred for a further 48hrs. TLC (system A) showed 75% conversion and so the mixture was filtered, quenched with a further addition of Na_2CO_3 and filtered again. The filtrate was evaporated to give crude product (29.95g). Purification was with silica gel column chromatography (CH_2Cl_2/MeOH, 0-10%). Fractions were combined to yield a colourless oil **7**, 23.08g (76%).

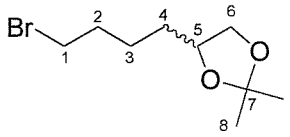
R_f (system A) 0.40.

^{1}H NMR ($CDCl_3$) δ: 4.14-4.01 (m, 2H, C**H**$_2^6$), 3.59 (t, 2H, J = 7.0 Hz, C**H**$_2^1$), 3.45 (t, 1H, J = 7.0 Hz, C**H**^{5}), 2.11 (s, 1H, O**H**), 1.73-1.38 (m, 6H, C**H**$_2^{2,3,4}$), 1.38 (s, 3H, C**H**$_3^8$), 1.31 (s, 3H, C**H**$_3^8$).

^{13}C NMR ($CDCl_3$) δ: 109.08 (**C**^{7}), 76.23 (**C**H^5), 69.79 (**C**H$_2^6$), 62.82 (**C**H$_2^1$), 33.46 (**C**H$_2^2$), 32.96 (**C**H$_2^4$), 27.29 (**C**H$_3^8$), 26.08 (**C**H$_3^8$), 22.42 (**C**H$_2^3$).

ES$^+$/MS: 174 $(M+H)^+$.

Analytical data was consistent with that found in the literature.[167]



8 $C_9H_{17}O_2Br$

Racemic 5,6-isopropylidenedioxy-1-bromohexane (8).

Bromination was carried out by the method described by Bates *et al.* [168]

A solution of *N*-bromosuccinimide (20.19g, 113mmol, 3 eq.) in CH_2Cl_2 (anhyd. 20ml) was stirred at room temperature under an atmosphere of argon. Triphenylphosphine (27.74g, 106mmol, 2.8eq.) also dissolved in CH_2Cl_2 (anhyd. 20ml) was added slowly, as the yellow suspension turned into a dark brown solution. After a period of 5mins, pyridine (anhyd. 3.6g, 40mmol, 1.2 eq.) was added which turned the solution a darker colour brown and finally a solution of the alcohol **7** (6.58g, 38mmol) in anhydrous CH_2Cl_2 (10ml) was added dropwise. The solution remained dark in colour and was stirred for 5hrs. TLC (system A) showed consumption of starting material and so the reaction was diluted with sat. sodium bicarbonate (250ml) and the organic layer extracted with CH_2Cl_2. The organic layer was kept and evaporated to dryness. Initial purification consisted of a short silica column, which removed the brown baseline material. The mixture was then dissolved in hexane and the solution filtered, removing the orange by-product. Finally the two components remaining (product and triphenylphosphine oxide) were separated by silica gel column chromatography (100% CH_2Cl_2), to yield a clear oil **8**, 5.69g (64%).

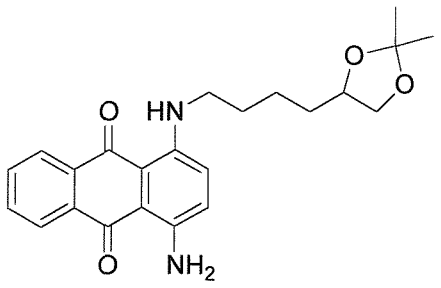
R_f (system A) 0.75.

^{1}H NMR ($CDCl_3$) δ: 4.06-3.94 (m, 2H, CH_2^6), 3.49-3.41 (m, 1H, CH^5), 3.33 (t, 2H, J = 7.0 Hz, CH_2^1), 1.85 (quintet, 2H, J = 6.5 Hz, CH_2^2), 1.35 (s, 3H, CH_3^8), 1.30 (s, 3H, CH_3^8), 1.30-1.75 (m, 4H, $CH_2^{3,4}$).

^{13}C NMR ($CDCl_3$) δ: 108.37 (C^7), 76.19 (CH^5), 69.79 (CH_2^6), 33.83 (CH_2^1), 33.13 (CH_2^2), 33.07 (CH_2^4), 27.35 (CH_3^8), 26.11 (CH_3^8), 22.85 (CH_2^3).

ES$^+$/MS: 238 $(M+H)^+$.

Analytical data was consistent with that found in the literature.[167]

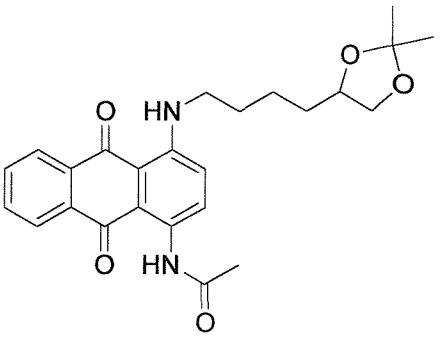


9 $C_{23}H_{26}N_2O_4$

1-((4-(2,2-Dimethyl-1,3-dioxolan-4-yl)butyl)amino)-4-amino-anthraquinone (9).

1,4-Diaminoanthraquinone (1.5g, 6.3mmol) was dissolved in triethylamine (anhyd. 40ml) and bromoacetal **8** (1.6g, 6.8mmol) was added. The mixture was refluxed at 130°C for 24hrs and studied by TLC (system A). A further 0.5 equivalents of bromoacetal **8** were added to consume the starting material, but no further reaction was observed by TLC. DMF was added for better solubility of anthraquinone, but again no further reaction was observed. The mixture was evaporated, redissolved in CH_2Cl_2 (200ml) and washed with sodium bicarbonate (10%, 200ml), dried with anhydrous Na_2SO_4 and filtered. Upon evaporating to dryness a crude mixture of anthraquinone starting material and product was recovered (2.1g). Attempts were made at purifying the resultant mixture but without success. Instead it was decided to react this on as a crude mixture.

R_f (system A) 0.65.

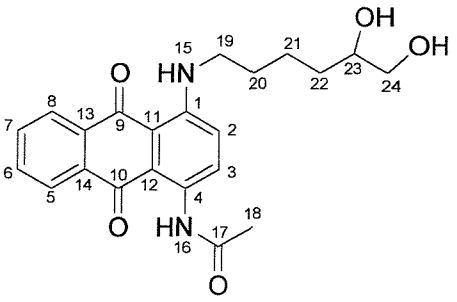


10 $C_{25}H_{28}N_2O_5$

1-((4-(2,2-Dimethyl-1,3-dioxolan-4-yl)butyl)amino)-4-(acetylamino)-anthraquinone (10).

The crude mixture **9** (0.68g, 1.8mmol) was dissolved in pyridine (30ml) and imidazole (0.2g, 3mmol) was added, followed by slow addition of acetic anhydride (2ml, 1.082g/ml, 21mmol). The reaction was stirred at room temperature for 5hrs and progress of the reaction was slow, so it was heated to 50°C for 2.5hrs. TLC (system A) showed consumption of starting materials and the mixture was evaporated to dryness, re-dissolved in CH_2Cl_2 (200ml) and washed with sat. sodium bicarbonate (200ml), dried with sodium sulfate (anhyd.), filtered and the solvent removed by evaporation to yield crude **10** (0.81g). This was not purified at this stage.

R_f (system A) 0.75.



11 $C_{22}H_{24}N_2O_5$

1-((5,6-Dihydroxyhexyl)amino)-4-(acetylamino)-anthraquinone (11).

Deprotection was carried out by the method described by Will. [167]

Crude **10** (0.81g) was dissolved in THF (10ml) and to this was added H_2O (4ml) and c.HCl (1ml). The mixture was stirred for 1hr at room temperature. TLC (system A) showed consumption of starting material and the mixture was evaporated, re-dissolved in CH_2Cl_2 (200ml) and washed with sat. sodium bicarbonate (200ml); dried with anhydrous sodium sulfate; filtered and the solvent evaporated to yield crude product. This was purified by column chromatography eluting with CH_2Cl_2/MeOH (0-15%) to yield **11** as a coloured solid, 100mg (3 steps: 4%).

R_f (system A) 0.45.

^{1}H NMR (DMSO-d_6) δ: 12.30 (s, 1H, N**H**^{16}), 10.20 (br s, 1H, N**H**^{15}), 8.85 (d, 1H, J = 9.7 Hz, Ar**H**^{3}), 8.16 (d, 1H, J = 8.1 Hz, Ar**H**^{5}), 8.15 (d, 1H, J = 8.1 Hz, Ar**H**^{8}), 7.64 (t, 1H, J = 7.5 Hz, Ar**H**^{6}), 7.61 (t, 1H, J = 7.5 Hz, Ar**H**^{7}), 7.05 (d, 1H, J = 9.7 Hz, Ar**H**^{2}), 3.74-3.58 (m, 2H, C**H**$_2^{24}$), 3.45-3.39 (m, 1H, C**H**^{23}), 3.30 (dt, 2H, J = 12.1 Hz, J = 7.0 Hz, C**H**$_2^{19}$), 2.18 (s, 3H, C**H**$_3^{18}$), 1.82-1.41 (m, 6H, C**H**$_2^{20,21,22}$).

^{13}C NMR (DMSO-d_6) δ: 187.49 (**C**^{10}), 184.06 (**C**^{9}), 170.31 (**C**^{17}), 149.35 (**C**^{14}), 134.82 (**C**^{13}), 134.40 (**C**H^3), 134.13 (**C**^{4}), 133.62 (**C**^{1}), 133.15 (**C**H^6), 130.30 (**C**H^7), 127.10 (**C**H^5), 126.73 (**C**H^8), 121.67 (**C**H^2), 117.18 (**C**^{12}), 110.51 (**C**^{11}), 72.40 (**C**H^{23}), 67.18 (**C**H$_2^{24}$), 43.15 (**C**H$_2^{19}$), 33.16 (**C**H$_2^{20}$), 26.14 (**C**H$_3^{18}$), 25.59 (**C**H$_2^{22}$), 23.58 (**C**H$_2^{21}$).

IR ν_{max}/cm^{-1}: 3269 (s), 2938 (m), 2867 (m), 1689 (s), 1571.1 (s), 1509 (m).

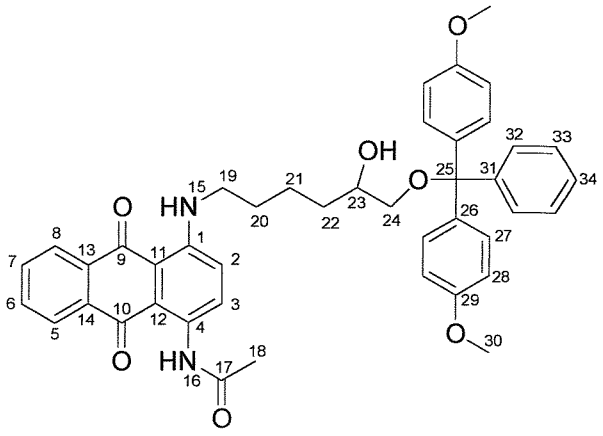
UV/vis (CH_2Cl_2): 563nm (8.01x10^{6}cm^{2}mol^{-1}), 597nm (6.83x10^{6}cm^{2}mol^{-1}).

ES$^+$/MS: 397.2 (M+H)$^+$.

HRMS (ES$^+$) for $C_{22}H_{24}N_2O_5$ (M+Na)$^+$: calculated 419.1577, found 419.1584.

Mpt. 134-138°C.

Elem. Anal. ($C_{22}H_{24}N_2O_5$): Calculated C: 66.65, H: 6.10, N: 7.06; found C: 66.69, H: 6.36, N: 6.87.



12 $C_{43}H_{42}N_2O_7$

1-((6-(4,4'-Dimethoxytrityloxy)-5-hydroxyhexyl)amino)-4-(acetylamino)-anthraquinone (12).

Diol **11** (0.1g, 0.25mmol) was dissolved in dry pyridine (5ml) and a solution of dimethoxytrityl chloride (0.09g, 0.26mmol) in dry pyridine (5ml) was added slowly, with stirring. The reaction was left for 15hrs and TLC (system D) showed consumption of the starting diol. The solution was quenched with methanol (2ml) and the mixture evaporated to dryness. The residue was redissolved in CH_2Cl_2 (100ml) and then washed with sat. KCl (2x 100ml), dried with Na_2SO_4 (anhydrous) and filtered. The organic layer was then evaporated and purified by silica gel column chromatography (CH_2Cl_2/MeOH, 0-5% with 1% NEt_3) to yield the tritylated product 0.15g (85%) as a blue/purple solid.

R_f (system D) 0.90.

^{1}H NMR ($CDCl_3$) δ: 12.50 (s, 1H, N**H**^{16}), 10.26 (br, 1H, N**H**^{15}), 8.90 (d, 1H, J = 9.6 Hz, Ar**H**^{3}), 8.26-8.18 (m, 2H, Ar**H**^{5,8}), 7.80-7.66 (d, 2H, J = 8.1 Hz, Ar**H**^{6,7}), 7.35-7.10 (m, 9H, Ar**H**^{28,32,33,34}), 7.12 (d, 1H, J = 9.6 Hz, Ar**H**^{2}), 6.83 (d, 4H, J = 9.6 Hz, Ar**H**^{27}), 3.81-3.74 (m, 1H, C**H**^{23}), 3.72 (s, 6H, C**H**$_3{}^{30}$), 3.29 (dt, 2H, J = 12.5 Hz, J = 7.0 Hz, C**H**$_2{}^{19}$), 3.20 (dd, 1H, J = 9.6 Hz, J = 3.7 Hz, C**H**^{24}), 3.05 (dd, 1H, J = 7.4 Hz, J = 9.6 Hz, C**H**^{24}), 2.47 (d, 1H, J = 3.7 Hz, O**H**), 2.18 (s, 3H, C**H**$_3{}^{18}$), 1.80-1.31 (m, 6H, C**H**$_2{}^{20,21,22}$).

^{13}C NMR ($CDCl_3$) δ: 187.26 (**C**^{10}), 183.67 (**C**^{9}), 170.00 (**C**^{17}), 158.63 (**C**^{29}), 149.03 (**C**^{14}), 144.97 (**C**^{31}), 136.12 (**C**^{26}), 134.58 (**C**^{13}), 134.13 (**C**^{4}), 133.86 (**C**H^3), 133.34 (**C**^{1}), 132.83 (**C**H^6), 130.19 (**C**H^7), 129.95 (**C**H^{27}), 128.28 (**C**H^{32}), 128.01 (**C**H^{33}), 126.99 (**C**H^5), 126.80 (**C**H^8), 126.48 (**C**H^{34}), 121.50 (**C**H^2), 116.85 (**C**^{12}), 113.28 (**C**H^{28}), 110.15 (**C**^{11}), 86.24

(**C**^{25}), 70.89 (**CH**^{23}), 67.69 (**CH**$_2^{24}$), 55.37 (**CH**$_3^{30}$), 42.98 (**CH**$_2^{19}$), 33.13 (**CH**$_2^{20}$), 29.40 (**CH**$_2^{22}$), 25.93 (**CH**$_3^{18}$), 23.34 (**CH**$_2^{21}$).

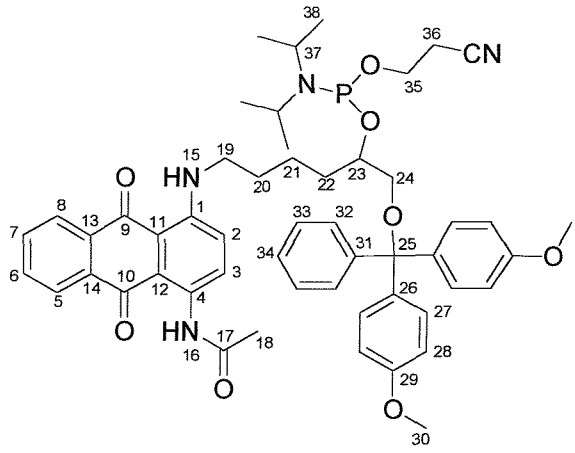
IR ν_{max}/cm^{-1}: 3422 (m), 2931 (m), 2857 (m), 1690 (m), 1571 (s), 1507 (s).

UV/vis (CH_2Cl_2): 565nm ($3.4x10^6 cm^2mol^{-1}$), 599nm ($2.9x10^6 cm^2mol^{-1}$).

ES$^+$/MS: 721.0 $(M+Na)^+$.

HRMS (ES^+) for $C_{43}H_{42}N_2O_7$ $(M+Na)^+$: calculated 721.2884, found 721.2893.

Mpt. 82-84°C.



13 $C_{52}H_{59}N_4O_7P$

1-((6-(4,4'-Dimethoxytrityloxy)-5-(2-cyanoethyl-*N,N*-diisopropylphosphoramidyl)hexyl)amino)-4-(acetylamino)-anthraquinone (13).

Phosphitylation was carried out by the method described by Caruthers *et al.* [6]

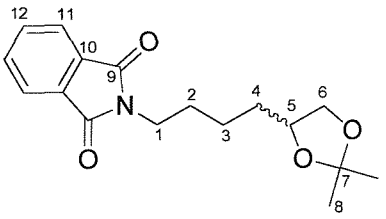
Compound **12** (0.1g, 0.14mmol) was dissolved in dry THF (2ml) to which DIPEA (0.037g, 0.29mmol) and 2-cyanoethoxy-*N,N*-diisopropylamine chlorophosphine (0.038g, 0.17mmol) were added under argon. The mixture was stirred for 1.5hrs and observed by TLC (system E), showing only partial consumption, so a further 0.1 eq. of the 2-cyanoethoxy-*N,N*-diisopropylamine chlorophosphine was added to drive the reaction to completion. The mixture was diluted with EtOAc (50ml), washed with sat. KCl (50 ml), dried with sodium sulfate (anhydrous), filtered and evaporated to yield crude product. This was purified with silica gel column chromatography under argon, eluting with EtOAc (100%) to yield **13** (0.1g, 0.81%) as a dark blue solid. This product was dissolved in acetonitrile (anhyd. 5ml), filtered and evaporated to check solubility for use as a monomer in automated synthesis.

R_f (system E) 0.9.

^{31}P NMR ($CDCl_3$) δ: 149.31, 148.79.

ES$^+$/MS: 899.2 $(M+H)^+$, 921.2 $(M+Na)^+$.

The extreme air sensitivity of this compound prevented the accumulation of other data.

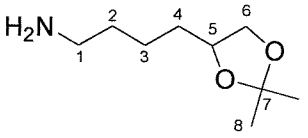


14 $C_{17}H_{21}NO_4$

Racemic *N*-phthaloyl-5,6-isopropylidenedioxyhexylamine (14).[167]

Alcohol **7** (9.6g, 55mmol), PPh_3 (15.2g, 58mmol) and phthalimide (8.5g, 58mmol) were dissolved in dry THF (50ml). A solution of DEAD (10.4g, 58mmol) in THF (dry, 20ml) was added dropwise. The reaction was stirred for 1.5hrs. TLC (system C) showed consumption of starting material and the mixture was evaporated to dryness. The residue was dissolved in ether and filtered. The filtrate was evaporated to dryness yielding the crude phthalimide **14** as a waxy white solid 24.0g.

R_f (system C) 0.5.



15 $C_9H_{19}NO_2$

Racemic 5,6-isopropylidenedioxyhexylamine (15).[167]

The crude phthalimide **14** (23.55g, 78mmol) was dissolved in dry methanol (100ml) and hydrazine monohydrate (4.26g, 133mmol) was added slowly. The mixture was left stirring for 15hrs and followed by TLC (system D). On consumption of starting material the solvent was removed by evaporation and the residual solid dissolved in ether (100ml), which was washed with NaOH (2M, 200ml) and the aqueous phase extracted with ether (3x 20ml). The organic phases were combined, evaporated and purified by Kugelrohr distillation to yield a colourless oil **15**, 6.1g (64%, 2 steps).

R_f (system D) 0.15.

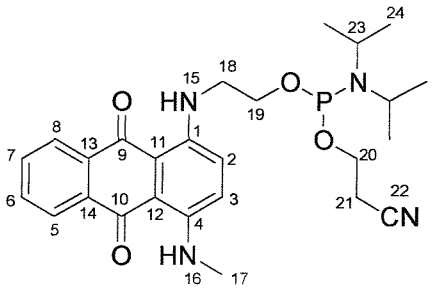
^{1}H NMR ($CDCl_3$) δ: 4.15-4.01 (m, 2H, CH_2^6), 3.55 (tt, 1H, J = 7.5 Hz, J = 2.5 Hz, CH^5), 2.78-2.69 (m, 2H, CH_2^1), 1.43 (s, 3H, CH_3^8), 1.32 (s, 3H, CH_3^8), 1.73-1.17 (m, 6H, $CH_2^{2,3,4}$).

^{13}C NMR ($CDCl_3$) δ: 108.88 (C^7), 76.42 (CH^5), 69.86 (CH_2^6), 42.46 (CH_2^1), 34.19 (CH_2^2), 33.85 (CH_2^4), 27.34 (CH_3^8), 26.12 (CH_3^8), 23.52 (CH_2^3).

IR ν_{max}/cm^{-1}: 2975 (m), 2852 (m), 1738 (s), 1369 (s).

ES$^+$/MS: 174 $(M+H)^+$.

Analytical data was consistent with that found in the literature.[167]



16 $C_{26}H_{31}N_4O_4P$

1-(((2-Cyanoethyl-*N,N*-diisopropylphosphoramidyl)ethyl)amino)-4-(methylamino)-anthraquinone (16).

Phosphitylation was carried out by the method described by Caruthers *et al.* [6]

Disperse blue 3 (5.0g) was purified by silica gel column chromatography, eluting with EtOAc/Hexane (1:1) to yield pure starting material, 1-((hydroxyethyl)amino)-4-(methylamino)-anthraquinone (0.3g, 1.0mmol, ~6%). This was dissolved in dry CH_2Cl_2 (20ml), to which DIPEA (0.65g, 5.2mmol) and 2-cyanoethoxy-*N,N*-diisopropylamine chlorophosphine (0.28g, 1.2mmol) were added under argon. The mixture was stirred for 3hrs and observed with TLC (system B) showing only partial consumption so a further 0.1 eq. of 2-cyanoethoxy-*N,N*-diisopropylamine chlorophosphine were added to drive the reaction to completion. The mixture was diluted with EtOAc (50ml), washed with sat. KCl (50 ml), dried (anhydrous sodium sulfate), filtered and evaporated to yield crude product (2.1g) as a blue/purple solid. This was purified with silica gel column chromatography

under argon, eluting with EtOAc (100%). The solid product was very sensitive to air and decomposed on TLC rapidly. This product was dissolved in acetonitrile (anhyd. 5ml), filtered and evaporated to confirm solubility for use as a monomer. A resultant yield of 0.32g (64%) was obtained for **16**.

R_f (system B) 0.7.

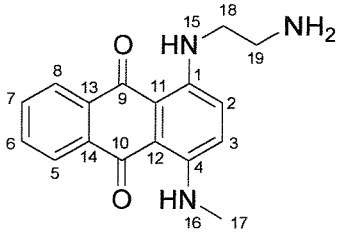
^{1}H NMR ($CDCl_3$) δ: 10.85-10.78 (m, 1H, N**H**^{15}), 10.55-10.48 (m, 1H, N**H**^{16}), 8.26-8.22 (m, 2H, Ar**H**^{6,7}), 7.62-7.57 (m, 2H, Ar**H**^{5,8}), 7.20 (d, 1H, J = 9.5 Hz, Ar**H**^{3}), 7.15 (d, 1H, J = 9.5 Hz, Ar**H**^{2}), 3.95-3.72 (m, 4H, C**H**^{23}, CH_2^{18}), 3.59-3.41 (m, 4H, CH_2^{19,20}), 3.00 (d, 3H, J = 5.2 Hz, CH_3^{17}), 2.55 (t, 2H, J = 6.6 Hz, CH_2^{21}), 1.15-1.05 (m, 12H, CH_3^{24}).

^{13}C NMR ($CDCl_3$) δ: 182.63 (**C**^{10}), 173.79 (**C**^{9}), 147.13 (**C**^{14}), 145.98 (**C**^{13}), 134.60 (**CH**^{4,1}), 132.21 (**CH**^{6}), 132.17 (**CH**^{7}), 126.19 (**CH**^{5}), 126.14 (**CH**^{8}), 123.72 (**CH**^{3}), 123.03 (**CH**^{2}), 117.93 (**C**^{22}), 110.37 (**C**^{12}), 110.15 (**C**^{11}), 62.23 (CH_2^{19}), 58.58 (CH_2^{20}), 43.78 (CH_2^{18}), 43.21 (**CH**^{23}), 29.67 (CH_3^{17}), 24.74 (CH_3^{24}), 20.55 (CH_2^{21}).

^{31}P NMR ($CDCl_3$) δ: 148.96.

ES$^+$/MS: 497.3 $(M+H)^+$, 519.3 $(M+Na)^+$.

IR and UV/vis of this compound were unavailable due to extreme air sensitivity.



17 $C_{17}H_{17}N_3O_2$

1-((2-Aminoethyl)amino)-4-(methylamino)-anthraquinone (17).

1-((Hydroxyethyl)amino)-4-(methylamino)-anthraquinone (0.10g, 0.33mmol), phthalimide (0.06g, 0.37mmol) and triphenylphosphine (0.10g, 0.37mmol) were dissolved in dry THF (20ml). To this a solution of DEAD (0.07g, 0.37mmol) in dry THF (2ml), was added slowly. The reaction was stirred for 1.5hrs, evaporated to dryness *in vacuo* and the residue dissolved in CH_2Cl_2:MeOH (1:2, 20ml). Hydrazine monohydrate (0.083g, 1.65mmol) was

added and the mixture was stirred for 15hrs, after which the solvent was removed *in vacuo* and the blue solid dissolved in CH_2Cl_2 (50ml) and washed with NaOH (2M, 2x 50ml). The product was removed from the organic layer by washing with citric acid solution (10%, 100ml). The blue aqueous layer was then neutralised (sat. $NaHCO_3$, 2x 100ml) and the product removed by washing with CH_2Cl_2 (150ml). The organic layer was dried (anhydrous Na_2SO_4) and evaporated to dryness *in vacuo*. Purification by silica gel column chromatography, eluting with CH_2Cl_2:MeOH (9:1) gave the title compound as a blue solid (0.09g, 90%) after drying with the high vacuum.

R_f (system A) 0.1.

^{1}H NMR ($CDCl_3$) δ: 10.79 (s, 1H, N**H**^{15}), 10.51 (s, 1H, N**H**^{16}), 8.30-8.20 (m, 2H, Ar**H**^{6,7}), 7.65-7.55 (m, 2H, Ar**H**^{5,8}), 7.12 (d, 1H, J = 9.5 Hz, Ar**H**^{3}), 7.08 (d, 1H, J = 9.5 Hz, Ar**H**^{2}), 3.39-3.28 (m, 4H, C**H**$_2^{18,19}$), 3.00 (d, 1H, J = 5.0 Hz, C**H**$_3^{17}$), 1.32 (br s, 2H, N**H**$_2$).

^{13}C NMR ($CDCl_3$) δ: 182.89 (**C**^{10}), 182.68 (**C**^{9}), 147.31 (**C**^{14}), 146.45 (**C**^{13}), 134.89 (**C**^{4}), 134.85 (**C**^{1}), 132.39 (**C**H^6), 132.35 (**C**H^7), 126.43 (**C**H^5), 126.33 (**C**H^8), 123.82 (**C**H^3), 123.33 (**C**H^2), 110.45 (**C**H^{12}), 110.31 (**C**^{11}), 46.31 (**C**H$_2^{18}$), 37.88 (**C**H$_2^{19}$), 29.86 (**C**H$_3^{17}$).

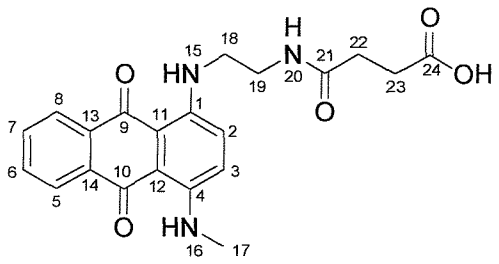
IR ν_{max}/cm^{-1}: 3325 (s), 2916 (m), 2857 (m), 1647 (m), 1561 (s), 1518 (s).

UV/vis (CH_2Cl_2): 560nm ($4.3x10^6 cm^2 mol^{-1}$), 598nm ($7.9x10^6 cm^2 mol^{-1}$), 645nm ($8.5x10^6 cm^2 mol^{-1}$).

ES$^+$/MS: 296.0 $(M+H)^+$.

HRMS (ES^+) for $C_{17}H_{17}N_3O_2$ $(M+H)^+$: calculated 296.1394, found 296.1388.

Mpt. 148-151°C.



18 $C_{21}H_{21}N_3O_5$

1-(2-Succinamido-ethylamine)-4-(methylamino)-anthraquinone (18).

Amine **17** (0.09g, 0.34mmol), succinic anhydride (0.04g, 0.38mmol) and DMAP (0.01g, 0.09mmol) were dissolved in dry pyridine (20ml). The reaction was stirred for 1.5hrs, evaporated to dryness *in vacuo*, and purified by silica gel column chromatography, eluting with CH_2Cl_2:MeOH (9:1). The good fractions were combined and evaporated to dryness to give the title compound as a blue solid (0.09g, 90%).

R_f (system A) 0.25.

^{1}H NMR (DMSO-d_6) δ: 10.81 (s, 1H, N**H**^{15}), 10.62 (s, 1H, N**H**^{16}), 8.36-8.27 (m, 2H, Ar**H**^{6,7}), 8.30-8.20 (m, 1H, N**H**^{20}), 7.78-7.65 (m, 2H, Ar**H**^{5,8}), 7.59 (d, 1H, J = 9.4 Hz, Ar**H**^{3}), 7.42 (d, 1H, J = 9.4 Hz, Ar**H**^{2}), 3.61-3.20 (m, 4H, C**H**$_2$^{18,19}), 3.08 (d, 3H, J = 5.2 Hz, C**H**$_3$^{17}), 2.45 (t, 2H, J = 7.5 Hz, C**H**$_2$^{23}), 2.34 (t, 2H, J = 7.5 Hz, C**H**$_2$^{22}).

^{13}C NMR (DMSO-d_6) δ: 180.89 (**C**^{10}), 179.85 (**C**^{9}), 173.07 (**C**^{24}), 170.85 (**C**^{21}), 146.07 (**C**^{14}), 145.16 (**C**^{13}), 134.89 (**CH**^{4}), 134.85 (**CH**^{1}), 131.51 (**CH**^{6,7}), 124.93 (**CH**^{5,8}), 123.77 (**CH**^{3}), 123.45 (**CH**^{2}), 107.85 (**C**^{12}), 107.31 (**C**^{11}), 54.13 (CH$_2$^{18}), 41.77 (**CH**$_2$^{19}), 39.28 (**CH**$_2$^{23}), 29.31 (**CH**$_2$^{22}), 28.50 (**CH**$_3$^{17}).

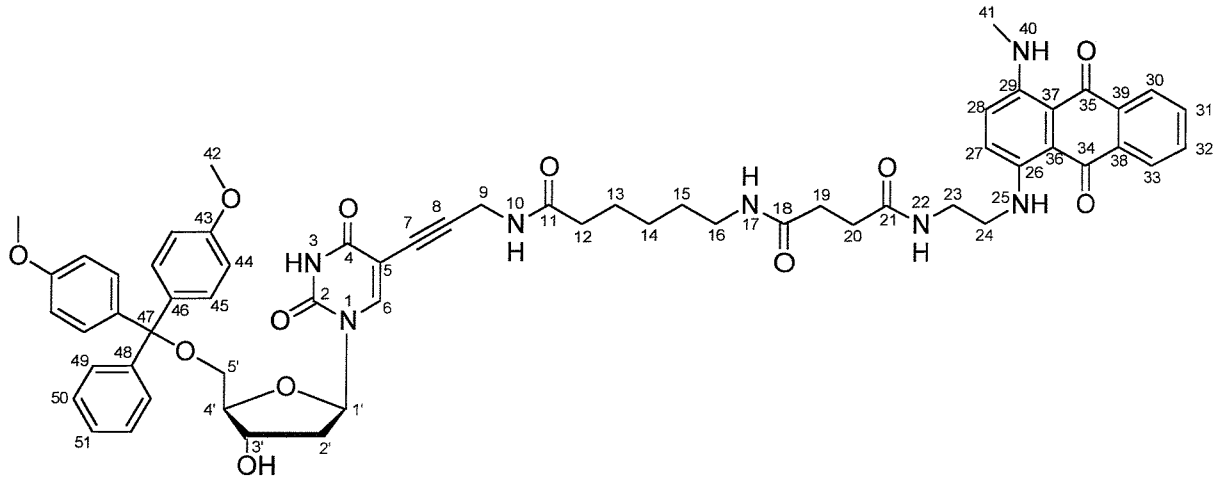
IR ν_{max}/cm^{-1}: 3302 (s), 2921 (m), 1650 (m), 1564 (s).

UV/vis (CH_2Cl_2): 561nm ($4.2x10^6 cm^2mol^{-1}$), 595nm ($7.8x10^6 cm^2mol^{-1}$), 642nm ($8.7x10^6 cm^2mol^{-1}$).

ES$^+$/MS: 396.0 $(M+H)^+$.

HRMS (ES^+) for $C_{21}H_{21}N_3O_5$ $(M+H)^+$: calculated 396.1554, found 396.1545.

Mpt. 179-182°C.



19 $C_{60}H_{63}N_7O_{12}$

5'-*O*-(4,4'-Dimethoxytrityl)-5-(3-(6-*N1*-(2-((4-(methylamino)-anthraquinone)amino)ethyl)succinamidohexamide)propynyl)-2'-deoxyuridine (19). **18** (0.3g, 0.78mmol), EDC (0.23g, 1.2mmol) and HOBT (0.16g, 1.2mmol) were dissolved in dry DMF (20ml) and stirred for 10min. A separate solution of 5'-*O*-(4,4'-dimethoxytrityl)-5-(*N*-(2-propynyl)-6-amino-hexanamide)-2'-deoxyuridine (0.6g, 0.87mmol) and DIPEA (0.16g, 1.2mmol) in dry DMF (10ml) was made and added to the first. The combined mixture was stirred at room temperature for 18hrs, until TLC (system A) showed completion. The solvent was removed and dissolved in CH_2Cl_2, washed with H_2O and $NaHCO_3$, dried with Na_2SO_4 and evaporated to dryness. The residue was purified by column chromatography with silica gel (CH_2Cl_2/MeOH, 9:1) to yield the title compound 0.38g (45%), as a blue solid.

R_f (system A) 0.4.

^{1}H NMR ($CDCl_3$) δ: 10.64 (s, 1H, N**H**^{25}), 10.50 (s, 1H, N**H**^{40}), 8.16-8.07 (m, 2H, Ar**H**^{31,32}), 7.95 (m, 1H, N**H**^{3}), 7.55-7.45 (m, 2H, Ar**H**^{30,33}), 7.40-7.05 (m, 11H, Ar**H**^{44,49,50,51}, C**H**^{6}, N**H**^{17,22}), 7.00-6.87 (m, 2H, C**H** Ar^{27,28}), 6.72 (d, 4H, J = 8.5 Hz, Ar**H**^{45}), 6.35 (s, 1H, NH^{10}), 6.18 (t, 1H, J = 6.5 Hz, C**H**$^{1'}$), 4.49-4.41 (m, 1H, C**H**$^{3'}$), 4.09-3.96 (m, 1H, C**H**$^{4'}$), 3.90-3.70 (m, 2H, C**H**$_2$^{9}), 3.65 (s, 6H, C**H**$_3$^{42}), 3.42-3.31 (m, 4H, C**H**$_2$^{23,24}), 3.29-3.16 (m, 2H, C**H**$^{5'}$), 3.12-2.99 (m, 2H, C**H**$_2$^{16}), 2.87 (d, 3H, J = 5.0 Hz, C**H**$_3$^{41}), 2.45-2.31 (m, 4H, C**H**$_2$^{19,20}), 2.25-1.85 (m, 4H, C**H**$_2$$^{12,2'}$), 1.56-1.12 (m, 6H, C**H**$_2$^{13,14,15}).

^{13}C NMR ($CDCl_3$) δ: 181.73 (**C**^{35}), 181.29 (**C**^{34}), 172.90 (**C**^{21}), 172.34 (**C**^{18}), 172.23 (**C**^{11}), 162.70 (**C**^{4}), 158.14 (**C**^{43}), 149.53 (**C**^{2}), 146.59 (**C**^{39}), 145.50 (**C**^{38}), 144.18 (**C**^{48}), 142.48 (**CH**^{6}), 135.18, 133.97, 133.73 (**C**^{26,29,46}), 131.56, 131.45 (**CH**^{31,32}), 129.59 (**CH**^{45}), 127.60 (**CH**^{49}), 127.53 (**CH**^{50}), 126.52 (**CH**^{51}), 125.61 (**CH**^{30}), 125.50 (**CH**^{33}), 122.78 (**CH**^{27,28}), 112.79 (**CH**^{44}), 109.42 (**C**^{36}), 109.04 (**C**^{37}), 98.96 (**C**^{5}), 89.05 (**C**^{8}), 86.49 (**C**^{47}), 86.21 (**CH**$^{4'}$), 85.39 (**CH**$^{1'}$), 73.92 (**C**^{7}), 71.49 (**CH**$^{3'}$), 63.33 (**CH**$_2{}^{5'}$), 54.86 (**CH**$_3{}^{42}$), 52.50 (**CH**$_2{}^{24}$), 41.48 (**CH**$_2{}^{23}$), 41.25 (**CH**$_2{}^{16}$), 39.06, 38.82, 35.39, 31.35, 29.41 (**CH**$_2{}^{9,12,19,20,2'}$), 28.96 (**CH**$_3{}^{41}$); 28.51, 25.71, 24.51 (**CH**$_2{}^{13,14,15}$).

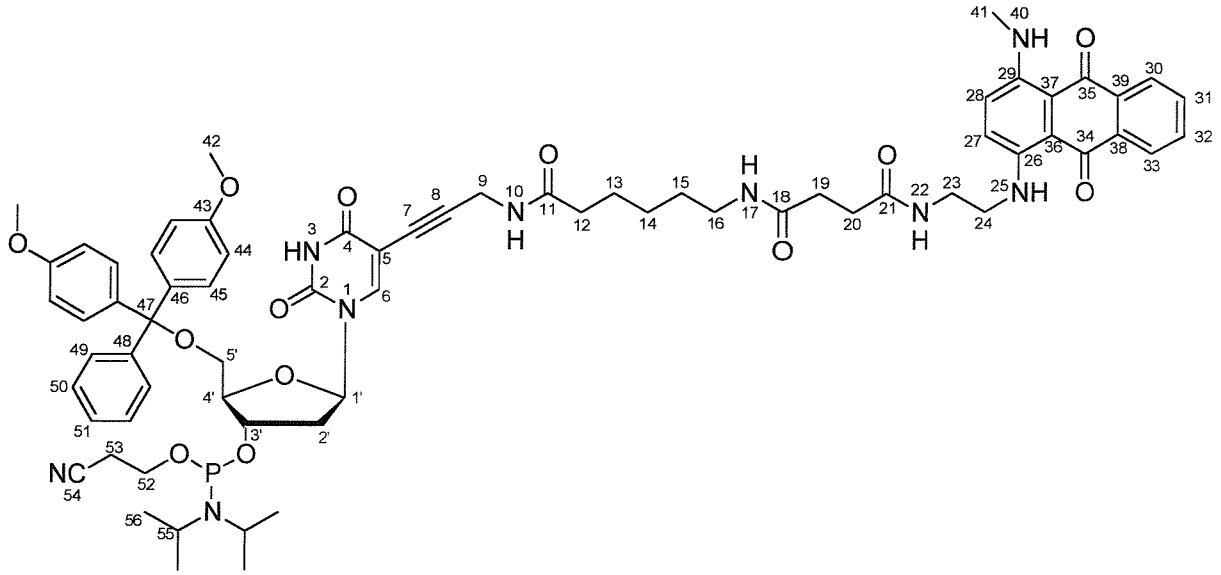
IR ν_{max}/cm^{-1}: 3317 (s), 3073 (m), 2941 (m), 1650 (s), 1574 (m).

UV/vis (CH_2Cl_2): 560nm ($6.3x10^6 cm^2mol^{-1}$), 596nm ($12.2x10^6 cm^2mol^{-1}$), 644nm ($13.6x10^6 cm^2mol^{-1}$).

ES$^+$/MS: 1096.5 $(M+Na)^+$.

HRMS (ES^+) for $C_{60}H_{63}N_7O_{12}$ $(M+H)^+$: calculated 1074.4636, found 1074.4607.

Mpt. 138-144°C.



20 $C_{69}H_{81}N_9O_{13}P$

5'-*O*-(4,4'-Dimethoxytrityl)-5-(3-(6-*N1*-(2-((4-(methylamino)-anthraquinone)amino)ethyl)succinamidohexamide)propynyl)-3'-*O*-(2-cyanoethyl-*N,N*-diisopropylphosphoramidyl)-2'-deoxyuridine (20).

Phosphitylation was carried out by the method described by Caruthers *et al.* [6]

19 (0.35g, 0.33mmol) was dissolved in dry CH_2Cl_2 (30ml) and dry DIPEA (0.23ml, 1.32mmol) was added. 2-Cyanoethoxy-*N,N*-diisopropylamine chlorophosphine (0.113ml, 0.50mmol) was added and the mixture was stirred under argon for 2.5hrs. TLC (system A) showed completion of the reaction and the mixture was reduced to near dryness before redissolving in EtOAc (degassed with argon) and then washing with sat. KCl (degassed), drying with sodium sulfate and reducing to dryness to yield crude product. This was purified by silica gel column chromatography under argon to yield product **20**, 0.36g (93%).

R_f (system A) 0.4.

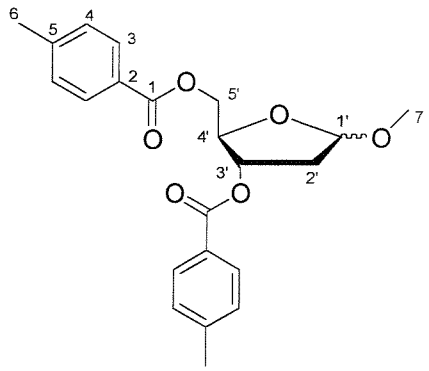
^{1}H NMR ($CDCl_3$) δ: 10.73 (s, 1H, N**H**^{25}), 10.61-10.48 (m, 1H, N**H**^{40}), 8.31-8.15 (m, 2H, Ar**H**^{31,32}), 8.10-7.98 (m, 1H, N**H**^{3}), 7.64-7.50 (m, 2H, Ar**H**^{30,33}), 7.41-7.01 (m, 13H, C**H**^{6}, N**H**^{17,22}, Ar**H**^{27,28,44,49,50,51}), 6.81-6.65 (d, 4H, J = 8.5 Hz, Ar**H**^{45}), 6.67-6.54 (s, 1H, N**H**^{10}), 6.23 (t, 1H, J = 6.5 Hz, C**H**$^{1'}$), 4.59-4.48 (m, 1H, C**H**$^{3'}$), 4.27-4.16 (m, 1H, C**H**$^{4'}$), 4.15-3.90 (m, 2H, CH_2^{9}), 3.90-3.21 (m, 14H, CH_3^{42}, CH_2^{23,24,52}, ^{i}Pr**H**^{55}), 3.19-3.08 (m, 2H,

$CH_2^{5'}$), 2.98 (d, 3H, J = 5.0 Hz, CH_3^{41}), 2.65-2.41 (m, 6H, $CH_2^{19,20,53}$), 2.38-1.88 (m, 4H, $CH_2^{2',12}$), 1.61-0.97 (m, 18H, $CH_2^{13,14,15}$, $^{i}PrCH_3^{56}$).

^{31}P NMR ($CDCl_3$) δ: 149.60, 149.28.

ES$^+$/MS: 1296.5 $(M+Na)^+$.

Further characterisation was not possible due to the air sensitivity of the material.



21 $C_{22}H_{24}O_6$

3',5'-*O*-Ditoluoyl-1'-methoxy-2'-deoxy-D-ribose (21). [171]

2-Deoxy-D-ribose (26.14g, 193mmol) was dissolved in 0.1% HCl/MeOH (500ml) and stirred at room temperature for 30 minutes. Silver carbonate (7.5g, 26mmol) was added to neutralise the mixture, which was then filtered and reduced to dryness to give a yellow oil. This was co-evaporated with freshly distilled pyridine three times, before dissolving in dry pyridine (150ml). The solution was stirred under argon, cooled on ice and 4-toluoyl chloride (65g, 56ml, 418mmol) was added dropwise. The vessel was warmed to room temperature and then heated to 65°C for 6 hours. The mixture was cooled to room temperature and water was added, which was then extracted with ether three times. The organic layers were combined and washed with water, 5% HCl, then sat. $NaHCO_3$, before drying (Na_2SO_4) and evaporation to dryness. The residue was co-evaporated with toluene three times and the residue was purified by column chromatography on silica gel eluting with CH_2Cl_2 to give a white waxy solid **21** (44.9g, 71%).

R_f (system E) 0.64, 0.54 (2 anomers).

^{1}H NMR ($CDCl_3$) δ: 8.01-7.91 (m, 4H, Ar**H**^{3}), 7.32-7.25 (m, 4H, Ar**H**^{4}), 5.64-5.58 (m, 0.4H, C**H**$^{3'}$α), 5.42 (dd, 0.6H, *J*= 3.7 Hz, *J* = 2.2 Hz, C**H**$^{3'}$β), 5.23 (dd, 0.4H, *J* = 2.2 Hz, *J* = 5.5 Hz, C**H**$^{1'}$α), 5.19 (d, 0.6H, *J* = 4.4 Hz, C**H**$^{1'}$β), 4.68-4.44 (m, 3H, C**H**$^{4'}$, C**H**$_2^{5'}$), 3.50 (s, 2H, C**H**$_3^7$β), 3.40 (s, 1H, C**H**$_3^7$α), 2.62-2.31 (m, 8H, C**H**$_2^{2'}$, C**H**$_3^6$).

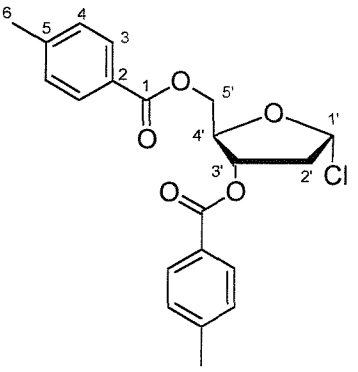
^{13}C NMR ($CDCl_3$) δ: 166.89, 166.74, 166.68, 166.52 (**C**^{1}), 144.42, 144.33, 144.18, 143.08 (**C**^{5}), 130.22, 130.10 (**C**^{3}), 129.52, 129.50 (**C**^{4}), 127.66, 127.55, 127.48, 127.34 (**C**^{2}), 105.76 (**C**H$^{1'}$α), 105.23 (**C**H$^{1'}$ β), 82.06 (**C**H$^{4'}$ α), 81.16 (**C**H$^{4'}$ β), 75.57 (**C**H$^{3'}$ α), 74.80 (**C**H$^{3'}$ β), 65.30 (**C**H$_2^{5'}$ α), 64.48 (**C**H$_2^{5'}$ β), 55.60 (**C**H$_3^7$α), 55.49 (**C**H$_3^7$β), 39.69 (**C**H$_2^{2'}$), 22.07 (**C**H$_3^6$).

IR ν_{max}/cm^{-1}: 2927.6 (m), 1709.2 (s), 1610.3 (m).

ES$^+$/MS: 790.9 $(2M+Na)^+$.

HRMS (ES^+) for $C_{22}H_{24}O_6$ $(M+H)^+$: calculated 407.1465, found 407.1454.

Data was consistent with that in the literature.[171]



22 $C_{21}H_{21}O_5Cl$

3',5'-*O*-Ditoluoyl-1'-chloro-2'-deoxy-D-ribose (22). [171]

Glacial acetic acid (81ml, 1400mmol), acetyl chloride (16.3ml, 230mmol), water (4ml, 230mmol) were mixed to make an 'HCl' solution *in-situ*. This solution (2.5ml) was added slowly to **21** (1g, 2.6mmol) in glacial acetic acid (2ml). The solution was stirred in an ice bath and warmed to room temperature. A further addition of acetyl chloride (2.5ml) was slowly added and shortly after a white precipitate formed. The mixture was filtered and washed with ether (100ml) and then dried *in vacuo* over KOH, to yield **22** (0.8g, 80.0%) as a white solid.

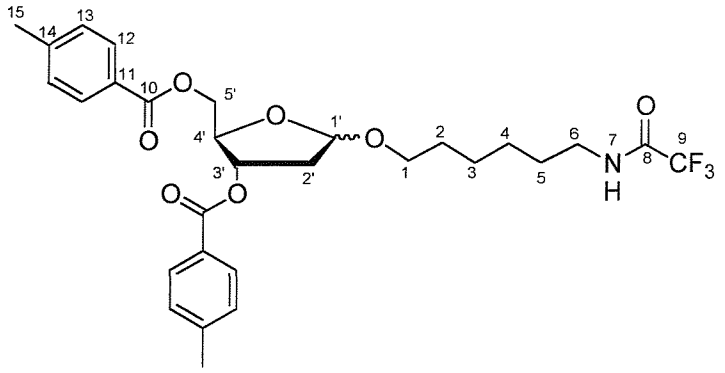
R_f (system E) 0.4.

^{1}H NMR ($CDCl_3$) δ: 8.50 (d, 2H, J = 8.5 Hz, Ar**H**^{3}), 7.50 (d, 2H, J = 8.0 Hz, Ar**H**^{3}), 7.31 (d, 2H, J = 8.5 Hz, Ar**H**^{4}), 7.28 (d, 2H, J = 8.0 Hz, Ar**H**^{4}), 6.52 (d, 1H, J = 5.5 Hz, C**H**$^{1'}$), 5.64-5.50 (m, 1H, C**H**$^{3'}$), 4.91 (q, 1H, J = 3.5 Hz, C**H**$^{4'}$), 4.80-4.60 (m, 2H, C**H**$_2^{5'}$), 2.98-2.70 (m, 2H, C**H**$_2^{2'}$), 2.48 (s, 3H, C**H**$_3^6$), 2.35 (s, 3H, C**H**$_3^6$).

^{13}C NMR ($CDCl_3$) δ: 166.81, 166.47 (**C**^{1}), 144.70, 144.46 (**C**^{5}), 130.33, 130.28 (C^3), 129.64, 129.62, (**C**H^4), 127.24, 127.11 (**C**^{2}), 95.73 (**C**H$^{1'}$), 85.10 (**C**H$^{4'}$), 73.97 (**C**H$^{3'}$), 63.91 (**C**H$^{5'}$), 44.87 (**C**H$_2^{2'}$), 22.12 (**C**H$_3^6$).

ES$^-$/MS: 385 (M-H$^+$).

Analytical data was consistent with that found in the literature.[171]



23 $C_{29}H_{34}FNO_7$

3',5'-*O*-Ditoluoyl-1'-*O*-(6-trifluoroacetylamidohexyl)-2'-deoxy-D-ribose (23).[171]

6-Trifluoroacetylamidohexan-1-ol (**30**) (1.7g, 8mmol) was co-evaporated with dry THF and then dissolved in THF (20ml). DMAP (0.22g, 1.8mmol) was added, followed by **22** (3.4g, 8.7mmol) and the reaction was stirred for three hours. The mixture was evaporated to dryness and co-evaporated three times with CH_2Cl_2. The residue was then purified by silica gel column chromatography eluting with CH_2Cl_2:EtOAc (9:1), to give a colourless oil **23** (3.6g, 79.7%).

R_f (system E) 0.50, 0.44 (2 anomers).

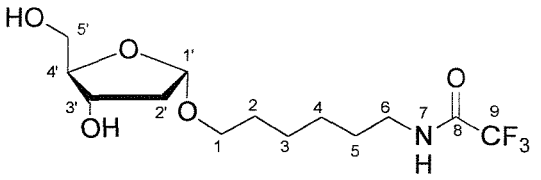
^{1}H NMR ($CDCl_3$) δ: 7.92-7.79 (m, 4H, Ar**H**^{12}), 7.21-7.09 (m, 4H, Ar**H**^{13}), 6.54-6.22 (m, 1H, NH), 5.55-5.31 (m, 1H, C**H**$^{3'}$), 5.27-5.19 (m, 1H, C**H**$^{1'}$), 4.60-4.35 (m, 3H, C**H**$^{4'}$, $CH_2^{5'}$), 3.75-3.62 (m, 1H, C**H**^{1}), 3.40-3.18 (m, 3H, C**H**^{1}, CH_2^{6}), 2.53-2.20 (m, 8H, $CH_2^{2'}$, CH_3^{15}), 1.60-1.19 (m, 8H, $CH_2^{2,3,4,5}$).

^{13}C NMR ($CDCl_3$) δ: 164.64, 164.54, 164.44, 164.27 (**C**^{10}), 142.19, 142.14, 142.02, 141.97 (**C**^{14}), 127.88, 127.83, 127.79 (**CH**^{12}), 127.24, 127.19 (**CH**^{13}), 125.17, 124.97 (**CH**^{11}), 102.81, 101.94 (**CH**$^{1'}$), 79.86, 79.12 (**CH**$^{4'}$), 73.80, 72.87 (**CH**$^{3'}$), 65.86, 65.33 (CH_2^{1}), 63.57, 62.45 ($CH_2^{5'}$), 37.99 (CH_2^{6}), 37.41, 37.34 ($CH_2^{2'}$), 27.62, 27.34 (CH_2^{2}), 27.03, 26.90 (CH_2^{5}), 24.50, 24.32 (CH_2^{3}), 23.88, 23.80 (CH_2^{4}), 19.78 (CH_3^{15}).

ES$^+$/MS: 588.2 $(M+Na)^+$, 1153.3 $(2M+Na)^+$.

HRMS (ES^+) for $C_{29}H_{34}FNO_7$ $(M+Na)^+$: calculated 588.2179, found 588.2183.

Analytical data was consistent with that found in the literature.[171]



24 $C_{13}H_{22}F_3NO_5$

1'-*O*-(6-Trifluoroacetylamidohexyl)-2'-deoxy-D-ribose (24).[171]

23 (3.55g, 6.30mmol) was dissolved in freshly prepared 0.5M NaOMe/MeOH solution (50ml) and the reaction was stirred for 1hr. TLC (system A) showed completion of the reaction and the solvent was removed. The residue was pre-absorbed on to silica and then purified with column chromatography eluting with CH_2Cl_2:MeOH (9:1) to yield product **24** (1.1g, 53%) as a pale yellow oil.

R_f (system A) 0.32

^{1}H NMR ($CDCl_3$) δ: 6.61-6.42 (m, 1H, N**H**^{7}), 5.16-5.12 (m, 1H, C**H**$^{1'}$α), 4.50-3.97 (m, 2H, C**H**$^{3'}$, O**H**), 3.72-3.49 (m, 4H, C**H**$^{4'}$, C**H**$_2{}^1$), 3.35-3.18 (m, 4H, C**H**$_2{}^{5'}$, C**H**$_2{}^6$), 2.39-1.88 (m, 2H, C**H**$_2{}^{2'}$), 1.55-1.46 (m, 4H, C**H**$_2{}^{2,5}$), 1.33-1.27 (m, 4H, C**H**$_2{}^{3,4}$).

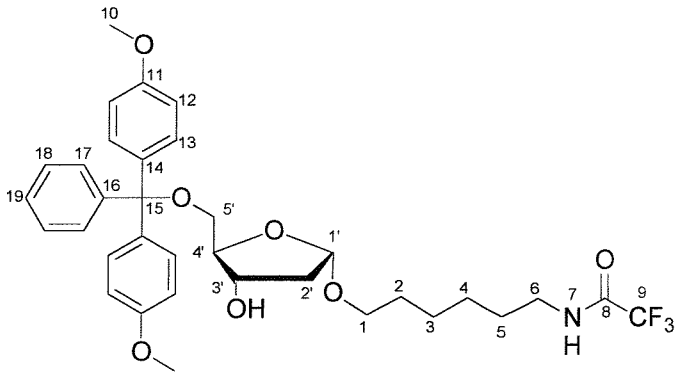
^{13}C NMR ($CDCl_3$) δ: 181.80, 181.01 (**C**^{8}), 144.18, 144.08 (**C**$F_3{}^9$), 104.86 (**C**H$^{1'}$α), 104.63 (**C**H$^{1'}$β), 87.94 (**C**H$^{4'}$α), 87.86 (**C**H$^{4'}$β), 73.30 (**C**H$^{3'}$α), 72.58 (**C**H$^{3'}$β), 68.41, 67.54 (**C**H$_2{}^1$), 63.71, 63.51 (**C**H$^{5'}$), 43.14, 41.81 (**C**H$_2{}^6$), 40.09, 39.95 (**C**H$^{2'}$), 29.61 (**C**H$_2{}^2$), 29.16, 29.08 (**C**H$_2{}^5$), 26.54, 26.44 (**C**H$_2{}^3$), 26.00, 25.86 (**C**H$_2{}^4$).

IR ν_{max}/cm^{-1}: 3294 (s), 3097 (w), 2935 (m), 2863 (m), 1702 (s), 1560 (m).

ES$^+$/MS: 352.1 $(M+Na)^+$; 680.9 $(2M+Na)^+$.

HRMS (ES^+) for $C_{13}H_{22}F_3NO_5$ $(M+K)^+$: calculated 368.1082, found 368.1080.

Analytical data was consistent with that found in the literature.[171]



25 $C_{34}H_{40}F_3NO_7$

5'-*O*-(4,4'-Dimethoxytrityl)-1'-*O*-(6-trifluoroacetylamidohexyl)-2'-deoxy-D-ribose (25).[171]

Sugar **24** (3.00g, 9.10mmol) was dissolved in dry pyridine (30ml) and 4,4'-dimethoxytrityl chloride (3.40g, 10.00mmol) was added. The mixture was stirred at room temperature for 12hrs, then the reaction was quenched with methanol (15ml) and evaporated to dryness *in vacuo*. The residue was dissolved in CH_2Cl_2 (200ml), washed with sat. $NaHCO_3$ (200ml), dried (anhydrous Na_2SO_4) and evaporated to dryness *in vacuo*. Co-evaporation three times with toluene gave an oil which was purified by silica gel column chromatography eluting with CH_2Cl_2:MeOH (9:1) to yield the foam product as two separable anomers: α (1.7g, 30%); β (1.1g, 20%). The α anomer was used in this synthesis and characterisation is given below.

R_f (system G) α 0.40; β 0.24.

^{1}H NMR ($CDCl_3$) δ: 7.49-7.08 (m, 9H, Ar**H**^{12,17,18,19}), 6.76 (d, J = 9.0 Hz, 4H, Ar**H**^{13}), 6.30 (s, 1H, N**H**^{7}), 5.17 (d, J = 4.5 Hz, 1H, C**H**$^{1'}$), 4.16-4.08 (m, 2H, C**H**$^{3'}$, C**H**$^{4'}$), 3.73-3.58 (m, 7H, C**H**$_3$^{10}, C**H**^{1}), 3.39-3.18 (m, 3H, C**H**^{1}, C**H**$_2$^{6}), 3.09 (dd, 1H, J= 10.0Hz, J= 5.0Hz, 2H, C**H**$^{5'}$), 3.01 (dd, 1H, J= 10.0Hz, J= 5.0Hz, C**H**$^{5'}$), 2.12-1.88 (m, 2H, C**H**$_2$$^{2'}$), 1.56-1.48 (m, 4H, C**H**$_2$^{2,5}), 1.35-1.28 (m, 4H, C**H**$_2$^{3,4}).

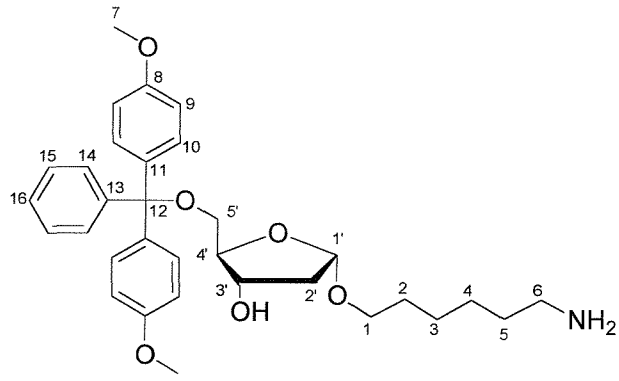
^{13}C NMR ($CDCl_3$) δ: 171.30 (**C**^{8}), 158.60 (**C**^{11}), 144.94 (**C**F$_3$^{9}), 143.80 (**C**^{16}), 136.14 (**C**^{14}), 130.20 (**C**H^{13}), 128.28 (**C**H^{17}), 127.97 (**C**H^{18}), 126.93 (**C**H^{19}), 113.05 (**C**H^{12}), 104.50 (**C**H$^{1'}$), 87.03 (**C**H$^{4'}$), 86.23 (**C**^{15}), 73.71 (**C**H$^{3'}$), 67.29 (**C**H$_2$$^{5'}$), 64.16 (**C**H$_2$^{1}), 55.37 (**C**H$_3$^{10}), 41.02 (**C**H$_2$ $^{2'}$), 39.94 (**C**H$_2$^{6}), 29.53 (**C**H$_2$^{2}), 29.03 (**C**H$_2$^{5}), 26.44 (**C**H$_2$^{4}), 25.90 (**C**H$_2$^{3}).

IR ν_{max}/cm^{-1}: 3305 (s), 2933 (m), 2861 (m), 1706 (s), 1607 (m), 1563 (m).

ES$^+$/MS: 654.0 $(M+Na)^+$.

HRMS (ES^+) for $C_{34}H_{40}F_3NO_7$ $(M+H)^+$: calculated 654.2649, found 654.2626.

Analytical data was consistent with that found in the literature.[171]



26 $C_{32}H_{41}F_3NO_6$

5'-*O*-(4,4'-Dimethoxytrityl)-1'-*O*-(6-aminohexyl)-2'-deoxy-D-ribose (26).

Tritylated sugar **25** (0.63g, 1.00mmol) was dissolved in a solution of NaOH (0.20g, 5.00mmol) in water (1.5ml) and methanol (6ml). The mixture was stirred at room temperature for 15hrs, then evaporated to dryness *in vacuo* and purified by column chromatography eluting with (CH_2Cl_2:MeOH: Et_3N, 9:1:1) to yield the title product as a pale yellow oil (0.32g, 60%).

R_f (system G) 0.2.

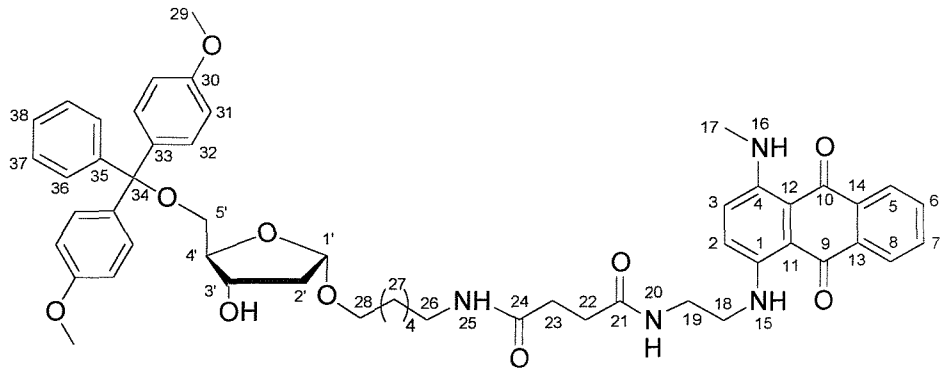
^{1}H NMR ($CDCl_3$) δ: 7.47-7.14 (m, 9H, Ar**H**^{9,14,15,16}), 6.82 (d, 4H, J = 9.0 Hz, Ar**H**^{10}), 5.24 (d, 1H, J = 4.4 Hz, C**H**$^{1'}$), 4.26-4.17 (m, 2H, C**H**$^{3'}$, C**H**$^{4'}$), 3.84-3.79 (m, 7H, C**H**$_3$^{7}, C**H**^{1}), 3.45-3.38 (m, 1H, C**H**^{1}), 3.20-3.09 (m, 2H, C**H**$^{5'}$), 2.68-2.60 (m, 2H, C**H**$_2$^{6}), 2.29-2.01 (m, 2H, C**H**$^{2'}$), 1.66-1.25 (m, 8H, C**H**$_2$^{2,3,4,5}).

^{13}C NMR ($CDCl_3$) δ: 157.51 (**C**^{8}), 143.85 (**C**^{13}), 135.06, 134.98 (**C**^{11}), 129.09 (**C**H^{10}), 127.19 (**C**H^{14}), 126.83 (**C**H^{15}), 125.79 (**C**H^{16}), 112.15 (**C**H^9), 103.45 (**C**H$^{1'}$), 85.74 (**C**^{12}), 85.11 (**C**H$^{4'}$), 72.38 (**C**H$^{3'}$), 66.41 (**C**H$_2$^{1}), 63.13 (**C**H$^{5'}$), 54.22 (**C**H$_3$^{7}), 40.02 (**C**H$_2$^{6}), 39.34 (**C**H$_2$$^{2'}$), 28.28 (**C**H$_2$^{2}), 27.97 (**C**H$_2$^{5}), 25.10 (**C**H$_2$^{3}), 24.72 (**C**H$_2$^{4}).

IR ν_{max}/cm^{-1}: 3357 (w), 2932 (m), 2863 (m), 1676 (m), 1606 (m), 1507 (s).

ES$^+$/MS: 536.3 $(M+H)^+$.

HRMS (ES^+) for $C_{32}H_{41}F_3NO_6$ $(M+H)^+$: calculated 558.2826, found 558.2836.



27 $C_{53}H_{60}N_4O_{10}$

5'-*O*-(4,4'-Dimethoxytrityl)-1'-*O*-(6-*N1*-(2-((4-(methylamino)-anthraquinone)amino)ethyl)succinamidohexyl)-2'-deoxy-D-ribose (27).

18 (0.26g, 0.67mmol), EDC (0.13g, 0.67mmol) and HOBT (0.09g, 0.67mmol) were dissolved in DMF (10ml) and stirred for 10mins. **26** (0.32g, 0.60mmol) was dissolved in DMF (5ml) and DIPEA (0.5ml) was added and this solution was then added to the acid mixture and stirred for 15hrs. Upon completion of the reaction the solvent was removed *in vacuo* and the solid was redissolved in CH_2Cl_2 (100ml). This was washed with water (100ml), sat. $NaHCO_3$ (100ml), dried (anhydrous Na_2SO_4) and evaporated to dryness *in vacuo* to give the title compound as a blue solid (0.16g, 30%).

R_f (system A) 0.40.

^{1}H NMR ($CDCl_3$) δ: 10.68-10.62 (m, 1H, N**H**^{15}), 10.53-10.48 (m, 1H, N**H**^{16}), 8.23-8.19 (m, 2H, Ar**H**^{5,8}), 7.62-7.56 (m, 2H, Ar**H**^{6,7}), 7.39-7.09 (m, 11H, Ar**H**^{2,3,31,36,37,38}), 6.72 (d, 4H, J = 9.0 Hz, Ar**H**^{32}), 6.69-6.64 (m, 1H, N**H**^{20}), 6.01-5.96 (m, 1H, N**H**^{25}), 5.12 (d, 1H, J = 4.5 Hz, C**H**$^{1'}$), 4.16-4.09 (m, 2H, C**H**$^{4'}$, C**H**$^{3'}$), 3.70 (s, 6H, C**H**$_3$^{29}), 3.69-3.60 (m, 1H, C**H**^{28}), 3.49-3.44 (m, 4H, C**H**$_2$^{19,26}), 3.33-3.27 (m, 1H, C**H**^{28}), 3.12-3.01 (m, 4H, C**H**$_2$^{18}, C**H**$_2$$^{5'}$), 3.00 (d, 3H, J = 5.0 Hz, C**H**$_3$^{17}), 2.52-2.41 (m, 4H, C**H**$_2$^{22,23}), 2.15-1.90 (m, 2H, C**H**$_2$$^{2'}$), 1.50-1.21 (m, 8H, C**H**$_2$^{27}).

^{13}C NMR ($CDCl_3$) δ: 182.55 (**C**^{10}), 182.09 (**C**^{9}), 171.93 (**C**^{21}), 171.86 (**C**^{24}), 158.31 (**C**^{30}), 146.93 (**C**^{14}), 145.71 (**C**^{13}), 144.66 (**C**^{35}), 135.78 (**C**^{33}), 134.36 (**C**^{4}), 134.16 (**C**^{1}), 132.00,

131.84 (**C**H^{6,7}), 130.06, 129.90 (**C**H^{32}), 128.16 (**C**H^{36}), 128.00 (**C**H^{37}), 127.64 (**C**H^{38}), 126.60, 125.89 (**C**H^{5,8}), 123.24 (**C**H^{3}), 123.02 (**C**H^{2}), 112.95 (**C**H^{31}), 110.10 (**C**^{12}), 109.68 (**C**^{11}), 104.17 (**C**H$^{1'}$), 86.57 (**C**H$^{4'}$), 85.91 (**C**^{34}) 73.33 (**C**H$^{3'}$), 67.20 (**C**H$_2$^{28}), 63.94 (**C**H$_2$$^{5'}$), 55.04 (**C**H$_3$^{29}), 45.98, 41.79, 40.79 (**C**H$_2$^{18,19,26}), 39.34 (**C**H$_2$$^{2'}$), 31.68, 29.33 (**C**H$_2$^{27}), 29.26, 29.27 (**C**H$_2$^{22,23}, **C**H$_3$^{17}), 26.40, 25.71 (**C**H$_2$^{27}).

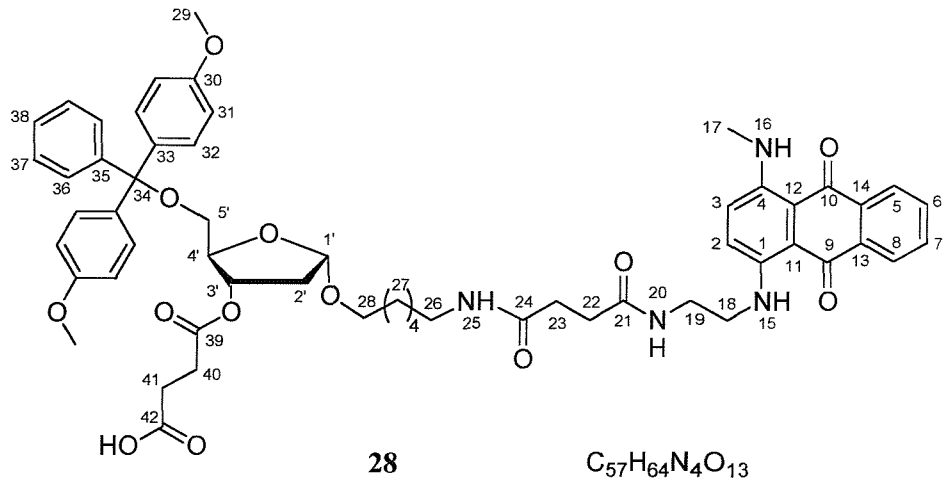
IR ν_{max}/cm^{-1}: 3293 (s), 2927 (m), 2868 (m), 1636 (m), 1572 (s), 1551 (s), 1513 (s).

UV/vis (CH_2Cl_2): 562nm ($11.1x10^6 cm^2mol^{-1}$), 595nm ($19.6x10^6 cm^2mol^{-1}$), 642nm ($20.7x10^6 cm^2mol^{-1}$).

ES$^+$/MS: 913.0 $(M+H)^+$, 935.0 $(M+Na)^+$.

HRMS (ES^+) for $C_{53}H_{60}N_4O_{10}$ $(M+H)^+$: calculated 913.4382, found 913.4364.

Mpt. 150-152°C.



28 $C_{57}H_{64}N_4O_{13}$

5'-*O*-(4,4'-Dimethoxytrityl)-1'-*O*-(6-*N1*-(2-((4-(methylamino)-anthraquinone)amino)ethyl)succinamidohexyl)-2'-deoxy-3'-*O*-oxobutanoic acid-D-ribose (28).

27 (0.15g, 0.16mmol) was dissolved in dry pyridine (5ml) and succinic anhydride (0.02g, 0.20mmol) and DMAP (20.00mg) were added. The mixture was then stirred for 18hrs at room temperature before a further equivalent of succinic anhydride (0.15g, 0.20mmol) was added and the reaction heated at 60°C for 5hrs. The pyridine was removed *in vacuo* and the residue dissolved in CH_2Cl_2 (150ml), washed with sat. KCl (150ml), dried (anhydrous Na_2SO_4) and evaporated to dryness. Purification by column chromatography eluting with CH_2Cl_2:MeOH:AcOH (99:1:0 to 80:20:0 to 79:20:1) gave the title product as a blue solid (0.15g, 94%).

R_f (system D) 0.1.

^{1}H NMR ($CDCl_3$) δ: 10.50-10.40 (m, 2H, N**H**^{15,16}), 8.15-8.08 (m, 2H, Ar**H**^{5,8}), 7.55-7.49 (m, 2H, Ar**H**^{6,7}), 7.35-7.06 (m, 11H, Ar**H**^{2,3,31,36,37,38}), 6.90-6.79 (m, 2H, N**H**^{20,25}), 6.71 (d, 4H, J = 8.5 Hz, Ar**H**^{32}), 5.14 (d, 1H, J = 4.5 Hz, C**H**$^{1'}$), 5.07 (d, 1H, J = 6.0 Hz, C**H**$^{3'}$), 4.13-4.09 (m, 1H, C**H**$^{4'}$), 3.68 (s, 6H, CH_3^{29}), 3.62-3.33 (m, 4H, CH_2^{28}, $CH_2$$^{5'}$), 3.31-3.11 (m, 4H, CH_2^{19,26}), 3.12-3.02 (m, 3H, CH_3^{17}), 2.96-2.86 (m, 2H, CH_2^{18}), 2.60-2.42 (m, 8H, CH_2^{22,23,40,41}), 2.30-1.85 (m, 2H, $CH_2$$^{2'}$), 1.70-1.00 (m, 8H, CH_2^{27}).

^{13}C NMR ($CDCl_3$) δ: 182.55 (**C**^{10}), 182.09 (**C**^{9}), 171.86 (**C**^{21}), 171.93 (**C**^{24}), 157.30 (**C**^{30}), 145.84 (**C**^{14}), 144.69 (**C**^{13}), 143.69 (**C**^{35}), 134.87, 134.76 (**C**^{33}), 133.23 (**C**^{4}), 132.89 (**C**^{1}), 130.90, 130.66 (**CH**^{6,7}), 128.91 (**CH**^{32}), 127.02 (**CH**^{36}), 126.62 (**CH**^{37}), 125.60 (**CH**^{38}), 124.92, 124.77 (**CH**^{5,8}), 122.25 (**CH**^{3}), 121.96 (**CH**^{2}), 111.95 (**CH**^{31}), 110.10 (**C**^{12}), 109.68 (**C**^{11}), 102.69 (**CH**$^{1'}$), 84.94 (**CH**$^{4'}$), 82.09 (**C**^{34}), 74.42 (**CH**$^{3'}$), 65.61 (CH_2^{28}), 62.91

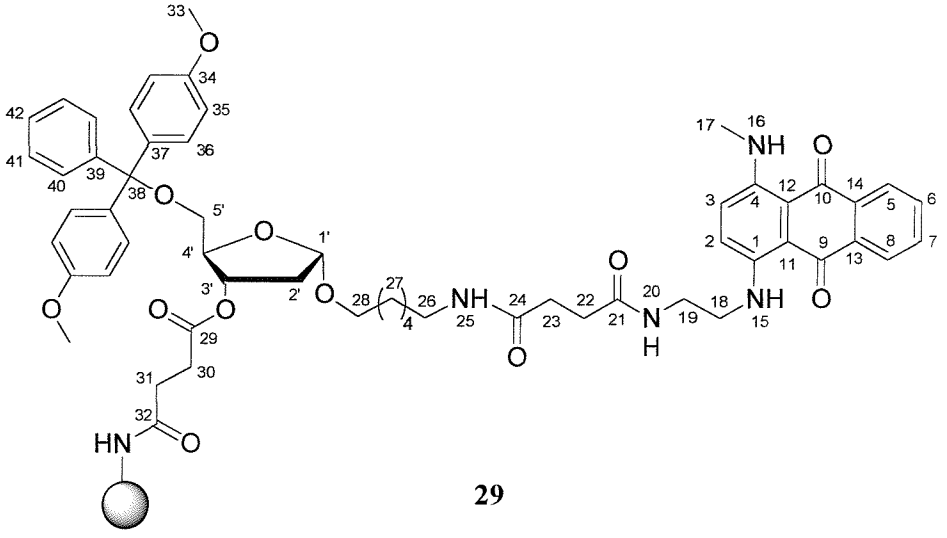
($CH^{5'}$), 54.04 (CH_3^{29}), 45.98, 41.79, 40.79 ($CH_2^{18,19,26}$), 39.34 ($CH_2^{2'}$), 31.68, 29.33 (CH_2^{27}), 29.27, 29.26 ($CH_2^{22,23,40,41}$, CH_3^{17}), 26.40, 25.71 (CH_2^{27}).

IR ν_{max}/cm^{-1}: 3322 (s), 2941 (m), 2606 (s), 1655 (s), 1574 (s).

UV/vis (CH_2Cl_2): 597nm ($10.6x10^6 cm^2mol^{-1}$), 642nm ($9.7x10^6 cm^2mol^{-1}$).

ES^+/MS: 1034.9 $(M+Na)^+$, 1051.4 $(M+K)^+$.

Mpt. Decomposed at 80°C.



29

5'-*O*-(4,4'-Dimethoxytrityl)-1'-*O*-(6-*N1*-(2-((4-(methylamino)-anthraquinone)amino)ethyl)succinamidohexyl)-2'-deoxy-3'-*O*-oxobutanoic acid-D-ribose LCAA-CPG (29).

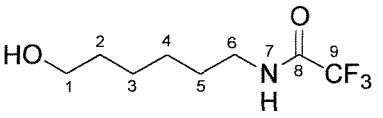
All glassware was soaked in $TMSCl:CH_2Cl_2$ (9.5:0.5) solution for 0.5hr and then rinsed with acetone, water, acetone and dried. The resin (0.50g) was washed with a solution of CH_2Cl_2:DIPEA (99:1) to liberate free amine and then dried under argon. **28** (0.05g, 0.05mmol) was dissolved in CH_2Cl_2:DIPEA (99:1) solution (0.5ml) and EDC (47.00mg, 0.25mmol) was added. The resin (0.50g) was added to this mixture and the mixture agitated gently for 2hrs. A small sample of the resin was taken and washed with CH_2Cl_2:DIPEA solution (99:1, 3x 10ml), Et_2O (3x 10ml) and dried. A sample of this (5.00mg) was dissolved in HCl:EtOH solution (3:2 v:v, 25ml) and shaken for 5min. The absorbance of the orange solution (1ml) was measured at 495nm and the loading of the resin was then calculated:

abs (1ml)	=	0.216
abs (25ml)	=	5.400
abs (1μmol) DMT^+	=	71.700 (25ml, 495nm)
∴ 5.4 ÷ 71.7	=	0.076μmol of product for 0.005g of resin
∴ Resin loading	=	0.076 ÷ 0.005
	=	$25.300\mu molg^{-1}$

The remaining resin was filtered and washed with CH_2Cl_2:DIPEA solution (99:1, 3x 10ml). The resin was then soaked in (capA) acetic anhydride, 2,6-lutidine in THF: (capB) 1-methylimidazole in THF (1:1) for 1hr, washed with CH_2Cl_2:DIPEA (99:1, 3x 10ml) solution and finally with Et_2O (3x 20mL). The resin **29** was dried and tested for DMT^+ loading as before.

abs (1ml)	=	0.156
abs (25ml)	=	3.900
abs (1μmol) DMT^+	=	71.700 (25ml, 495nm)
∴ 5.4 ÷ 71.7	=	0.054μmol of product for 0.002g of resin
∴ Resin loading	=	0.054 ÷ 0.002
	=	$24.700\mu molg^{-1}$

Columns (Glen research) were prepared for oligonucleotide synthesis (0.2μmol synthesis scale) each containing 9.00mg of functionalised resin.



30 $C_8H_{14}F_3NO_2$

6-Trifluoroacetamidohexan-1-ol (30).[171]

6-Aminohexan-1-ol (10.00g, 93.46mmol) was dissolved in freshly distilled CH_2Cl_2 (50ml) and dry triethylamine (15ml) was added. The solution was stirred under argon and ethyltrifluoroacetate (18.20g, 144.44mmol) was added. The reaction showed completion after 24hrs, and the mixture was washed with citric acid solution (10%, 100ml), dried (anhydrous Na_2SO_4) and evaporated to dryness *in vacuo* to yield a yellow foam. This was crystallised from toluene:chloroform (4:1) and the white crystals were dried over P_2O_5 to yield the title compound (12.8g, 70.3%).

R_f (system B) 0.5.

^{1}H NMR ($CDCl_3$) δ: 6.79 (s, 1H, N**H**^{7}), 3.69-3.59 (m, 2H, C**H**$_2^6$), 3.46-3.25 (m, 2H, C**H**$_2^1$), 1.95 (s, 1H, O**H**), 1.65-1.45 (m, 4H, C**H**$_2^{2,5}$), 1.45-1.30 (m, 4H, C**H**$_2^{3,4}$).

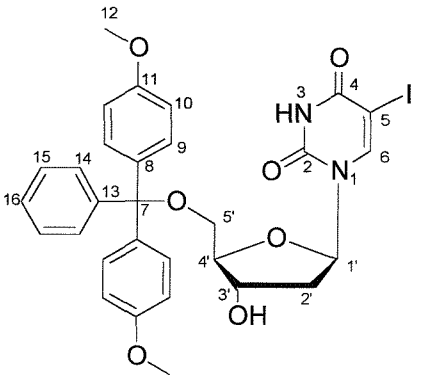
^{13}C NMR ($CDCl_3$) δ: 157.13 (**C**^{8}), 117.93 (**C**F$_3^9$), 62.72 (**C**H$_2^1$), 39.94 (**C**H$_2^6$), 32.47 (**C**H$_2^2$), 28.94 (**C**H$_2^5$), 26.44 (**C**H$_2^3$), 25.34 (**C**H$_2^4$).

ES$^+$/MS: 449.0 $(2M+Na)^+$.

HRMS (ES^+) for $C_8H_{14}F_3NO_2$ $(M+Na)^+$: calculated 236.0868, found 236.0870.

Mpt. 49-51°C.

Analytical data was consistent with that found in the literature.[171]



31 $C_{30}H_{29}N_2O_7I$

5'-*O*-(4,4'-Dimethoxytrityl)-5-iodo-2'-deoxyuridine (31).

5-Iodo-2'-deoxyuridine (4.95g, 14.0mmol) was dissolved in double distilled dry pyridine (60ml). To this solution 4,4'-dimethoxytrityl chloride (5.21g, 15.4mmol) was added in aliquots over 10mins. The reaction mixture was then allowed to stir at room temperature and its progress was followed by TLC (system A). TLC showed starting material still present after 6hrs, so DMAP (10mg, catalytic) was added and the reaction was left overnight (14hrs). At completion methanol (8ml) was added and the solvents were removed under vacuum. The residue was redissolved in dichloromethane (400ml), washed with sat. potassium chloride (2x 200ml), dried with sodium sulfate (anhydrous) and the solvent was removed. The crude yellow product was purified by silica gel column chromatography, eluting with dichloromethane/methanol (0-5%), to yield the compound **31** as a brilliant white foam (5.8g, 70%).

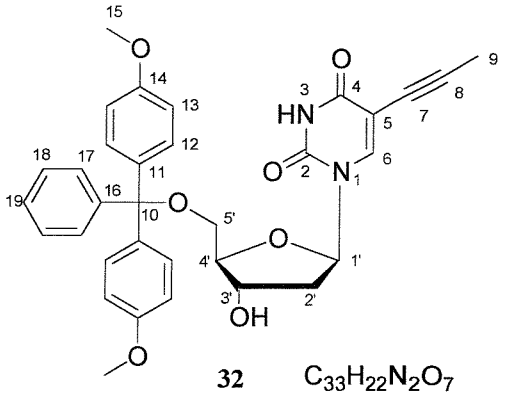
R_f (system A) 0.6.

^{1}H NMR ($CDCl_3$) δ: 8.09 (s, 1H, C**H**^{6}), 7.17-7.48 (m, 9H, Ar**H**^{10,14,15,16}), 6.85 (d, 4H, J = 8.8 Hz, Ar**H**^{9}), 6.38 (dd, 1H, J = 8.1 Hz, J = 5.9 Hz, C**H**$^{1'}$), 4.51 (q(5), 1H, J = 2.9 Hz, C**H**$^{3'}$), 4.08 (q, 1H, J = 2.9 Hz, C**H**$^{4'}$), 3.78 (s, 6H, C**H**$_3{}^{12}$), 3.41-3.37 (m, 2H, C**H**$_2{}^{5'}$), 2.45 (ddd, 1H, J = 14.0 Hz, J = 5.9 Hz, J = 2.9 Hz, C**H**$^{2'}$), 2.25 (ddd, 1H, J = 14.0 Hz, J = 8.1 Hz, J = 5.9 Hz, C**H**$^{2'}$).

^{13}C NMR ($CDCl_3$) δ: 162.94 (**C**^{4}), 158.77 (**C**^{11}), 152.20 (**C**^{2}), 144.56 (**C**^{13}), 143.94 (**CH**^{6}), 135.72, 135.60 (**C**^{8}), 130.27 (**CH**^{9}), 128.23 (**CH**^{14,15}), 127.17 (**CH**^{16}), 113.50 (**CH**^{10}), 87.06 (**C**^{7}), 86.44 (**CH**$^{1'}$), 85.50 (**CH**$^{4'}$), 72.38 (**CH**$^{3'}$), 70.28 (**C**^{5}), 63.77 (**CH**$_2{}^{5'}$), 55.43 (**CH**$_3{}^{12}$), 41.64 (**CH**$_2{}^{2'}$).

ES$^+$/MS: 679.2 $(M+Na)^+$.

Analytical data was consistent with that found in the literature.[176]



32 $C_{33}H_{22}N_2O_7$

5'-*O*-(4,4'-Dimethoxytrityl)-5-(1-propynyl)-2'-deoxyuridine (32). [176,177]

5'-*O*-(4,4'-Dimethoxytrityl)-5-iodo-2'-deoxyuridine (3.91g, 6.1mmol) was dissolved in freshly distilled DMF (8ml) which was degassed by flushing with argon. To this was added copper(I) iodide (0.23g, 1.22mmol), dry triethylamine (3.5ml, 24.7mmol), propyne (1.0ml, 18.3mmol) condensed by dry ice/acetone into dry DMF (4ml) and finally tetrakis(triphenylphosphine)palladium(0) (0.79g, 0.61mmol). The reaction was stirred for 15 hrs and closely followed by TLC. Another 3 equivalents of propyne in DMF were added after a period of 3 hrs and 12 hrs, which was found to be necessary after studying the TLC (system B). The mixture was evacuated and redissolved in ethyl acetate, washed with sat. potassium chloride, dried with sodium sulfate and the solvent was removed to yield a yellow oil. This was purified with silica gel column chromatography, eluting with CH_2Cl_2/MeOH (0-3%). The product was obtained as an off-white foam (0.39g, 12%).

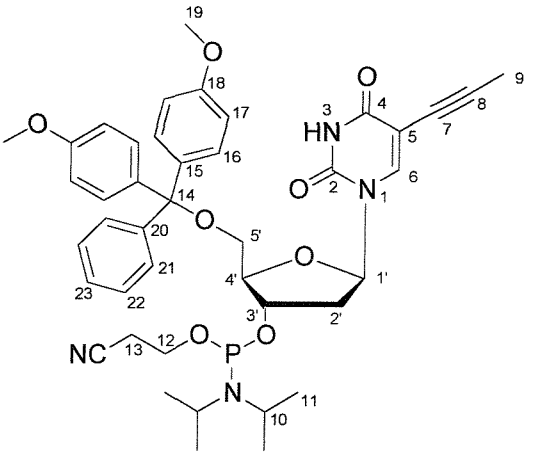
R_f (system B) 0.5.

^{1}H NMR ($CDCl_3$) δ: 7.99 (s, 1H, C**H**^{6}); 7.16-7.49 (m, 9H, Ar**H**^{13,17,18,19}), 6.82 (d, 4H, J = 8.8 Hz, Ar**H**^{12}), 6.36 (dd, 1H, J = 8.0 Hz, J = 5.8 Hz, C**H**$^{1'}$), 4.60-4.52 (m, 1H, C**H**$^{3'}$), 4.12 (q, 1H, J = 2.9 Hz, C**H**$^{4'}$), 3.75 (s, 6H, C**H**$_3{}^{15}$), 3.38-3.29 (m, 2H, C**H**$_2{}^{5'}$), 2.50 (ddd, 1H, J = 13.2 Hz, J = 5.2 Hz, J = 2.2 Hz, C**H**$^{2'}$), 2.31-2.20 (m, 1H, C**H**$^{2'}$), 1.72 (s, 3H, C**H**$_3{}^9$).

^{13}C NMR ($CDCl_3$) δ: 162.99 (**C**^{4}), 158.71 (**C**^{14}), 150.10 (**C**^{2}), 144.71 (**C**^{16}), 141.86 (**C**H^6), 135.79, 135.65, (**C**^{11}), 130.16 (**C**H^{12}), 128.16 (**C**H^{17}), 128.05 (**C**H^{18}), 127.05 (**C**H^{19}),

113.42 ($\mathbf{C}H^{13}$), 101.20 ($\mathbf{C}^{5}$), 90.79 ($\mathbf{C}^{10}$), 87.12 ($\mathbf{C}^{8}$), 86.69 ($\mathbf{C}H^{1'}$), 85.70 ($\mathbf{C}H^{3'}$), 72.35 ($\mathbf{C}H^{4'}$), 70.42 ($\mathbf{C}^{7}$), 63.79 ($\mathbf{C}H_2{}^{5'}$), 55.40 ($\mathbf{C}H_3{}^{15}$), 41.63 ($\mathbf{C}H_2{}^{2'}$), 4.61 ($\mathbf{C}H_3{}^{9}$),
ES$^+$/MS: 569.3 $(M+H)^+$.

Analytical data was consistent with that found in the literature.[176]



33 $C_{42}H_{49}N_4O_8P$

5'-*O*-(4,4'-Dimethoxytrityl)-5-(1-propynyl)-3'-*O*-(2-cyanoethyl-N,N-diisopropylphosphoramidyl)-2'-deoxyuridine (33).[176]

To 5'-O-(4,4'-dimethoxytrityl)-5-[1-propynyl]-2'-deoxyuridine (0.39g, 0.68mmol) dissolved in dry THF (10ml) was added dry diisopropylethylamine (0.49ml, 2.27mmol) and 2-cyanoethoxy-*N,N*-diisopropylamine chlorophosphine (0.18ml, 0.75mmol) and the solution was stirred under argon, for 1.5 hrs. Ethyl acetate (50ml) was then added and the organic layer was washed with sat. potassium chloride (50ml), dried over sodium sulfate and evaporated to dryness to yield an off white foam. This was then redissolved in dichloromethane (4ml, anhydrous) and purified by precipitation in hexane (500ml, dry-ice/acetone). The hexane was decanted off and the dichloromethane was removed by evaporation to yield the product, 0.47g (89%).

R_f (system B) 0.8.

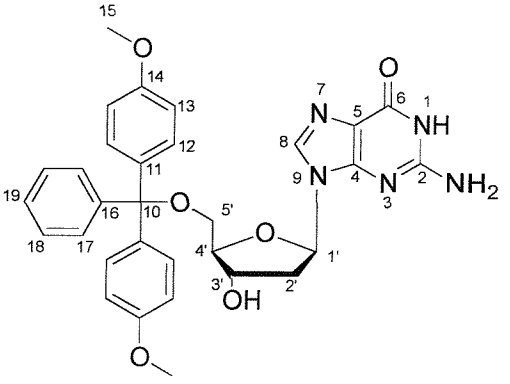
^{1}H NMR ($CDCl_3$) δ: 8.45 (s, 1H, N**H**), 7.98 (s, 1H, $\mathbf{C}H^{6}$), 7.40-7.15 (m, 9H, $\mathbf{Ar}H^{17,21,22,23}$), 6.85-6.76 (m, 4H, $\mathbf{Ar}H^{16}$), 6.27-6.18 (m, 1H, $\mathbf{C}H^{1'}$), 4.61-4.53 (m, 1H, $\mathbf{C}H^{3'}$), 4.19-4.06

(m, 1H, C**H**$^{4'}$), 3.72 (s, 6H, C**H**$_3{}^{19}$), 3.60-3.20 (m, 6H, C**H**$_2{}^{5'}$,C**H**$_2{}^{13}$, C**H**^{10}), 2.50-2.19 (m, 4H, C**H**$_2{}^{12}$, C**H**$_2{}^{2'}$), 1.63-1.58 (m, 3H, C**H**$_3{}^{9}$), 1.21-0.96 (m, 12H, C**H**$_3{}^{11}$).

^{31}P NMR ($CDCl_3$) δ: 149.19, 149.57.

ES$^+$/MS: 769.8 $(M+H)^+$.

Analytical data was consistent with that found in the literature.[176]



34 $C_{31}H_{31}N_5O_6$

5'-*O*-(4,4'-Dimethoxytrityl)-2'-deoxyguanosine (34).[122]

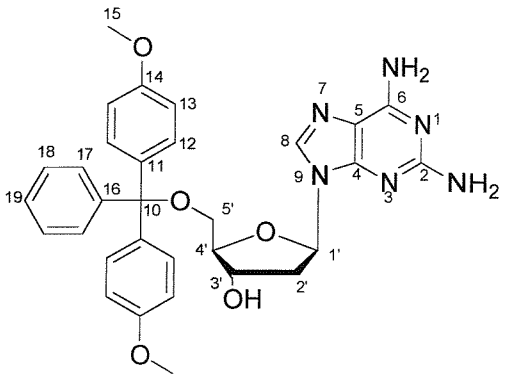
5'-DMT-N^6-isobutyryl-2'-deoxyguanosine (5.0g, 8.0mmol) was dissolved in dioxane (100ml) and conc. ammonia (100ml) was added. The mixture was sealed and stirred for 5 days and its progress was monitored with TLC (system A). The dioxane was removed *in vacuo*, and the mixture filtered to yield 5'-*O*-(4,4'-dimethoxytrityl)-2'-deoxyguanosine (3.5g, 79%).

R_f (system A) 0.3.

^{1}H NMR ($CDCl_3$) δ: 10.72 (s, 1H, N**H**), 7.94-7.89 (m, 1H, C**H**^{8}), 7.21-7.60 (m, 9H, Ar**H**^{13,17,18,19}), 6.87-6.96 (m, 4H, Ar**H**^{12}), 6.60-6.54 (br, 2H, N**H**$_2$), 6.24 (t, 1H, J = 6.5 Hz, C**H**$^{1'}$), 5.41 (d, 1H, J = 4.5 Hz, O**H**), 4.49-4.42 (m, 1H, C**H**$^{3'}$), 4.05-3.97 (m, 1H, C**H**$^{4'}$), 3.80 (s, 6H, C**H**$_3{}^{15}$), 3.24-3.17 (m, 2H, C**H**$_2{}^{5'}$), 2.69 (dt, 1H, J = 12.9 Hz, J = 6.5 Hz, C**H**$^{2'}$), 2.42-2.35 (m, 1H, C**H**$^{2'}$).

ES$^+$/MS: 570.3 $(M+H)^+$.

Analytical data was consistent with that found in the literature.[122]



35 $C_{31}H_{32}N_6O_5$

5'-*O*-(4,4'-Dimethoxytrityl)-2-amino-2'-deoxyadenosine (35).[122]

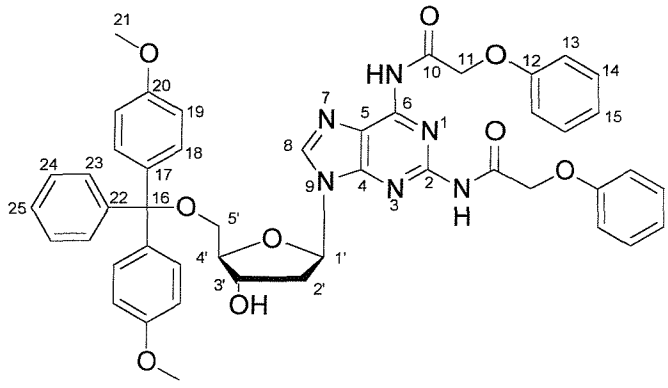
5'-*O*-(4,4'-Dimethoxytrityl)-2'-deoxyguanosine (3.5g, 6.15mmol) was dissolved in pyridine (doubly distilled, 60ml), the mixture cooled to 0°C and covered to protect from light. Trifluoroacetic anhydride (6ml) was added slowly with stirring and the flask was maintained at 0°C for 15 min. Anhydrous ammonia was bubbled in for 2 hrs and the reaction mixture kept at -20°C for 2 days. The reaction was monitored with TLC (system A) for the duration of the experiment. On completion, dichloromethane (100ml) was added carefully and the mixture was then washed with sat. $NaHCO_3$ (3x 200ml). The organic layer was recovered and dried with sodium sulfate (anhydrous) and evaporated to dryness yielding a dark solid (3.2g crude). This was purified with silica gel column chromatography (CH_2Cl_2/MeOH (1-10%)), and fractions were combined and evaporated to yield the product (1.08g, 30%).

R_f (system A) 0.25.

^{1}H NMR ($CDCl_3$) δ: 7.69 (s, 1H, C**H**^{8}), 7.18-7.45 (m, 9H, Ar**H**^{13,17,18,19}), 6.78 (d, 4H, J = 9.6 Hz, Ar**H**^{12}), 6.28 (t, 1H, J = 6.6 Hz, C**H**$^{1'}$), 5.59-5.51 (br, 2H, N**H**$_2$), 5.35-5.29 (m, 1H, O**H**), 4.78-4.70 (br, 2H, N**H**$_2$), 4.68-4.63 (m, 1H, C**H**$^{3'}$), 4.21-4.13 (m, 1H, C**H**$^{4'}$), 3.79 (s, 6H, C**H**$_3^{15}$), 3.38 (d, 2H, J = 5.2 Hz, C**H**$_2^{5'}$), 2.82 (dt, 1H, J = 13.2 Hz, J = 6.6 Hz, C**H**$^{2'}$), 2.45 (ddd, 1H, J = 13.2 Hz, J = 5.9 Hz, J = 3.7 Hz, C**H**$^{2'}$).

ES$^+$/MS: 569.1 $(M+H)^+$.

Analytical data was consistent with that found in the literature.[122]



36 $C_{47}H_{44}N_6O_9$

5'-*O*-(4,4'-Dimethoxytrityl)-N^2,N^6-bisphenoxyacetyl-2-amino-2'-deoxyadenosine (36).[122]

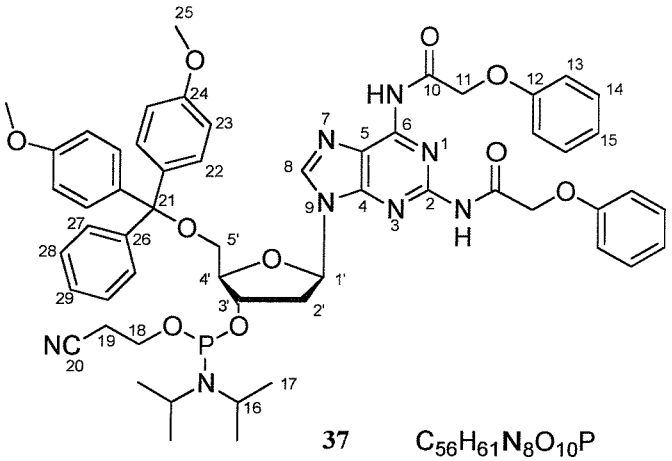
5'-*O*-(4,4'-Dimethoxytrityl)-2-amino-2'-deoxyadenosine (0.95g, 1.67mmol) was dissolved in dry pyridine (doubly distilled, 250ml) and cooled to 0°C in an ice bath. Trimethylsilylchloride (1.6ml, 16.7mmol) was added slowly and the mixture was stirred for half an hour whilst slowly warming to room temperature. Phenoxyacetic anhydride (2.5g, 8.36mmol) was added and the mixture was stirred overnight whilst monitoring with TLC. Excess anhydride was hydrolysed with water (2ml) and after 3 hrs, the mixture was diluted with dichloromethane (25ml), washed with sat. $NaHCO_3$ (2x 100ml), then sat. sodium chloride solution (100ml). The organic layer was dried (Na_2SO_4) and the solvent removed (co-evaporating with toluene to remove pyridine) to yield the crude product (2.18g). This crude product was purified with silica gel column chromatography, eluting with CH_2Cl_2/Acetone (10-30%). Pure fractions were combined and evaporated to yield 5'-*O*-(4,4'-Dimethoxytrityl)-N^2,N^6-bisphenoxyacetyl-2-amino-2'-deoxyadenosine (0.31g, 22%).

R_f (system H) 0.4.

^{1}H NMR (d_6-DMSO) δ: 9.56 (s, 1H, N**H**), 9.31 (s, 1H, N**H**), 8.12 (s, 1H, C**H**^{8}), 6.70-7.41 (m, 23H, Ar**H**^{13,14,15,18,19,23,24,25}), 6.57 (t, 1H, J = 6.5 Hz, C**H**$^{1'}$), 5.91-5.83 (m, 1H, O**H**), 4.95-4.88 (br, 2H, C**H**$_2^{11}$), 4.75-4.69 (br, 3H, C**H**$_2^{11}$, C**H**$^{3'}$), 4.25-4.19 (m, 1H, C**H**$^{4'}$), 3.71 (s, 6H, C**H**$_3^{21}$), 3.40-3.33 (m, 2H, C**H**$_2^{5'}$), 2.71 (dt, 1H, J = 13.2 Hz, J = 6.5 Hz, C**H**$^{2'}$), 2.63-2.58 (m, 1H, C**H**$^{2'}$).

ES$^+$/MS: 837 $(M+H)^+$.

Analytical data was consistent with that found in the literature.[122]



37 $C_{56}H_{61}N_8O_{10}P$

5'-*O*-(4,4'-Dimethoxytrityl)-N^2,N^6-bisphenoxyacetyl-2-amino-3'-*O*-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidyl)-2'-deoxyadenosine (37).[6]

To 5'-*O*-(4,4-dimethyoxytrityl)-*N*,*N*-bisphenoxyacetyl-2-amino-2'-deoxyadenosine (0.36g, 0.4mmol) dissolved in dry THF (15ml) was added dry diisopropylethylamine (0.28ml, 1.6mmol) and 2-cyanoethoxy-*N*,*N*-diisopropylamine chlorophosphine (0.09ml, 0.44mmol) and the solution was stirred under argon, for 1.5 hrs. Ethyl acetate (50ml) was added and the organic layer was washed with sat. potassium chloride (50ml), dried over sodium sulfate and evaporated to dryness to yield the crude product. This was then redissolved in dichloromethane (5ml, anhydrous) and purified by precipitation in hexane (500ml, dry-ice/acetone). The hexane was decanted off and the dichloromethane was removed. The resulting residue was purified further with a short silica column to yield the product (0.46g, 90%).

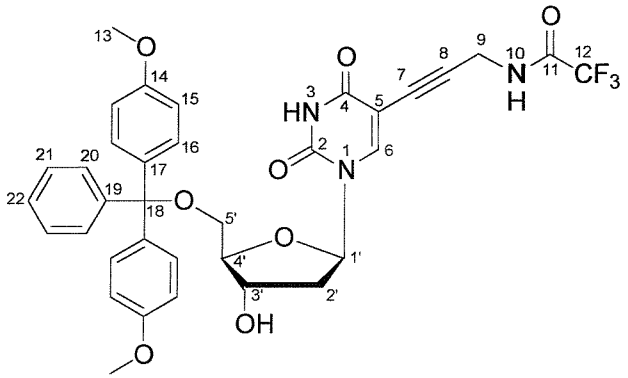
R_f (system A) 0.8.

^{1}H NMR ($CDCl_3$) δ: 9.20 (s, 1H, N**H**), 8.80 (s, 1H, N**H**), 8.09-8.01 (m, 1H, C**H**^{8}), 7.40-6.90 (m, 19H, Ar**H**^{13,14,15,23,27,28,29}), 6.71-6.63 (m, 4H, Ar**H**^{22}), 6.40 (t, 1H, J = 6.6 Hz, C**H**$^{1'}$), 4.90 (br, 2H, CH_2^{11}), 4.80-4.61 (m, 3H, C**H**$^{3'}$, CH_2^{11}), 4.27-4.18 (m, 1H, C**H**$^{4'}$), 3.70 (s, 6H, CH_3^{25}), 3.65-3.48 (m, 4H, CH_2^{19}, C**H**^{16}), 3.34 (d, 1H, J = 3.7 Hz, C**H**$^{5'}$), 3.29 (d, 1H, J = 4.4 Hz, C**H**$^{5'}$), 2.90 (dt, 1H, J = 13.2 Hz, J = 6.6 Hz, C**H**$^{2'}$), 2.65-2.35 (m, 3H, C**H**$^{2'}$, CH_2^{18}), 1.10-1.01 (m, 12H, CH_3^{17}).

^{31}P NMR ($CDCl_3$) δ: 149.57, 149.62.

ES$^+$/MS: 1037 $(M+H)^+$.

Analytical data was consistent with that found in the literature.[122]



38 $C_{35}H_{32}F_3N_3O_8$

5'-*O*-(4,4'-Dimethoxytrityl)-5-(3-*N*-(2,2,2-trifluoroacetyl)amino-1-propynyl)-2'-deoxyuridine (38).[118]

TFA-propargylamine (0.5g, 3.3mmol) was dissolved in anhydrous DMF (12ml) and to this was added copper (I) iodide (0.12g, 0.6mmol), triethylamine (2ml, 15mmol), 5'-DMT-5-iodo-2'-deoxyuridine (2g, 3mmol) and finally $Pd(PPh_3)_4$ (0.36g, 0.3mmol). The mixture was stirred for 4hrs and protected from direct sunlight. TLC (system D) showed completion of the reaction and so the mix was evaporated to dryness, redissolved in CH_2Cl_2 (100ml) and washed with 5% EDTA solution (100ml), sat. $NaHCO_3$ (100ml), sat. KCl (100ml) and dried over anhydrous Na_2SO_4. The filtrate was concentrated *in vacuo* and the residue was purified by silica gel column chromatography eluting with EtOAc, which on evaporation gave a white foam: 1.42g (69%).

R_f (system D) 0.4.

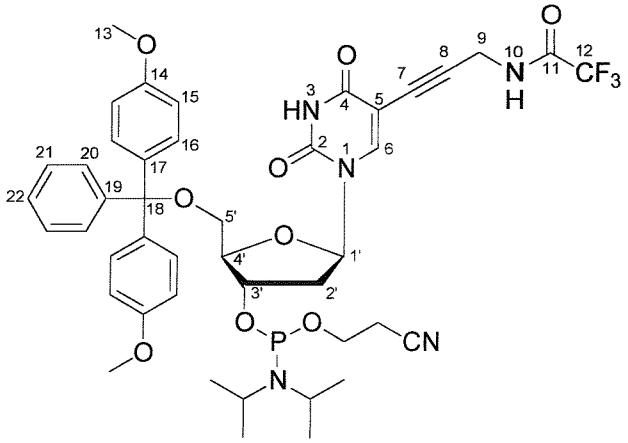
^{1}H NMR ($CDCl_3$) δ: 8.15 (s, 1H, N**H**^{3}), 7.51-7.10 (m, 11H, C**H**^{6}, Ar**H**^{10,15,20,21,22}), 6.79 (d, 4H, J = 8.0 Hz, Ar**H**^{16}), 6.29-6.22 (m, 1H, C**H**$^{1'}$), 4.54-4.49 (m, 1H, C**H**$^{3'}$), 4.11-4.03 (m, 1H, C**H**$^{4'}$), 3.95-3.81 (m, 2H, C**H**$_2^9$), 3.68 (s, 6H, C**H**$_3^{13}$), 3.29-3.22 (m, 2H, C**H**$_2^{5'}$), 2.54-2.19 (m, 2H, C**H**$_2^{2'}$).

^{13}C NMR ($CDCl_3$) δ: 163.07 (**C**^{4}), 158.78 (**C**^{2}), 156.91 (q, J = 36.8 Hz, **C**^{11}), 149.77 (**C**^{14}), 144.91 (**C**^{19}), 143.97 (**CH**^{6}), 133.89 (**C**^{17}), 130.43 (**CH**^{16}), 128.92, 128.30, 127.42

(**CH**^{20,21,22}), 114.62 (q, J = 287.8 Hz, **C**F_3^{12}), 113.77 (**CH**^{15}), 99.40 (**C**^{5}), 87.86 (**C**^{8}), 87.50 (**C**^{7}), 87.37 (**CH**$^{1'}$), 86.44 (**CH**$^{3'}$), 75.73 (**C**^{18}), 72.67 (**CH**$^{4'}$), 63.95 (**C**$H_2$$^{5'}$), 55.67 (**C**$H_3$^{13}), 42.00 (**C**$H_2$^{9}), 30.79 (**C**$H_2$$^{2'}$).

ES$^+$/MS: 704.5 $(M+H)^+$.

Analytical data was consistent with that found in the literature.[176]



39 $C_{44}H_{49}F_3N_5O_9$

5'-*O*-(4,4'-Dimethoxytrityl)-5-(3-*N*-(2,2,2-trifluoroacetyl)amino-1-propynyl)-3'-*O*-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidyl)-2'-deoxyuridine (39).

Phosphitylation was carried out by the method described by Caruthers *et al.* [6]

Propargylamino-dU **38** (1g, 1.5mmol) was dissolved in THF (anhydrous, 20ml). To this was added dry DIPEA (0.42g, 3.2mmol) and 2-cyanoethoxy-*N*,*N*-diisopropylamine chlorophosphine (0.38g, 1.6mmol) under an atmosphere of argon. The reaction was stirred at room temperature under argon for 2hrs and the mixture was checked by TLC (system A). TLC showed completion of reaction and a white precipitate had formed in the pale yellow solution. To the THF was added EtOAc (30ml) and sat. KCl (50ml). The mixture was shaken and the organic layer removed, dried over anhydrous Na_2SO_4, filtered, evaporated to dryness and purified by silica gel column chromatography under argon, eluting with EtOAc which gave a white foam on evaporation *in vacuo*: 0.94g (73%). All

phosphoramidites synthesised were stored under argon, at -20°C, in bottles suitable for an ABI 394 DNA synthesiser.

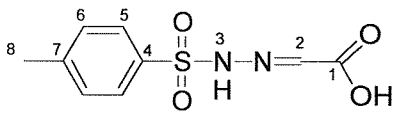
R_f (system A) 0.6.

^{1}H NMR ($CDCl_3$) δ: 8.79 (br s, 1H, N**H**^{3}), 8.20 (s, 1H, C**H**^{6}), 7.42-7.10 (m, 9H, Ar**H**^{15,20,21,22}), 6.82-6.71 (m, 4H, Ar**H**^{16}), 6.42-6.18 (m, 1H, N**H**^{10}), 6.21 (dd, 1H, J = 11.0 Hz, J = 5.8 Hz, C**H**$^{1'}$), 4.64-4.55 (m, 1H, C**H**$^{3'}$), 4.16-4.05 (m, 1H, C**H**$^{4'}$), 3.89-3.71 (dd, 2H, J = 9.6 Hz, J = 4.4 Hz, C**H**$_2$^{9}), 3.71 (s, 6H, C**H**$_3$^{13}), 3.70-3.19 (m, 6H, C**H**^{25}, C**H**$_2$^{23}, C**H**$_2$$^{5'}$), 2.59-2.27 (m, 4H, C**H**$^{2'}$, C**H**$_2$^{24}), 1.19-0.95 (m, 12H, C**H**$_3$^{26}).

^{31}P NMR ($CDCl_3$) δ: 149.57, 149.34.

ES$^+$/MS: 879.3 $(M+H)^+$.

Analytical data was consistent with that found in the literature.[176]



40 $C_9H_{10}N_2O_4S$

2-((4-Methylphenyl)sulfonyl)hydrazonoacetic acid (40). [187,188]

A solution of glyoxylic acid monohydrate (4.55g, 49mmol) in water (50ml) was heated to 60°C in a water bath. A warm solution of *p*-toluenesulfonyl hydrazide (9.33g, 50.1mmol) in 2.5M $HCl_{(aq)}$ (25ml) was added and the mixture stirred vigourously for 5min. Initially an oil formed, but on further stirring white crystals precipitated. The mixture was then cooled for 12hrs, before filtering to yield the crude product, which was dried in a vacuum desiccator (over P_2O_5) for 48hrs. This was recrystallised from EtOAc/CCl_4 (1:2) and dried as before to yield pure white crystals 5.1g (50%).

R_f (system A) 0.1.

^{1}H NMR ($CDCl_3$) δ: 12.39 (s, 1H, N**H**^{3}), 7.70 (d, 2H, J = 8.2 Hz, Ar**H**^{5}), 7.42 (d, 2H, J = 8.2 Hz, Ar**H**^{6}), 7.17 (s, 1H, C**H**^{2}), 2.41 (s, 3H, C**H**$_3$^{8}).

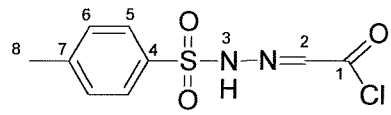
^{13}C NMR ($CDCl_3$) δ: 163.68 (**C**^{1}), 144.13 (**C**^{7}), 137.55 (**CH**^{2}), 135.78 (**C**^{4}), 130.01 (**CH**^{6}), 127.19 (**CH**^{5}), 21.12 (**CH$_3$**^{8}).

IR ν_{max}/cm^{-1}: 3207 (s), 2902 (m), 1701 (s), 1593 (m).

ES$^+$/MS: 265.0 $(M+Na)^+$.

Mpt. 153-155°C (lit. 148-154°C).

Analytical data was consistent with that found in the literature.[187,188]



41 $C_9H_9N_2O_3Cl$

2-((Methylphenyl)sulfonyl)hydrazonoethanoyl chloride (41). [187,188]

The acid **40** (5.0g, 20.6mmol) was suspended in toluene (25ml) and thionyl chloride was added (4.9g, 41.2mmol). The mixture was heated at reflux for 2hrs and the resulting mixture was filtered through celite before evaporating to dryness. The residue was resuspended in toluene and filtered. The precipitate was washed with cold toluene and dried in the vacuum desiccator for 12hrs. The filtrate was kept and recrystallised a second time. Product was dried in a vacuum desiccator over P_2O_5 to yield 1.4g (26%). This is a highly reactive molecule and characterisation was plagued by the readiness of this product to react with water.

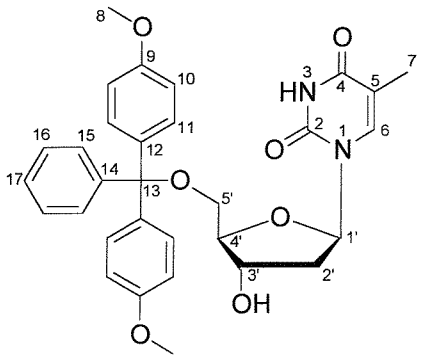
^{1}H NMR ($CDCl_3$) δ: 8.60 (s, 1H, N**H**^{3}), 7.79 (d, 2H, J = 8.1 Hz, Ar**H**^{5}), 7.32 (d, 2H, J = 8.1 Hz, Ar**H**^{6}), 7.16 (s, 1H, C**H**^{2}), 2.41 (s, 3H, **CH$_3$**^{8}).

^{13}C NMR ($CDCl_3$) δ: 165.48 (**C**^{1}), 146.13 (**C**^{7}), 136.32 (**CH**^{2}), 134.59 (**C**^{4}), 130.52 (**CH**^{6}), 128.54 (**CH**^{5}), 22.11 (**CH$_3$**^{8}).

IR ν_{max}/cm^{-1}: 3145 (s), 2888 (m), 1742 (s), 1595 (m), 1576 (m).

Mpt. 105-109°C (lit. 104-108°C).

Analytical data was consistent with that found in the literature.[187,188]



42 $C_{31}H_{32}N_2O_7$

5'-*O*-(4,4'-Dimethoxytrityl)-2'-deoxythymidine (42).

Thymidine (5.0g, 21.0mmol) and 4,4'-dimethoxytrityl chloride (7.7g, 1.1eq., 22.0mmol) were added to a dry r.b. flask and left drying in a vacuum desiccator for 48hrs. The flask was opened under argon and pyridine (double distilled, 50ml) was added. The mixture was stirred at room temperature and studied with TLC (system A). After 3hrs the reaction mixture was quenched with MeOH (50ml) and the solvent was evaporated. The residue was redissolved in CH_2Cl_2, washed with sat. $NaHCO_3$, dried with anhydrous Na_2SO_4 and evaporated to dryness. The residue was purified by column chromatography (pre-equilibrated with 0.1% NEt_3) eluting with CH_2Cl_2/MeOH (0-10%) to yield 9.7g (86.7%) as a white foam. This was dried overnight in a vacuum desiccator (silica gel).

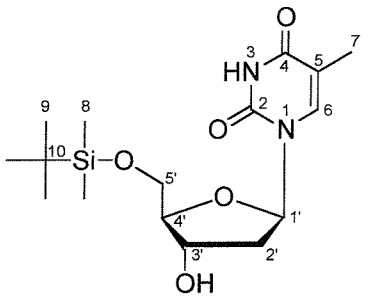
R_f (system A) 0.4.

^{1}H NMR ($CDCl_3$) δ: 7.50 (s, 1H, C**H**^{6}), 7.35-7.11 (m, 9H, Ar**H**^{10,15,16,17}), 6.76 (d, 4H, J = 8.0 Hz, Ar**H**^{11}), 6.31 (m, 1H, C**H**$^{1'}$), 4.50-4.44 (m, 1H, C**H**$^{3'}$), 4.02-3.97 (m, 1H, C**H**$^{4'}$), 3.69 (s, 6H, C**H**$_3{}^8$), 3.43-3.26 (m, 2H, C**H**$_2{}^{5'}$), 2.39-2.18 (m, 2H, C**H**$_2{}^{2'}$), 1.40 (s, 3H, C**H**$_3{}^7$).

^{13}C NMR ($CDCl_3$) δ: 164.31(**C**^{4}), 159.14 (**C**^{2}), 150.95 (**C**^{9}), 144.76 (**C**^{14}), 136.09 (**C**H^6), 135.85 (**C**^{12}), 130.50 (**C**H^{11}), 128.55 (**C**H^{15}), 128.40 (**C**H^{16}), 127.55 (**C**H^{17}), 113.70 (**C**H^{10}), 111.67 (**C**^{5}), 87.35 (**C**^{13}), 86.67 (**C**H$^{4'}$), 85.19 (**C**H$^{1'}$), 72.80 (**C**H$^{3'}$), 64.02 (**C**H$_2{}^{5'}$), 55.67 (**C**H$_3{}^8$), 41.40 (**C**H$_2{}^{2'}$), 12.24 (**C**H$_3{}^7$).

ES$^+$/MS: 567.2 $(M+Na)^+$.

This data matches that of the commercially available compound.



43 $C_{16}H_{28}N_2O_5Si$

5'-*O*-(*tert*-Butyldimethylsilyl)-2'-deoxythymidine (43).[197]

Thymidine (5.0g, 20mmol) and TBDMSCl (3.3g, 21mmol) were added to a dry r.b. flask and pyridine (distilled, 40ml) was added. The mixture was stirred at room temperature for 5hrs. TLC (system A) showed completion of reaction and the solvent was removed. The residue was redissolved in CH_2Cl_2 and washed with 2M HCl and sat. KCl. The organic layer was extracted and dried (anhydrous Na_2SO_4), before evaporating to dryness to yield a white solid. This was purified by silica gel column chromatography, eluting with CH_2Cl_2/MeOH (0-10%), to yield a white solid 6.1g (82.9%).

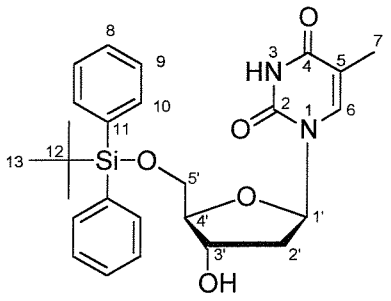
R_f (system A) 0.5.

^{1}H NMR ($CDCl_3$) δ: 9.39 (s, 1H, N**H**^{3}), 7.45 (s, 1H, C**H**^{6}), 6.29 (dd, 1H, J = 6.0 Hz, J = 2.5 Hz, C**H**$^{1'}$), 4.41-4.34 (m, 1H, C**H**$^{3'}$), 4.01-3.92 (m, 1H, C**H**$^{4'}$), 3.82-3.68 (m, 2H, C**H**$_2^{5'}$), 3.10-2.60 (br, 1H, O**H**$^{3'}$), 2.34-1.91 (m, 2H, C**H**$_2^{2'}$), 1.79 (s, 3H, C**H**$_3^7$), 0.80 (s, 9H, C**H**$_3^9$), 0.01 (s, 6H, C**H**$_3^8$).

^{13}C NMR ($CDCl_3$) δ: 164.19 (**C**^{4}), 150.79 (**C**^{2}), 135.68 (**C**H^6), 111.16 (**C**^{5}), 87.53 (**C**H$^{4'}$), 85.21 (**C**H$^{1'}$), 72.76 (**C**H$^{3'}$), 63.80 (**C**H$_2^{5'}$), 41.29 (**C**H$_2^{2'}$), 26.07 (**C**H$_3^9$), 18.51 (**C**^{10}), 12.71 (**C**H$_3^7$), -4.98 (**C**H$_3^8$).

ES$^+$/MS: 735.2 $(2M+Na)^+$.

Analytical data was consistent with that found in the literature.[197]



44 $C_{26}H_{32}N_2O_5Si$

5'-*O*-(*tert*-Butyldiphenylsilyl)-2'-deoxythymidine (44). [179]

Thymidine (2.5g, 10.3mmol) was added to a r.b. flask containing pyridine (20ml, distilled). TBDMSCl (3.1g, 11.3mmol) was added slowly *via* a syringe. The reaction was stirred for 15hrs and TLC (system A) showed completion. The mixture was reduced to dryness *in vacuo* and the residue was redissolved in CH_2Cl_2 and washed with sat. KCl, dried over anhydrous Na_2SO_4 then evaporated. The crude product was purified by silica gel column chromatography eluting with CH_2Cl_2/MeOH (0-10%), to yield a white foam 3.6g (72.4%).

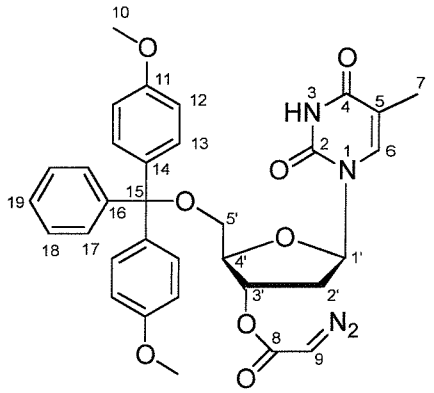
R_f (system A) 0.5.

^{1}H NMR ($CDCl_3$) δ: 9.10 (s, 1H, N**H**^{3}), 7.76 (d, 4H, J = 9.0 Hz, Ar**H**^{10}), 7.55-7.40 (m, 7H, C**H**^{6}, Ar**H**^{8,9}), 6.41 (dd, 1H, J = 8.0 Hz, J = 5.5 Hz, C**H**$^{1'}$), 4.64-4.59 (m, 1H, C**H**$^{3'}$), 4.09 (dd, 1H, J = 5.5 Hz, J = 2.5 Hz, C**H**$^{4'}$), 4.03 (dd, 1H, J = 11.5 Hz, J = 2.5 Hz, C**H**$^{5'}$), 4.03 (dd, 1H, J = 11.5 Hz, J = 2.5 Hz, C**H**$^{5'}$), 2.75 (s, 1H, **OH**), 2.49 (ddd, 1H, J = 13.6 Hz, J = 5.5 Hz, J = 2.5 Hz, C**H**$^{2'}$), 2.24 (ddd, 1H, J = 13.6 Hz, J = 8.0 Hz, J = 6.0 Hz, C**H**$^{2'}$), 1.70 (s, 3H, C**H**$_3^7$), 1.13 (s, 9H, C**H**$_3^{13}$).

^{13}C NMR ($CDCl_3$) δ: 164.18 (**C**^{4}), 150.88 (**C**^{2}), 135.96 (**C**H^{10}), 135.73 (**C**H^6), 133.31, 132.80 (**C**^{11}), 130.60, 130.49 (**C**H^9), 128.44, 128.39 (**C**H^8), 111.66 (**C**^{5}), 87.48 (**C**H$^{4'}$), 85.13 (**C**H$^{1'}$), 72.65 (**C**H$^{3'}$), 64.56 (**C**H$_2^{5'}$), 41.41 (**C**H$_2^{2'}$), 27.41 (**C**H$_3^{13}$), 19.77 (**C**^{12}), 12.51 (**C**H$_3^7$).

IR ν_{max}/cm^{-1}: 3382 (s), 3057 (m), 2929 (m), 2856 (m), 1680 (s), 1663 (s).

ES$^+$/MS: 983.3 $(2M+Na)^+$.



45 $C_{33}H_{32}N_4O_8$

5'-*O*-(4,4'-Dimethoxytrityl)-3'-(α-diazoacetyl)-2'-deoxythymidine (45).

This reaction was carried out following methods described in the literature. [187 189]

To an ice cold solution of dry DMT-dT (**42**) (0.5g, 0.92mmol) in CH_2Cl_2 (5ml), was added glyoxylic acid chloride p-toluenesulphonylhydrazone (0.45g, 1.73mmol) and dimethylaniline (0.2g, 1.69mmol) promptly, whilst flushing with argon. The solution turned a dark green colour and was stirred at 0°C for 15min under argon. Triethylamine (0.47g, 4.7mmol) was then added and the resulting dark orange suspension was stirred at 0°C for 10min, then warmed to room temperature and stirred for a further 15min. Water (4ml) was added and the mixture was concentrated *in vacuo*. The residue was dissolved in CH_2Cl_2 and washed with 10% citric acid solution (50ml). Aqueous washings were combined and extracted with CH_2Cl_2. Organic layers were combined and dried over Na_2SO_4, then evaporated to dryness. The crude product was purified by column chromatography (hexane/EtOAc, 1:1) and after reducing to dryness gave a pale yellow foam 0.4g (75%).

R_f (system I) 0.4.

^{1}H NMR ($CDCl_3$) δ: 11.46 (s, 1H, N**H**^{3}), 7.61 (s, 1H, C**H**^{6}), 7.55-7.36 (m, 9H, Ar**H**^{12,17,18,19},), 7.01 (d, 4H, J = 8.0 Hz, Ar**H**^{13}), 6.31 (dd, 1H, J = 6.0 Hz, J = 2.5 Hz, C**H**$^{1'}$), 5.79 (br s, 1H, C**H**^{9}), 5.51-5.47 (m, 1H, C**H**$^{3'}$), 4.17-4.12 (m, 1H, C**H**$^{4'}$), 3.81 (s, 6H, C**H**$_3$^{10}), 3.49-3.31 (m, 2H, C**H**$_2$$^{5'}$), 2.65-2.43 (m, 2H, C**H**$_2$$^{2'}$), 1.55 (s, 3H, C**H**$_3$^{7}).

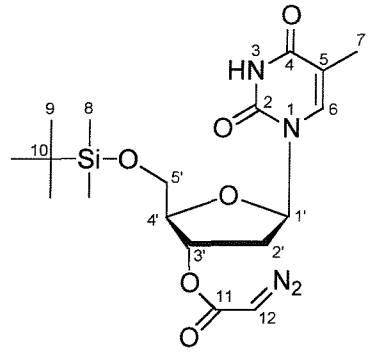
^{13}C NMR ($CDCl_3$) δ: 163.92 ($\mathbf{C}^4$), 158.56 ($\mathbf{C}^2$), 150.71 ($\mathbf{C}^{11}$), 144.92 ($\mathbf{C}^{16}$), 135.87 ($\mathbf{CH}^6$), 135.48 ($\mathbf{C}^{14}$), 130.06 ($\mathbf{CH}^{13}$), 128.28 ($\mathbf{CH}^{17}$), 128.01 ($\mathbf{CH}^{18}$), 127.21 ($\mathbf{CH}^{19}$), 113.63 ($\mathbf{CH}^{12}$), 110.28 ($\mathbf{C}^5$), 86.46 ($\mathbf{C}^{15}$), 84.04 ($\mathbf{CH}^{4'}$), 83.15 ($\mathbf{CH}^{1'}$), 75.17 ($\mathbf{CH}^{3'}$), 63.93 ($\mathbf{CH_2}^{5'}$), 55.41 ($\mathbf{CH_3}^{10}$), 46.70 ($\mathbf{CH}^9$), 36.84 ($\mathbf{CH_2}^{2'}$), 11.99 ($\mathbf{CH_3}^7$).

IR ν_{max}/cm^{-1}: 3187 (w), 3062 (m), 2932 (m), 2837 (m), 2121 (s), 1685 (s), 1605 (m), 1510 (s).

ES$^+$/MS: 630.0 $(M+NH_4)^+$, 635.2 $(M+Na)^+$, 651.2 $(M+K)^+$.

HRMS (ES^+) for $C_{33}H_{32}N_4O_8$ $(M+Na)^+$: calculated 635.2112, found 635.2117.

Mpt. 125-130°C.



46 $C_{18}H_{28}N_4O_6Si$

5'-*O*-(*tert*-Butyldimethylsilyl)-3'-(α-diazoacetyl)-2'-deoxythymidine (46).

This reaction was carried out following methods described in the literature [187,189]

TBDMSdT (**43**) (2.2g, 6.2mmol) was weighed into a dry r.b. flask and CH_2Cl_2 (20ml) and *p*-tosylhydrazone glyoxylic acid chloride (3.0g, 12.0mmol) was added. Dimethylaniline (1.5g, 1.5ml, 12.0mmol) was added and the mixture was stirred under argon at 0°C for 30mins. Triethylamine (distilled, 3.1g, 4.3ml, 32.0mmol) was added to the green/yellow coloured solution, which was stirred at 0°C for a further 1hr, then warmed to room temperature and stirred for 15mins. The reaction was quenched with water (50ml) and the mixture was evaporated to dryness. The residue was dissolved in CH_2Cl_2 (200ml) and washed with 10% citric acid solution (2x 200ml). Aqueous washings were combined and

extracted with CH_2Cl_2. The combined organic layers were dried over anhydrous sodium sulfate, then evaporated to dryness. The residue was purified by silica gel column chromatography, eluting with hexane/EtOAc (50-75%) and from this a white solid precipitated, yielding 0.9g (35%).

R_f (system K) 0.6.

^{1}H NMR ($CDCl_3$) δ: 8.81 (s, 1H, N**H**^{3}), 7.32 (s, 1H, C**H**^{6}), 6.20 (dd, 1H, J = 9.0 Hz, J = 5.3 Hz, C**H**$^{1'}$), 5.21 (d, 1H, J = 6.5 Hz, C**H**$^{3'}$), 4.70 (br s, 1H, C**H**^{12}), 4.03-3.96 (m, 1H, C**H**$^{4'}$), 3.82-3.75 (m, 2H, C**H**$_2{}^{5'}$), 2.30 (dd, 1H, J = 14.1 Hz, J = 5.0 Hz, C**H**$^{2'}$), 1.98 (ddd, 1H, J = 14.1 Hz, J = 9.0 Hz, J = 6.5 Hz, C**H**$^{2'}$), 1.70 (s, 3H, C**H**$_3{}^7$), 0.80 (s, 9H, C**H**$_3{}^9$), 0.01 (s, 6H, C**H**$_3{}^8$).

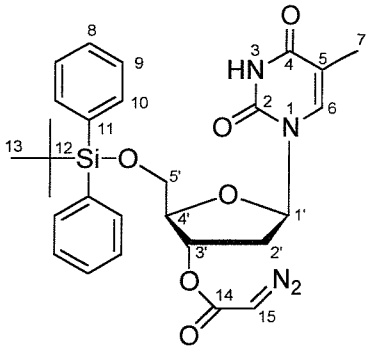
^{13}C NMR ($CDCl_3$) δ: 164.05 (**C**^{4}), 150.82 (**C**^{2}), 135.39 (**C**H^6), 111.68 (**C**^{5}), 85.84 (**C**H$^{4'}$), 85.08 (**C**H$^{1'}$), 76.33 (**C**H$^{3'}$), 63.95 (**C**H$_2{}^{5'}$), 47.08 (**C**H^{12}), 38.53 (**C**H$_2{}^{2'}$), 26.33 (**C**H$_3{}^9$), 18.74 (**C**^{10}), 12.89 (**C**H$_3{}^7$), -4.97 (**C**H$_3{}^8$).

IR ν_{max}/cm^{-1}: 3233 (s), 3110 (m), 2950 (m), 2927 (m), 2854 (m), 2131 (s), 1700 (s), 1683 (s), 1660 (s).

ES$^+$/MS: 871.7 $(2M+Na)^+$.

HRMS (ES^+) for $C_{18}H_{28}N_4O_6Si$ $(M+Na^+)$: calculated 447.1669, found 447.1670.

Mpt. 166-170°C.



47 $C_{28}H_{32}N_4O_6Si$

5'-*O*-(*tert*-Butyldiphenylsilyl)-3'-(α-diazoacetyl)-2'-deoxythymidine (47).

This reaction was carried out following methods described in the literature [187,189]

TBDPSdT (**44**) (4.6g, 9.6mmol) was dissolved in CH_2Cl_2 (distilled, 40ml) and glyoxylic acid chloride *p*-toluenesulfonylhydrazone (5.0g, 19.2mmol) and *N,N*-dimethylaniline (2.2ml, 17.35mmol) were added slowly to the cool solution, whilst in an ice bath at 0°C. This mixture was stirred for 15min under an atmosphere of argon, turning an orange colour initially, then dark brown, until finally a dark green colour was observed. Triethylamine (distilled, 6.8ml, 48mmol) was added and the resulting dark orange suspension was stirred at 0°C for 10mins, then warmed to room temperature and stirred for a further 15mins. Water (50ml) was added to quench the reaction, the mixture was evaporated to dryness and the residue redissolved in CH_2Cl_2. This was washed with citric acid (10%) and the aqueous washings were each extracted with CH_2Cl_2 and combined. The organic layer was dried (anhydrous Na_2SO_4) and filtered, before evaporating to dryness to yield a crude yellow solid. This was purified by silica gel column chromatography, eluting with EtOAc/Hexane (1:1), to yield an off white foam 4.0g (76.2%).

R_f (system I) 0.6.

^{1}H NMR ($CDCl_3$) δ: 8.66 (s, 1H, N**H**^{3}), 7.60 (t, 4H, J = 7.0 Hz, Ar**H**^{10}), 7.50-7.20 (m, 7H, C**H**^{6}, Ar**H**^{8,9}), 6.45 (dd, 1H, J = 9.0 Hz, J = 5.3 Hz, C**H**$^{1'}$), 5.60 (d, 1H, J = 6.5 Hz, C**H**$^{3'}$), 4.72 (br s, 1H, C**H**^{15}), 4.11-4.07 (m, 1H, C**H**$^{4'}$), 4.02-3.88 (m, 2H, C**H**$_2^{5'}$), 2.55 (dd, 1H, J = 14.0 Hz, J = 5.5 Hz, C**H**$^{2'}$), 2.35 (ddd, 1H, J = 14.0 Hz, J = 9.0 Hz, J = 6.5 Hz, C**H**$^{2'}$), 1.50 (s, 3H, C**H**$_3^7$), 1.03 (s, 9H, C**H**$_3^{13}$).

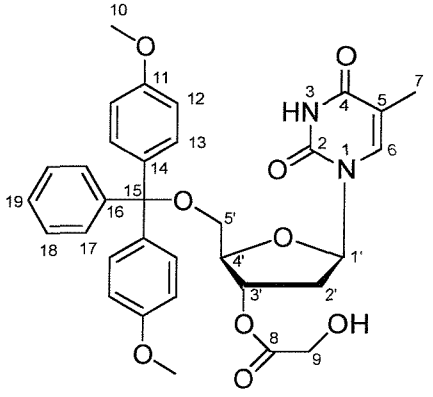
^{13}C NMR ($CDCl_3$) δ: 163.92 ($\mathbf{C}^4$), 150.77 ($\mathbf{C}^2$), 135.94, 135.62 ($\mathbf{CH}^{10}$), 135.33 ($\mathbf{CH}^6$), 133.37, 132.46 ($\mathbf{C}^{11}$), 130.64, 130.51 ($\mathbf{CH}^9$), 128.50, 128.42 ($\mathbf{CH}^8$), 112.02 ($\mathbf{C}^5$), 85.46 ($\mathbf{CH}^{4'}$), 84.69 ($\mathbf{CH}^{1'}$), 75.80 ($\mathbf{CH}^{3'}$), 64.57 ($\mathbf{CH_2}^{5'}$), 47.05 ($\mathbf{CH}^{15}$), 38.49 ($\mathbf{CH_2}^{2'}$), 27.44 ($\mathbf{CH_3}^{13}$), 19.84 ($\mathbf{C}^{12}$), 12.33 ($\mathbf{CH_3}^7$).

IR ν_{max}/cm^{-1}: 3233 (s), 3110 (m), 2929 (m), 2857 (m), 2113 (s), 1685 (s), 1649 (s).

ES$^+$/MS: 1120.3 $(2M+Na)^+$.

HRMS (ES^+) for $C_{28}H_{32}N_4O_6Si$ $(M+Na)^+$: calculated 571.1983, found 571.1985.

Mpt. 128-132°C.



48 $C_{33}H_{34}N_2O_9$

5'-*O*-(4,4'-Dimethoxytrityl)-3'-(2-hydroxyacetyl)-2'-deoxythymidine (48).

This reaction was carried out following the method described by Lim *et al.*[179]

To an ice cold solution of dry diazo-compound **45** (0.5g, 0.82mmol) in distilled CH_2Cl_2 (5ml), was added dirhodium(II) tetraacetate catalyst (8.0mg, 0.018mmol) and the mixture stirred at 0°C for 1.5hrs under argon. The green coloured reaction was followed by TLC (system A) and when no further reaction took place the mixture was quenched with water (2ml) turning the aqueous layer blue/green and the organic layer pale yellow. The mixture was diluted with CH_2Cl_2 (50ml) and washed with water (50ml). Aqueous washings were combined and extracted with CH_2Cl_2. Organic layers were combined and dried over Na_2SO_4 (anhydrous), then evaporated to dryness. The crude product was purified by

column chromatography (CH_2Cl_2:MeOH, 0-5%) and after evaporating to dryness gave a white solid 0.4g (82%).

R_f (system A) 0.4.

^{1}H NMR ($CDCl_3$) δ: 8.55 (s, 1H, N**H**^{3}), 7.49 (s, 1H, C**H**^{6}), 7.32-7.12 (m, 10H, Ar**H**^{12,17,18,19}, C**H**^{9}), 6.72 (d, 4H, J = 8.5 Hz, Ar**H**^{13}), 6.30 (dd, 1H, J = 8.5 Hz, J = 2.5 Hz, C**H**$^{1'}$), 5.48-5.43 (m, 1H, C**H**$^{3'}$), 4.19-4.11 (m, 2H, C**H**$_2$^{9}), 4.11-4.07 (m, 1H, C**H**$^{4'}$), 3.72 (s, 6H, C**H**$_3$^{10}), 3.42 (dd, 1H, J = 10.5 Hz, J = 2.5 Hz, C**H**$^{5'}$), 3.39 (dd, 1H, J = 10.5 Hz, J = 2.5 Hz, C**H**$^{5'}$), 2.48-2.35 (m, 3H, O**H**, C**H**$_2$$^{2'}$), 1.34 (s, 3H, C**H**$_3$^{7}).

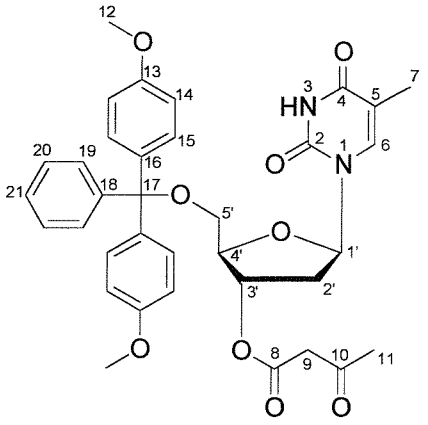
^{13}C NMR ($CDCl_3$) δ: 174.67 (**C**^{8}), 165.40 (**C**^{4}), 160.75 (**C**^{2}), 152.27 (**C**^{11}), 146.07 (**C**^{16}), 137.11 (**C**H^6), 137.04 (**C**^{14}), 132.00, 131.97 (**C**H^{13}), 130.02 (**C**H^{17}), 129.99 (**C**H^{18}), 129.20 (**C**H^{19}), 115.28 (**C**H^{12}), 113.69 (**C**^{5}), 89.22 (**C**^{15}), 86.23 (**C**H$^{4'}$), 85.74 (**C**H$^{1'}$), 78.40 (**C**H$^{3'}$), 65.46 (**C**H$_2$$^{5'}$), 62.61 (**C**H$_2$^{9}), 57.19 (**C**H$_3$^{10}), 39.83 (**C**H$_2$$^{2'}$), 13.59 (**C**H$_3$^{7}).

IR ν_{max}/cm^{-1}: 3192 (m), 2917 (m), 2837 (m), 1685 (s), 1606 (m), 1507 (s).

ES$^+$/MS: 602.2 $(M+H)^+$, 625.1 $(M+Na)^+$.

HRMS (ES^+) for $C_{33}H_{34}N_2O_9$ $(M+Na)^+$: calculated 625.2156, found 625.2168.

Mpt. 130-132°C.



49 $C_{35}H_{36}N_2O_9$

5'-*O*-(4,4'-Dimethoxytrityl)-3'-*O*-(β-ketoester)-2'-deoxythymidine (49).

This reaction was carried out following methods described in the literature.[192,193]

To a solution of dimethoxytritylthymidine (**42**) (0.1g, 0.18mmol) in xylenes (1ml) was added 2,2,6-trimethyl-4*H*-1,3-dioxin-4-one (0.029g, 1.1eq., 0.20mmol) in a 3ml open vial. The mixture was then placed directly into a preheated oil bath at 150°C and the solution stirred vigorously. Heating was continued for 15mins and gas was evolved. The reaction was cooled and concentrated *in vacuo* to remove excess xylenes. Purification was with silica gel column chromatography eluting with CH_2Cl_2/MeOH (0-3%). The title compound was obtained as a colourless oil (0.050g, 43%).

R_f (system A) 0.6.

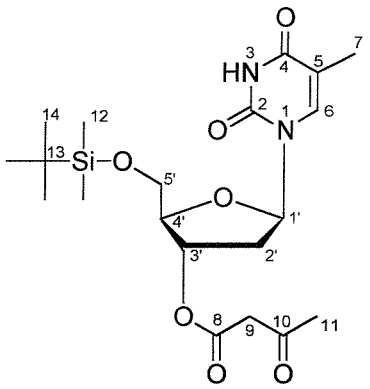
^{1}H NMR ($CDCl_3$) δ: 7.98 (s, 1H, N**H**^{3}), 7.51 (s, 1H, C**H**^{6}), 7.35-7.12 (m, 9H, Ar**H**^{14,19,20,21}), 6.76 (d, 4H, J = 8.5 Hz, Ar**H**^{15}), 6.32 (dd, 1H, J = 9.5 Hz, J = 5.5 Hz, C**H**$^{1'}$), 5.45 (d, 1H, J = 5.5 Hz, C**H**$^{3'}$), 4.08 (s, 1H, C**H**$^{4'}$), 3.71 (s, 6H, C**H**$_3{}^{12}$), 3.45-3.35 (m, 4H, C**H**$_2{}^{5'}$, C**H**$_2{}^9$), 2.39-2.32 (m, 2H, C**H**$_2{}^{2'}$), 2.18 (s, 3H, C**H**$_3{}^{11}$), 1.31 (s, 3H, C**H**$_3{}^7$).

^{13}C NMR ($CDCl_3$) δ: 202.50 (**C**^{10}), 165.73 (**C**^{8}), 162.40 (**C**^{4}), 158.04 (**C**^{13}), 149.33 (**C**^{2}), 143.39 (**C**^{18}), 134.54 (**CH**^{6}), 134.34 (**C**^{16}), 129.31, 129.28 (**CH**^{15}), 127.34 (**CH**^{19}), 127.26 (**CH**^{20}), 126.47 (**CH**^{21}), 112.56, 112.39 (**CH**^{14}), 110.85 (**C**^{5}), 87.68 (**C**^{17}), 83.52 (**CH**$^{4'}$), 83.20 (**CH**$^{1'}$), 75.50 (**CH**$^{3'}$), 62.87 (**CH**$_2{}^{5'}$), 54.47 (**CH**$_3{}^{12}$), 48.94 (**CH**$_2{}^9$), 37.01 (**CH**$^{2'}$), 29.53 (**CH**$_3{}^{11}$), 10.80 (**CH**$_3{}^7$).

IR ν_{max}/cm^{-1}: 3177 (m), 3052 (m), 2947 (m), 2837 (m), 1744 (m), 1686 (s), 1648 (s), 1607 (m), 1508 (s).

ES$^+$/MS: 651.1 $(M+Na)^+$, 1279.2 $(2M+Na)^+$.

HRMS (ES^+) for $C_{35}H_{36}N_2O_9$ $(M+Na^+)$: calculated 651.2313, found 651.2313.



50 $C_{20}H_{32}N_2O_7Si$

5'-*O*-(*tert*-Butyldimethylsilyl)-3'-*O*-(β-ketoester)-2'-deoxythymidine (50).

This reaction was carried out following methods described in the literature.[192,193]

To a solution of nucleoside **43** (0.05g, 0.14mmol) in xylenes (0.5ml) was added 2,2,6-trimethyl-4*H*-1,3-dioxin-4-one (0.022g, 1.1eq., 0.15mmol) in a 3ml open vial. The mixture was then placed directly into a preheated oil bath at 150°C and the solution stirred vigorously. Heating was continued for 20mins and gas was evolved. The reaction was cooled and concentrated *in vacuo* to remove excess xylenes. Purification was accomplished by column chromatography eluting with CH_2Cl_2/MeOH (0-3%). The title compound was obtained as a colourless oil (0.042g, 68%).

R_f (system A) 0.6.

^{1}H NMR ($CDCl_3$) δ: 8.21 (br s, 1H, N**H**^{3}), 7.39 (s, 1H, C**H**^{6}), 6.21 (dd, 1H, J = 9.5 Hz, J = 5.0 Hz, C**H**$^{1'}$), 5.19 (d, 1H, J = 6.5 Hz, C**H**$^{3'}$), 4.08 (s, 1H, C**H**$^{4'}$), 4.88-4.79 (m, 2H, C**H**$_2^{5'}$), 3.35 (s, 2H, C**H**$_2^9$), 2.34 (dd, 1H, J = 14.1 Hz, J = 5.0 Hz, C**H**$^{2'}$), 2.19 (s, 3H,

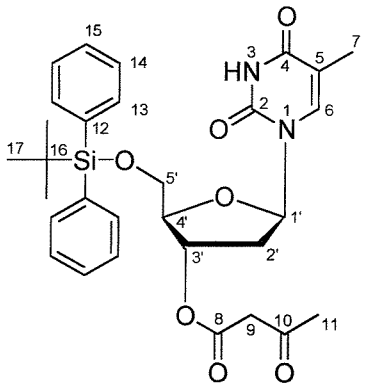
CH_3^{11}), 2.02 (ddd, 1H, J = 14.1 Hz, J = 9.5 Hz, J = 6.5 Hz, $CH^{2'}$), 1.80 (s, 3H, CH_3^{7}), 0.78 (s, 9H, CH_3^{14}), 0.01 (s, 6H, CH_3^{12}).

^{13}C NMR ($CDCl_3$) δ: 200.25 (C^{10}), 167.10 (C^{8}), 163.78 (C^{4}), 150.59 (C^{2}), 135.37 (CH^{6}), 111.65 (C^{5}), 85.70 ($CH^{4'}$), 85.07 ($CH^{1'}$), 75.29 ($CH^{3'}$), 63.97 ($CH_2^{5'}$), 50.14 (CH_2^{9}), 38.30 ($CH_2^{2'}$), 30.76 (CH_3^{11}), 26.33 (CH_3^{14}), 18.73 (C^{13}), 12.88 (CH_3^{7}), -4.98 (CH_3^{12}).

IR ν_{max}/cm^{-1}: 3233 (s), 3110 (m), 2929 (m), 2857 (m), 2113 (s), 1685 (s), 1649 (s).

ES$^+$/MS: 902.9 $(2M+Na)^+$.

HRMS (ES^+) for $C_{20}H_{32}N_2O_7Si$ $(2M+Na)^+$: calculated 903.3850, found 903.3865.



51 $C_{30}H_{36}N_2O_7Si$

5'-*O*-(*tert*-Butyldiphenylsilyl)-3'-*O*-(β-ketoester)-2'-deoxythymidine (51).

This reaction was carried out following methods described in the literature [192,193]

Nucleoside **44** (0.1g, 0.21mmol) was dissolved in xylenes (1ml) in a 3ml open vial and 2,2,6-trimethyl-4*H*-1,3-dioxin-4-one (0.033g, 1.1eq., 0.23mmol) was added. The mixture was then heated in an oil bath at 150°C and stirred vigorously for 20mins as gas was evolved. The reaction was cooled and concentrated *in vacuo* to remove excess xylenes. Purification was accomplished by column chromatography eluting with CH_2Cl_2/MeOH (0-3%). The title compound was obtained as a colourless oil (0.078g, 67%).

R_f (system A) 0.7.

^{1}H NMR ($CDCl_3$) δ: 7.92 (s, 1H, NH^{3}), 7.65-7.55 (m, 4H, ArH^{13}), 7.41-7.25 (m, 7H, $ArH^{14,15}$, CH^{6}), 6.31 (dd, 1H, J = 9.0 Hz, J = 5.0 Hz, $CH^{1'}$), 5.44 (d, 1H, J = 6.0 Hz, $CH^{3'}$),

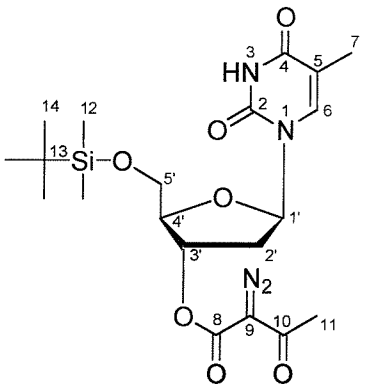
4.09-4.01 (m, 1H, $CH^{4'}$), 3.95-3.82 (ddd, 2H, J = 14.0 Hz, J = 11.5 Hz, J = 9.0 Hz, $CH_2^{5'}$), 3.45 (s, 2H, CH_2^{9}), 2.39 (dd, 1H, J = 14.1 Hz, J = 5.0 Hz, $CH^{2'}$), 2.26-2.15 (m, 4H, CH_3^{11}, $CH^{2'}$), 1.45 (s, 3H, CH_3^{7}), 0.98 (s, 9H, CH_3^{17}).

^{13}C NMR ($CDCl_3$) δ: 194.90 (C^{10}), 166.72 (C^{8}), 163.63 (C^{4}), 150.50 (C^{2}), 135.94 (CH^{6}), 135.61, 135.28 (CH^{13}), 133.65, 132.54 (C^{12}), 130.66, 130.53 (CH^{14}), 128.51, 128.43 (CH^{15}), 111.73 (C^{5}), 85.39 ($CH^{4'}$), 84.69 ($CH^{1'}$), 76.21 ($CH^{3'}$), 64.60 ($CH_2^{5'}$), 50.134 (CH_2^{9}), 38.22 ($CH_2^{2'}$), 30.75 (CH_3^{11}), 27.43 (CH_3^{17}), 19.83 (C^{16}), 12.32 (CH_3^{7}).

IR ν_{max}/cm^{-1}: 3177 (m), 3052 (m), 2947 (m), 2837 (m), 1744 (s), 1686 (s), 1648 (s), 1607 (m).

ES$^+$/MS: 587.1 $(M+Na)^+$.

HRMS (ES^+) for $C_{30}H_{36}N_2O_7Si$ $(M+Na)^+$: calculated 587.2184, found 587.2183.



52 $C_{20}H_{30}N_4O_7$

5'-*O*-(*tert*-Butyldimethylsilyl)-3'-*O*-(β-ketodiazoester)-2'-deoxythymidine (52).

β-Ketoester **50** (0.035g, 0.08mmol) in acetonitrile (0.5ml) was stirred at room temperature, DIPEA (0.024g, 0.183mmol) was added and the reaction stirred for 5mins. Following this *p*-carboxybenenesulphonyl azide (0.024g, 0.103mmol) was added and the mixture stirred for a further 18hrs. A white precipitate formed which was filtered and washed with diethyl ether. The filtrate was then diluted with ether and washed with water. The aqueous layer was extracted with ether twice and the combined organic layers were then washed with $NaHCO_3$ solution (sat.) and brine solution (sat.) before drying with Na_2SO_4 (anhydrous). The organic layer was filtered and evaporated to dryness, then applied to a silica column,

eluting with Hexane/EtOAc (4:1-1:1). Fractions were combined to yield the product 20mg (54%).

R_f (system J) 0.3.

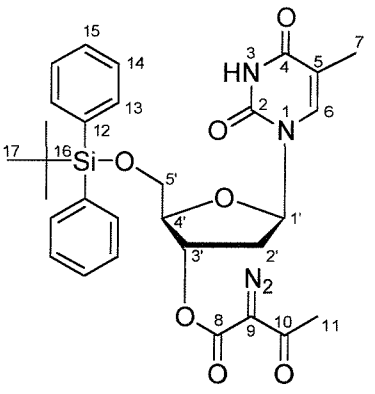
^{1}H NMR ($CDCl_3$) δ: 8.21 (br s, 1H, N**H**^{3}), 7.39 (s, 1H, C**H**^{6}), 6.21 (dd, 1H, J = 9.5 Hz, J = 5.0 Hz, 1H, C**H**$^{1'}$), 5.19 (d, 1H, J = 6.0 Hz, C**H**$^{3'}$), 4.08 (m, 1H, C**H**$^{4'}$), 3.85-3.78 (s, 2H, C**H**$_2{}^{5'}$), 2.38-2.30 (m, 4H, C**H**$_3{}^{11}$, C**H**$^{2'}$), 2.08 (ddd, 1H, J = 14.0 Hz, J = 9.5 Hz, J = 6.0 Hz, C**H**$^{2'}$), 1.80 (s, 3H, C**H**$_3{}^7$), 0.78 (s, 9H, C**H**$_3{}^{14}$), 0.01 (s, 6H, C**H**$_3{}^{12}$).

^{13}C NMR ($CDCl_3$) δ: 189.75 (**C**^{10}), 163.63 (**C**^{8}), 161.32 (**C**^{4}), 150.50 (**C**^{2}), 135.22 (**C**H^6), 111.73 (**C**^{5}), 85.62 (**C**H$^{4'}$), 85.10 (**C**H$^{1'}$), 77.41 (**C**H$^{3'}$), 63.90 (**C**H$_2{}^{5'}$), 38.57 (**C**H$_2{}^{2'}$), 28.69 (**C**H$_3{}^{11}$), 26.32 (**C**H$_3{}^{14}$), 18.74 (**C**^{13}), 12.90 (**C**H$_3{}^7$), -4.95 (**C**H$_3{}^{12}$).

IR ν_{max}/cm^{-1}: 3192 (m), 3068 (m), 2955 (m), 2930 (m), 2858 (m), 2144 (s), 1710 (s), 1691 (s), 1662 (s).

ES$^+$/MS: 954.9 $(2M+Na)^+$.

HRMS (ES^+) for $C_{20}H_{30}N_4O_7Si$ $(2M+Na)^+$: calculated 955.3660, found 955.3665.



53 $C_{30}H_{34}N_4O_7$

5'-*O*-(*tert*-Butyldiphenylsilyl)-3'-*O*-(β-ketodiazoester)-2'-deoxythymidine (53).

β-Ketoester **51** (0.05g, 0.089mmol) in acetonitrile (1ml) was stirred at room temp. with addition of DIPEA (0.035ml, 2.3eq., 0.20mmol). After 5mins *p*-carboxybenzenesulfonyl azide (0.026g, 0.12mmol) was added and the reaction stirred for 15hrs. TLC (system J) showed completion of the reaction and the white precipitate was filtered off and washed with diethyl ether. The filtrate and washings were combined and diluted with ether. This

organic mixture was washed with water, sat. sodium bicarbonate and brine. The organic layer was dried with sodium sulfate (anhydrous), filtered and evaporated to dryness. The crude product was purified by column chromatography with silica gel, eluting with EtOAc/hexane (1:2). Fractions were combined to yield a white solid, 0.039g (75%).

R_f (system J) 0.3.

^{1}H NMR ($CDCl_3$) δ: 7.98 (s, 1H, N**H**^{3}), 7.65-7.55 (m, 4H, Ar**H**^{13}), 7.41-7.25 (m, 7H, Ar**H**^{14,15}, C**H**^{6}), 6.30 (dd, 1H, J = 9.0 Hz, J = 5.5 Hz, C**H**$^{1'}$), 5.50 (d, 1H, J = 6.0 Hz, C**H**$^{3'}$), 4.10 (s, 1H, C**H**$^{4'}$), 3.97 (dd, 1H, J = 11.5 Hz, J = 2.0 Hz, C**H**$^{5'}$), 3.92 (dd, 1H, J = 11.5 Hz, J = 2.0 Hz, C**H**$^{5'}$), 2.45 (dd, 1H, J = 14.0 Hz, J = 5.5 Hz, C**H**$^{2'}$), 2.41 (s, 3H, C**H**$_3{}^{11}$), 2.28 (ddd, 1H, J = 14.0 Hz, J = 9.0 Hz, J = 6.0 Hz, C**H**$^{2'}$), 1.50 (s, 3H, C**H**$_3{}^{7}$), 1.01 (s, 9H, C**H**$_3{}^{17}$).

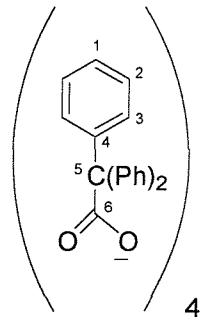
^{13}C NMR ($CDCl_3$) δ: 189.75 (**C**^{10}), 162.25 (**C**^{8}), 159.92 (**C**^{4}), 149.22 (**C**^{2}), 135.22 (**C**H^{6}), 134.39, 133.89 (**C**H^{13}), 131.16 (**C**^{12}), 129.48, 129.37 (**C**H^{14}), 127.29, 127.23 (**C**H^{15}), 110.84 (**C**^{5}), 83.95 (**C**H$^{4'}$), 83.44 (**C**H$^{1'}$), 75.16 (**C**H$^{3'}$), 63.20 (**C**H$_2{}^{5'}$), 38.28 (**C**H$_2{}^{2'}$), 27.47 (**C**H$_3{}^{11}$), 26.21 (**C**H$_3{}^{17}$), 18.60 (**C**^{16}), 11.15 (**C**H$_3{}^{7}$).

IR ν_{max}/cm^{-1}: 3187 (m), 3070 (m), 2957 (m), 2931 (m), 2858 (m), 2143 (s), 1707 (s), 1685 (s), 1662 (s).

ES$^+$/MS: 1203.6 $(2M+Na)^+$.

HRMS (ES^+) for $C_{30}H_{34}N_4O_7Si$ ($M+Na^+$): calculated 613.2089, found 613.2090.

2Rh (II)



Dirhodium(II) tetra(triphenylacetate) (54). [186]

A mixture of $Rh_2(OAc)_4$ (500mg, 1.13mmol), triphenylacetic acid (2.61g, 9.04mmol) and chlorobenzene (150ml) were heated at reflux with vigorous stirring, while the solvent was distilled off at a rate such that 30ml was removed per hour. The reaction was heated like this for 3hrs (168-172°C) and TLC (system A) showed the progress of the reaction. On completion the mixture was concentrated to 25ml and diluted with CH_2Cl_2 (100ml). The resulting dark green solution was washed with $NaHCO_3$ solution (sat.), water, brine and dried over Na_2SO_4, which was then filtered and evaporated *in vacuo* to yield a crystalline residue. This was recrystallised from CH_2Cl_2 resulting in green prisms, 732mg (48%).

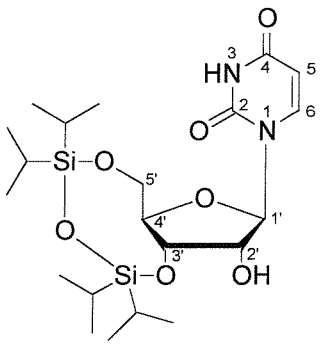
R_f (system A) 0.9.

^{1}H NMR ($CDCl_3$) δ: 7.01 (t, 12H, J = 7.7 Hz, Ar**H**^{1}), 6.79 (t, 24H, J = 7.7 Hz, Ar**H**^{2}), 6.79 (d, 24H, J = 7.7 Hz, Ar**H**^{3}).

^{13}C NMR ($CDCl_3$) δ: 193.46 (**C**^{6}), 143.11 (**C**^{4}), 130.65 (**C**^{2}), 127.48 (**C**^{3}), 126.84 (**C**^{1}), 69.27 (**C**^{5}).

IR ν_{max}/cm^{-1}: 3587 (m), 3057 (m), 1957 (w), 1880 (w), 1813 (w), 1677 (m), 1587 (s), 1576 (s), 1488 (m) 1443 (m), 1363 (s).

Analytical data was consistent with that found in the literature.[186]



55 $C_{21}H_{38}N_2O_7Si_2$

3',5'-*O*-(1,1,3,3-Tetraisopropyldisiloxy)uridine (55).[196]

Uridine (7.0g, 28.7mmol) was dissolved in freshly distilled pyridine (70ml) and dichlorotetraisopropylsiloxane (10.0g, 1.11eq. 31.7mmol) was added dropwise with stirring under an atmosphere of argon. The reaction was followed by TLC (system A) and showed completion after 2.5hrs. The mixture had formed a white precipitate. The solvent was removed and the residue redissolved in CH_2Cl_2 (200ml). This was washed with water (200ml), sat. $NaHCO_3$ (2x 200ml) and sat. KCl (2x 200ml), before drying with Na_2SO_4 and filtering. The solvent was removed and the colourless oil was applied to a silica column for purification eluting with CH_2Cl_2/MeOH (0-3%). Pure product was furnished as a colourless oil (12.22g, 87.6%).

R_f (system A) 0.7.

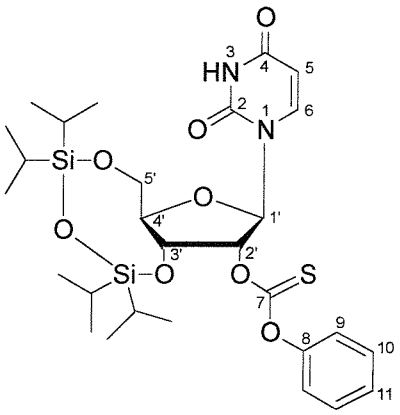
^{1}H NMR ($CDCl_3$) δ: 9.05 (s, 1H, N**H**^{3}), 7.58 (d, 1H, J = 8.5 Hz, C**H**^{6}), 5.60 (s, 1H, C**H**$^{1'}$), 5.56 (d, 1H, J = 8.5 Hz, C**H**^{5}), 4.22 (dd, 1H, J = 9.0 Hz, J = 5.0 Hz, C**H**$^{3'}$), 4.10-3.95 (m, 2H, C**H**$^{2'}$, C**H**$^{5'}$), 3.92 (dt, 1H, J = 9.0 Hz, J = 2.5 Hz, C**H**$^{4'}$), 3.81 (dd, 1H, J = 13.0 Hz, J = 2.5 Hz, C**H**$^{5'}$), 3.19 (s, 1H, O**H**), 1.08-0.80 (m, 28H, TIPS **H**).

^{13}C NMR ($CDCl_3$) δ: 163.68 (**C**^{4}), 150.43 (**C**^{2}), 140.40 (**C**H^6), 102.37 (**C**H^5), 91.37 (**C**H$^{1'}$), 82.35 (**C**H$^{4'}$), 75.58 (**C**H$^{2'}$), 69.37 (**C**H$^{3'}$), 60.70 (**C**H$_2$$^{5'}$), 17.85, 17.77, 17.68, 17.64, 17.44, 17.37, 17.32, 17.22 (**C**H$_3$^{TIPS}), 13.78, 13.34, 13.34, 12.92 (**C**H^{TIPS}).

IR ν_{max}/cm^{-1}: 2943 (m), 2867 (m), 1695 (s), 1681 (s).

ES$^+$/MS: 509.3 $(M+Na)^+$, 995.4 $(2M+Na)^+$.

Analytical data was consistent with that found in the literature.[196]



56 $C_{28}H_{42}N_2O_8SSi_2$

3',5'-*O*-(1,1,3,3-Tetraisopropyldisiloxy)-2'-deoxy-2'-α-*C*-(phenoxythioester)uridine (56).[181,194]

TIPS-uridine **55** (8.8g, 18.2mmol) and DMAP (2.4g, 19.7mmol) were added to a dry round bottom flask and distilled CH_2Cl_2 (50ml) was added under argon. Phenyl chlorothioformate (3.4g, 19.7mmol) and NEt_3 (2.0g, 19.7mmol) were added *via* syringe and the mixture was stirred for 18hrs. TLC (system I) showed completion and the mixture was diluted with CH_2Cl_2 (100ml), then washed with 2M HCl (200ml), water (200ml), sat. $NaHCO_3$ (200ml) and sat. KCl (200ml). The organic layer was removed and dried with anhydrous Na_2SO_4 then filtered and evaporated to dryness to yield crude product. Purification was with silica gel column chromatography eluting with Hexane/EtOAc (3:1-1:1) furnishing a white foam (7.6g, 60.4%).

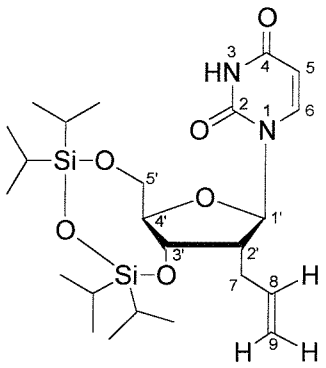
R_f (system E) 0.3.

^{1}H NMR ($CDCl_3$) δ: 8.55 (s, 1H, N**H**^{3}), 7.62 (d, 1H, J = 8.0 Hz, C**H**^{6}), 7.35 (t, 2H, J = 7.5 Hz, Ar**H**^{10}), 7.23 (t, 1H, J = 7.5 Hz, Ar**H**^{11}), 7.07 (d, 2H, J = 7.5 Hz, Ar**H**^{9}), 5.95 (d, 1H, J = 4.5 Hz, C**H**$^{2'}$), 5.89 (s, 1H, C**H**$^{1'}$), 5.70-5.61 (m, 1H, C**H**^{5}), 4.50 (dd, 1H, J = 9.5 Hz, J = 4.5 Hz, C**H**$^{3'}$), 4.20 (d, 1H, J = 13.6 Hz, C**H**$^{5'}$), 4.06 (d, 1H, J = 9.5 Hz, C**H**$^{4'}$), 3.93 (dd, 1H, J = 13.6 Hz, J = 2.5 Hz, C**H**$^{5'}$), 1.08-0.80 (m, 28H, TIPS **H**).

^{13}C NMR ($CDCl_3$) δ: 192.75 (**C**^{7}), 161.80 (**C**^{4}), 152.45 (**C**^{2}), 148.49 (**C**^{8}),138.49 (**C**H^6), 128.57 (**C**H^{10}), 125.70 (**C**H^{11}), 120.76 (**C**H^9), 101.32 (**C**H^5), 87.62 (**C**H$^{1'}$), 82.77 (**C**H$^{4'}$), 81.25 (**C**$^{2'}$), 67.16 (**C**H$^{3'}$), 58.50 (**C**H$_2^{5'}$), 16.44, 16.36,16.29, 16.22, 15.97, 15.88 (**C**H$_3^{TIPS}$), 12.37, 11.96, 11.90, 11.78 (**C**H^{TIPS}).

IR ν_{max}/cm^{-1}: 2943 (m), 2866 (m), 1686 (s).

ES$^+$/MS: 1297.3 $(M+Na)^+$.



57 $C_{24}H_{42}N_2O_6Si_2$

3',5'-*O*-(1,1,3,3-Tetraisopropyldisiloxy)-2'-deoxy-2'-α-*C*-(allyl)uridine (57).[181]

Phenoxythioester **56** (5.3g, 8.5mmol) was dissolved in anhydrous degassed benzene (50ml). AIBN (0.7g, 4.3mmol) and allylSnBu$_3$ (14.0g, 13.1ml, 42.3mmol) were added and the reaction stirred. A mercury lamp was used to illuminate the reaction, which was left stirring for 5hrs. TLC (system K) showed completion and the solvent was removed *in vacuo*. Purification was by column chromatography eluting with CH_2Cl_2/MeOH (0-3%). Fractions were combined to yield a white foam, 3.93g (90.6%).

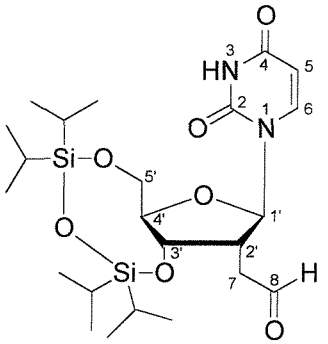
R_f (system L) 0.6.

^{1}H NMR ($CDCl_3$) δ: 8.62 (s, 1H, N**H**^{3}), 7.67 (d, 1H, J = 8.0 Hz, C**H**^{6}), 6.06-5.91 (m, 1H, C**H**^{8}), 5.75-5.65 (m, 1H, C**H**$^{1'}$), 5.62 (d, 1H, J = 8.0 Hz, C**H**^{5}), 5.09 (d, 1H, J = 17.0 Hz, C**H**^{9}), 5.01 (d, 1H, J = 7.0 Hz, C**H**^{9}), 4.41 (t, 1H, J = 8.0 Hz, C**H**$^{3'}$), 4.06 (dd, 1H, J = 13.0 Hz, J = 3.0 Hz, C**H**$^{5'}$), 3.93 (dd, 1H, J = 13.0 Hz, J = 3.0 Hz, C**H**$^{5'}$), 3.70 (dt, 1H, J = 8.0 Hz, J = 3.0 Hz, C**H**$^{4'}$), 2.59-2.05 (m, 3H, C**H**$^{2'}$, C**H**$_2^7$), 1.08-0.75 (m, 28H, TIPS **H**).

^{13}C NMR ($CDCl_3$) δ: 161.43 (**C**^{4}), 148.15 (**C**^{2}), 138.05 (**C**H^6), 133.89 (**C**H^8), 115.45 (**C**H$_2^9$), 100.02 (**C**H^5), 86.96 (**C**H$^{1'}$), 81.68 (**C**H$^{4'}$), 67.22 (**C**H$^{3'}$), 59.02 (**C**H$_2^{5'}$), 46.51 (**C**H$^{2'}$), 28.90 (**C**H$_2^7$), 15.79, 15.68, 15.61, 15.52, 15.37, 15.27, 15.24, 15.17 (**C**H$_3^{TIPS}$), 11.93, 11.71, 11.36, 11.29 (**C**H^{TIPS}).

IR ν_{max}/cm^{-1}: 2944 (m), 2867 (m), 1679 (s).

Analytical data was consistent with that found in the literature. [181]



58 $C_{23}H_{40}N_2O_7Si_2$

3',5'-*O*-(1,1,3,3-Tetraisopropyldisiloxy)-2'-deoxy-2'-α-*C*-(2-oxyethyl)uridine (58).[182]
Alkene **57** (3.60g, 7.1mmol) was dissolved in acetone/water (36ml/9ml) and *N*-methylmorpholine *N*-oxide (NMNO) was added (0.95g, 1.1eq, 7.8mmol). To this was added a 1% solution of OsO_4 (9ml). The mix was stirred for 1.5hrs and TLC (system K) showed completion. The mixture was quenched with saturated sodium thiosulfate solution (14ml) and water (14ml), then evaporated to a third of its volume. EtOAc was added and the organic layer washed with sat. $NaHCO_3$, sat. brine and then dried with Na_2SO_4 (anhydrous). The solution was filtered and evaporated to dryness then redissolved in dioxane/water (75ml/25ml). $NaIO_4$ (3.0g, 14.0mmol) was added and the reaction stirred for 45mins. TLC (system A) showed completion and the mixture was diluted with EtOAc. This was washed with sat. $NaHCO_{3(aq.)}$ and sat. $NaCl_{(aq.)}$ and dried over Na_2SO_4 (anhydrous) before evaporating to dryness. The product was purified by column chromatography eluting with CH_2Cl_2/MeOH (0-3%) to yield a white foam 2.55g (71%).

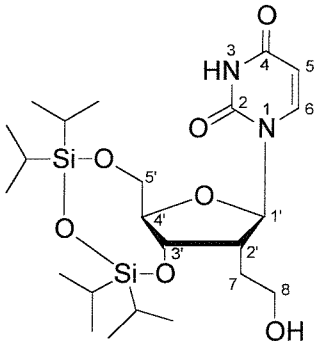
R_f (system A) 0.5.

^{1}H NMR ($CDCl_3$) δ: 9.72 (s, 1H, C**H**^{8}), 8.90 (s, 1H, N**H**^{3}), 7.55 (d, 1H, J = 8.0 Hz, C**H**^{6}), 5.71-5.65 (m, 2H, C**H**$^{1'}$, C**H**^{5}), 4.46 (t, 1H, J = 7.3 Hz, C**H**$^{3'}$), 4.06 (dd, 1H, J = 13.0 Hz, J = 4.0 Hz, C**H**$^{5'}$), 4.01 (dd, 1H, J = 13.0 Hz, J = 3.5 Hz, C**H**$^{5'}$), 3.85 (ddd, 1H, J = 7.3 Hz, J = 4.0 Hz, J = 3.5 Hz, C**H**$^{4'}$), 2.42-2.35 (m, 2H, C**H**$^{2',7}$), 2.66 (dd, 1H, J = 16.0 Hz, J = 4.0 Hz, C**H**^{7}), 1.08-0.75 (m, 28H, TIPS **H**).

^{13}C NMR ($CDCl_3$) δ: 199.83 (**C**H^{8}), 163.44 (**C**^{4}), 150.63 (**C**^{2}), 139.51 (**C**H^{6}), 102.67 (**C**H^{5}), 88.85 (**C**H$^{1'}$), 84.35 (**C**H$^{4'}$), 69.77 (**C**H$^{3'}$), 61.50 (**C**H$_2$$^{5'}$), 43.89 (**C**H$^{2'}$), 40.79 (**C**H$_2$^{7}), 17.87, 17.77, 17.71, 17.63, 17.34, 17.27, 17.25, 17.23 (**C**H$_3$^{TIPS}), 13.73, 13.47, 13.46, 13.03 (**C**H^{TIPS}).

ES$^+$/MS: 513.2 $(M+H)^+$, 1047.1 $(2M+Na)^+$.

Analytical data was consistent with that found in the literature.[182]



59 $C_{23}H_{42}N_2O_7Si_2$

3',5'-*O*-(1,1,3,3-Tetraisopropyldisiloxy)-2'-deoxy-2'-α-*C*-(2-hydroxyethyl)uridine (59).[182]

Aldehyde **58** (2.6g, 5.1mmol) was dissolved in cold methanol and added dropwise with stirring to a suspension of sodium borohydride (0.58g, 15.2mmol) in methanol at 0°C. After 1hr the reaction was neutralised with citric acid (10%), evaporated to dryness and redissolved in CH_2Cl_2. This was washed with water (200ml), sat. $NaHCO_3$ (200ml), sat. brine (200ml) and dried (Na_2SO_4), before filtering the organic layer and evaporating to dryness. Purification was with silica chromatography eluting with EtOAc/Hexane (1:1) to yield a white foam 2.32g (89%).

R_f (system I) 0.5.

^{1}H NMR ($CDCl_3$) δ: 9.46 (s, 1H, N**H**^{3}), 7.90 (d, 1H, J = 8.0 Hz, C**H**^{6}), 5.81 (s, 1H, C**H**$^{1'}$), 5.65 (d, 1H, J = 8.0 Hz, C**H**^{5}), 4.36 (dd, 1H, J = 9.0 Hz, J = 7.0 Hz, C**H**$^{3'}$), 4.16 (d, 1H, J = 13.6 Hz, C**H**$^{5'}$), 3.95-3.70 (m, 4H, C**H**$^{5'}$, C**H**$^{4'}$, C**H**$_2$^{8}), 3.40 (s, 1H, O**H**), 2.42-2.35 (ddd,

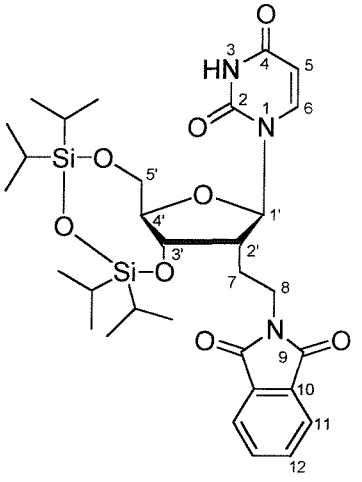
1H, J = 10.5 Hz, J = 7.0 Hz, J = 3.5 Hz, $CH^{2'}$), 2.12-1.50 (m, 2H, CH_2^{7}), 1.08-0.75 (m, 28H, TIPS **H**).

^{13}C NMR ($CDCl_3$) δ: 163.87 (C^{4}), 151.33 (C^{2}), 140.03 (CH^{6}), 102.18 (CH^{5}), 89.43 ($CH^{1'}$), 83.42 ($CH^{4'}$), 67.68 ($CH^{3'}$), 61.08 ($CH_2^{5'}$), 60.17 (CH_2^{8}), 47.18 ($CH^{2'}$), 28.26 (CH_2^{7}), 17.93, 17.82, 17.74, 17.63, 17.48, 17.40, 17.33, 17.28 (CH_3^{TIPS}), 13.80, 13.52, 13.31, 12.93 (CH^{TIPS}).

IR ν_{max}/cm^{-1}: 3428 (m), 2945 (m), 2867 (m), 1677 (s).

ES$^+$/MS: 1051.1 $(2M+Na)^+$.

Analytical data was consistent with that found in the literature.[182]



60 $C_{31}H_{43}N_3O_8Si_2$

3',5'-*O*-(1,1,3,3-Tetraisopropyldisiloxy)-2'-deoxy-2'-α-*C*-(2-*N*-phthaloylethyl)uridine (60).

The nucleoside **59** (0.5g, 0.97mmol), PPh_3 (0.28g, 1.0mmol), phthalimide (0.15g, 1.0mmol) were dissolved in freshly distilled THF (5ml). To this was added a solution of DEAD (0.18g, 0.16ml, 1.0mmol) in THF (3ml) slowly with stirring. The reaction was left stirring for 18hrs and TLC (system K) showed consumption of starting material and the mixture was evaporated to dryness *in vacuo*. This was redissolved in a minimum amount of diethyl ether and on standing at 4°C a white precipitate formed. This precipitate was filtered off and the filtrate was washed with sat. KCl, dried with Na_2SO_4 and filtered then

evaporated to dryness to yield crude product. This was purified with silica gel column chromatography eluting with EtOAc/hexane (1:3) to furnish a white foam 0.35g (55.8%).

R_f (system I) 0.5.

^{1}H NMR ($CDCl_3$) δ: 8.60 (s, 1H, N**H**^{3}), 7.78-7.75 (m, 2H, Ar**H**^{11}), 7.71 (d, 1H, J = 8.0 Hz, C**H**^{6}), 7.65-7.61 (m, 2H, Ar**H**^{12}), 5.83-5.75 (m, 1H, C**H**$^{1'}$), 5.62 (dd, 1H, J = 8.0 Hz, J = 2.0 Hz, C**H**^{5}), 4.41 (t, 1H, J = 8.0 Hz, C**H**$^{3'}$), 4.12 (d, 1H, J = 13.6 Hz, C**H**$^{5'}$), 3.95-3.70 (m, 4H, C**H**$^{5'}$, C**H**$^{4'}$, C**H**$_2^8$), 2.45-2.30 (m, 1H, C**H**$^{2'}$), 2.15-1.49 (m, 2H, C**H**$_2^7$), 1.20-0.82 (m, 28H, TIPS **H**).

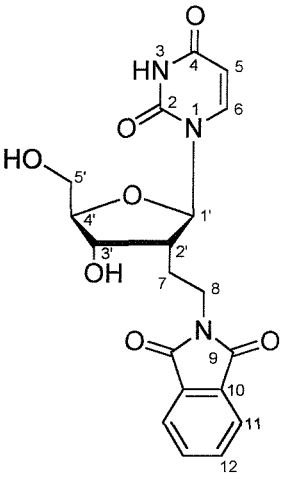
^{13}C NMR ($CDCl_3$) δ: 168.65 (**C**^{4}), 163.52 (**C**^{2}), 150.32 (**C**^{9}), 139.86 (**C**H^6), 134.30 (**C**H^{12}), 132.58 (**C**^{10}), 123.62 (**C**H^{11}), 102.12 (**C**H^5), 89.39 (**C**H$^{1'}$), 83.37 (**C**H$^{4'}$), 68.67 (**C**H$^{3'}$), 60.60 (**C**H$_2^{5'}$), 47.09 (**C**H$^{2'}$), 36.75 (**C**H$_2^8$), 25.69 (**C**H$_2^7$), 17.91, 17.81, 17.73, 17.64, 17.43, 17.39, 17.36, 17.27 (**C**H$_3^{TIPS}$), 13.80, 13.49, 13.38, 12.94 (**C**H^{TIPS}).

IR ν_{max}/cm^{-1}: 3428 (m), 2944 (m), 2867 (m), 1708 (s), 1664 (s).

ES$^+$/MS: 644.3 $(M+H)^+$, 666.3 $(M+Na)^+$, 1309.6 $(2M+Na)^+$.

HRMS (ES^+) for $C_{31}H_{43}N_3O_8Si_2$ $(M+H)^+$: calculated 644.2830, found 644.2818.

Mpt. 85-90°C.



61 $C_{19}H_{19}N_3O_7$

2'-Deoxy-2'-α-*C*-(2-*N*-phthaloylethyl)uridine (61).

To the TIPS-protected nucleoside **60** (1.0g, 1.5mmol) was added TBAF in THF (1M) (10ml, 10mmol). The reaction was stirred for 1hr and TLC (system A) showed completion of the reaction. The mixture was diluted with CH_2Cl_2 (200ml), washed with sat. $NaHCO_3$ (100ml), sat. KCl (200ml). In each case the aqueous washings were extracted with CH_2Cl_2 several times to maximise yield of deprotected nucleoside. TLC (staining with anisaldehyde) was used to indicate presence of sugar. The combined organic layers were dried over Na_2SO_4 and filtered, before evaporating to dryness to yield a pale-yellow oil. This was purified with silica gel column chromatography (CH_2Cl_2/MeOH 2-10%). Pure fractions were combined to yield a colourless oil 0.3g (48%).

R_f (system A) 0.2.

^{1}H NMR (CD_3OD) δ: 7.91 (d, 1H, J = 8.0 Hz, C**H**^{6}), 7.90-7.71 (m, 4H, Ar**H**^{11,12}), 6.07 (d, 1H, J = 8.5 Hz, C**H**$^{1'}$), 5.61 (d, 1H, J = 8.0 Hz, C**H**^{5}), 4.87 (s, 2H, C**H**$_2^{5'}$), 4.35 (dd, 1H, J = 5.0 Hz, J = 1.5 Hz, C**H**$^{3'}$), 3.99 (dd, 1H, J = 5.0 Hz, J = 3.0 Hz, C**H**$^{4'}$), 3.85-3.70 (m, 2H, C**H**$_2^8$), 2.39-2.21 (m, 1H, C**H**$^{2'}$), 2.25-1.72 (m, 2H, C**H**$_2^7$).

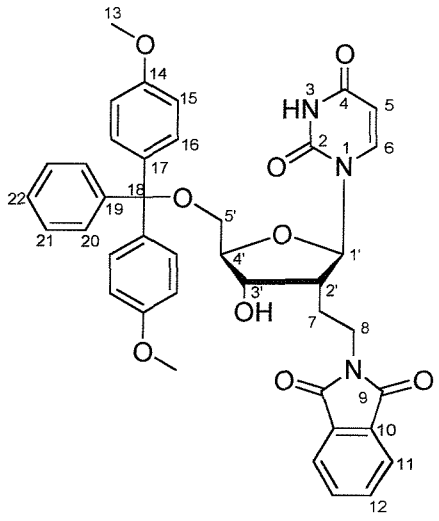
^{13}C NMR (CD_3OD) δ: 170.78 (**C**^{9}), 166.90 (**C**^{4}), 153.56 (**C**^{2}), 143.30 (**CH**^{6}), 136.29 (**CH**^{12}), 134.25 (**C**^{10}), 125.04 (**CH**^{11}), 104.195 (**CH**^{5}), 90.77 (**CH**$^{1'}$), 90.14 (**CH**$^{4'}$), 74.38 (**CH**$^{3'}$), 64.33 (**CH**$_2^{5'}$), 48.05 (**CH**$^{2'}$), 38.15 (**CH**$_2^8$), 24.95 (**CH**$_2^7$).

IR ν_{max}/cm^{-1}: 3429 (s), 3053 (m), 2936 (m), 2804 (m), 1686 (s).

ES$^+$/MS: 402.2 $(M+H)^+$, 424.1 $(M+Na)^+$.

HRMS *m/z* for $C_{19}H_{19}N_3O_7$ $(M+H)^+$: calculated 402.1291, found 402.1296.

Mpt. 199-203°C.



62 $C_{40}H_{37}N_3O_9$

5'-*O*-(4,4'-Dimethoxytrityl)-2'-deoxy-2'-α-*C*-(2-*N*-phthaloylethyl)uridine (62).

Deprotected nucleoside **61** (0.1g, 0.25mmol) and dimethoxytrityl chloride (0.1g, 0.28mmol) were placed in a round bottom flask and dried for 18hrs in a vacuum desiccator. The flask was opened under an atmosphere of argon and freshly distilled pyridine (3ml) was added. The reaction was left stirring for 18hrs and TLC (system A) showed consumption of starting material. The mixture was evaporated to dryness, dissolved in CH_2Cl_2 (50ml), washed with $NaHCO_3$ (50ml), sat. KCl (50ml) then dried over Na_2SO_4 (anhydrous), filtered and evaporated to yield a colourless oil. This was purified by column chromatography (CH_2Cl_2/MeOH 0-3%) to yield a white solid 0.12g, (69%)

R_f (system A) 0.6

^{1}H NMR (DMSO-d_6) δ: 11.39 (s, 1H, N**H**^{3}), 7.84-7.78 (m, 4H, Ar**H**^{11,12}), 7.62 (d, 1H, J = 8.0 Hz, C**H**^{6}), 7.43-7.22 (m, 9H, Ar**H**^{15,20,21,22}), 6.95-6.87 (dd, 4H, J = 11.5 Hz, J = 9.0 Hz, Ar**H**^{16}), 5.96 (d, 1H, J = 9.0 Hz, C**H**$^{1'}$), 5.50 (d, 1H, J = 5.5 Hz, O**H**), 5.31 (dd, 1H, J = 8.5 Hz, J = 2.0 Hz, C**H**^{5}), 4.38 (t, 1H, J = 4.5 Hz, C**H**$^{3'}$), 4.05-3.98 (m, 1H, C**H**$^{4'}$), 3.81-3.76 (m, 8H, CH_3^{13}, CH_2^{8}), 3.29 (dd, 1H, J = 10.5 Hz, J = 4.5 Hz, C**H**$^{5'}$), 3.16 (dd, 1H, J = 10.5 Hz, J = 3.5 Hz, C**H**$^{5'}$), 2.36-2.27 (m, 1H, C**H**$^{2'}$), 2.09-1.62 (m, 2H, CH_2^{7}).

^{13}C NMR (DMSO-d_6) δ: 168.50 (**C**^{9}), 163.26 (**C**^{4}), 158.56 (**C**^{2}), 151.17 (**C**^{14}), 144.94 (**C**^{19}), 140.55 (**CH**^{6}), 135.82, 135.49 (**C**^{17}), 134.78 (**CH**^{12}), 132.11 (**C**^{10}), 130.21, 130.16

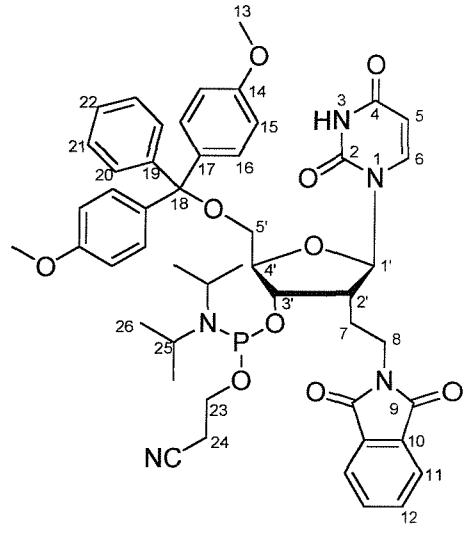
(**CH**[16]), 128.30 (**CH**[20]), 128.12 (**CH** [21]), 127.19 (**CH**[22]), 123.42 (**CH**[11]), 113.69, 113.65 (**CH**[15]), 102.39 (**CH**[5]), 87.90 (**CH**[1']), 86.46 (**C**[18]), 86.27 (**CH**[4']), 71.85 (**CH**[3']), 64.30 (**CH_2**[5']), 55.50 (**CH_3**[13]), 45.67 (**CH**[2']), 36.32 (**CH_2**[8]), 23.20 (**CH_2**[7]).

IR ν_{max}/cm^{-1}: 3414 (m), 3068 (m), 2936 (m), 2834 (m), 1708 (s), 1677 (s), 1606 (m).

ES$^+$/MS: 726.5 $(M+Na)^+$.

HRMS *m/z* for $C_{40}H_{37}N_3O_9$ $(M+Na)^+$: calculated 726.2422, found 726.2416.

Mpt. decomposed ~150°C.



63 $C_{49}H_{54}N_5O_{10}P$

5'-*O*-(4,4'-Dimethoxytrityl)-3'-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidyl)-2'-deoxy-2'-α-*C*-(2-*N*-phthaloylethyl)uridine (63).

Phosphitylation was carried out by the method described by Caruthers *et al.*[6]

Tritylated nucleoside **62** (0.1g, 0.14mmol) was dissolved in freshly distilled THF (3ml) and to this was added DIPEA (0.073g, 0.57mmol) and 2-cyanoethoxy-*N*,*N*-diisopropylamine chlorophosphine (0.038g, 0.16mmol) under an atmosphere of argon. TLC (system A) showed completion after 5hrs. The solvent was removed and redissolved in CH_2Cl_2 (50ml), which was washed with sat. KCl (50ml) and dried with anhydrous Na_2SO_4, then the organic layer was decanted off to a separate flask for the solvent to be

removed. The crude oil was purified by column chromatography under argon eluting with degassed hexane/EtOAc (1:1) to yield the product as a white foam, 0.089g (70%).

R_f (system A) 0.7.

^{1}H NMR (DMSO-d6) δ: 7.75 (s, 1H, N**H**^{3}), 7.70-7.56 (m, 5H, Ar**H**^{11,12}, C**H**^{6}), 7.31-7.08 (m, 9H, Ar**H**^{15,20,21,22}), 6.70 (dd, 4H, J = 11.5 Hz, J = 9.0 Hz, Ar**H**^{16}), 6.01 (d, 1H, J = 8.5 Hz, C**H**$^{1'}$), 5.18-5.06 (m, 1H, C**H**^{5}), 4.77 (dd, 1H, J = 11.5 Hz, J = 5.5 Hz, C**H**$^{3'}$), 4.18 (m, 1H, C**H**$^{4'}$), 3.95-3.48 (m, 12H, C**H**$_3$^{13}, C**H**^{25}, C**H**$_2$^{23}, C**H**$_2$^{8}), 3.37 (dd, 1H, J = 10.5 Hz, J = 3.0 Hz, C**H**$^{5'}$), 3.26 (dd, 1H, J = 10.5 Hz, J = 3.5 Hz, C**H**$^{5'}$), 2.63 (td, 2H, J = 6.5Hz, J = 2.0 Hz, C**H**$_2$^{24}), 2.45-2.25 (m, 1H, C**H**$^{2'}$), 2.09-1.69 (m, 2H, C**H**$_2$^{7}), 1.30-1.00 (m, 12H, C**H**$_3$^{26}).

^{31}P NMR ($CDCl_3$) δ: 149.08.

ES$^+$/MS: 904.3 $(M+H)^+$, 926.2 $(M+Na)^+$.

4.5 Preparation of synthetic oligonucleotides

All oligonucleotides were synthesised on an Applied Biosystems 394 automated DNA synthesisers using the standard cycle of acid-catalysed detritylation, coupling, capping and iodine oxidation procedures. Oligonucleotides were assembled on a 0.2μmole scale and stepwise coupling efficiencies and overall yields were calculated by the automated trityl cation conductivity monitoring facility. Standard DNA phosphoramidites, solid supports and additional reagents were purchased from Applied Biosystems, unless otherwise stated.

β-Cyanoethyl phosphoramidites were used for all standard monomers and were dissolved in anhydrous acetonitrile to a concentration of 0.1M immediately prior to use. Non-standard monomers were prepared in our laboratories and synthetic details are published elsewhere.[171] Cyanine dyes were added as funtionalised dT resins ie. a dT resin with a single addition of a Cy5 phosphoramidite (Pharmacia) to the 3'-OH, leaving the DMT group of the Cy5 monomer on (figure 4.4.1). Oligonucleotide synthesis could then be continued from the Cy5 as if it were a normal dT resin. All phosphoramidites synthesised were stored under argon, at -20°C, in bottles suitable for an ABI 394 DNA synthesiser.

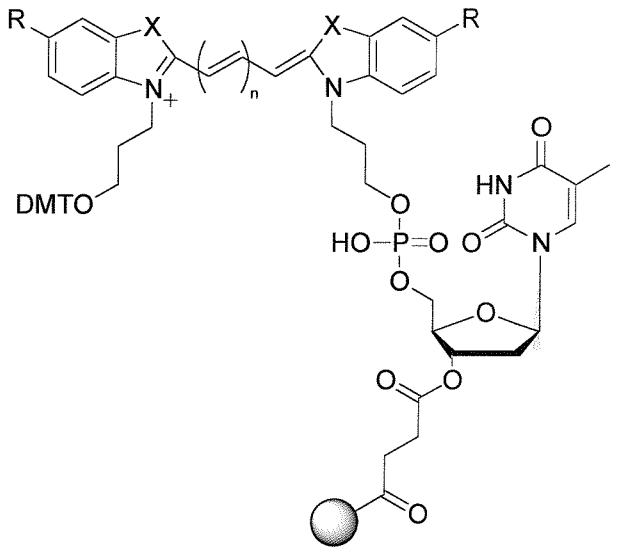


Figure 4.4.1: Cyanine dye attached to a dT resin.

Cleavage of the oligonucleotides from the solid support was achieved by exposure to concentrated ammonia solution (30 min at room temp.). All oligonucleotides containing monomers **20**, **29, 39** and Cy5 modifications requiring base deprotection were placed in a solution of water:methanol:^{t}butylamine (2:1:1) at 70°C for 2½hrs, or ammonium hydroxide:concentrated aqueous methylamine (1:1). Oligonucleotides containing monomers **5** or **13** were deprotected with concentrated ammonia at 25°C for 1hr. All other oligonucleotides were deprotected in concentrated ammonia at 55°C for 4hrs. Following deprotection the oligonucleotides were evaporated to dryness *in vacuo*.

Purification was effected using reversed phase HPLC on a Gilson system (column: ABI C8 (octyl) 8mm x 250mm, pore size = 300Å), controlled by Gilson 7.12 software. Buffer systems consisted of buffer A (0.1M ammonium acetate, pH 7.0) and buffer B (0.1M ammonium acetate with 40% acetonitrile, pH 7.0). Two programs were used regularly: a short program for simple unmodified oligonucleotides and a long program for doubly modified and more complex oligonucleotides. The short program ran for 23 minutes with a flow rate of 4ml/min and the following gradient: Time in mins (%Buffer B); 0 (0); 2 (0); 15 (70); 17 (100); 20 (100); 22 (0); 22.9 (0); 23 (0). The longer program ran for 30 minutes at a rate of 4ml/min with the gradient: Time in mins (%Buffer B); 0 (0); 3 (0); 5 (20); 21 (100); 25 (100); 27 (0); 30 (0). Elution of oligonucleotides was monitored by UV detection at appropriate wavelengths (260-290nm). Analytical injections were monitored at 265nm, 0.1 AUFS, and preparative injections were monitored at 280-290nm, 1.0 AUFS. The oligonucleotide peaks were collected manually. Oligonucleotides were desalted using disposable NAP 10 Sephadex columns (Pharmacia) and then aliquoted into separate eppendorf tubes. Aliquots were stored at -20°C.

4.6 Oligonucleotide sequences

Reference code	Oligo. No.	SEQUENCE	MOLECULAR ION	
			Expected	Found
X2	**ODN1**	QTT	-	-
X12	**ODN2**	QTTTTTTTTTTT	4031	4031
TB1669	**ODN3**	QGCAGCCGTTTCCTCCACTGTTGCGCTGC5	9775	9752
TB1673	**ODN4**	MGCAGCCGTTTCCTCCACTGTTGCGCTGC5	9862	9859
TB1368	**ODN5**	GCAACAGTGGAGGAAACG	5607	5609
	ODN6	BTTTTTTTTTT		
RS0466	**ODN7**	TTTTTTTTTB		
TB1780	**ODN8**	GGATCCAAGTTTTTC2	5718	5514
TB1778	**ODN9**	GGATCCAAGTTTTTC3	5669	5670
TB1776	**ODN10**	GGATCCAAGTTTTTC4	-	5566
TB1764	**ODN11**	GGATCCAAGTTTTTC5	5697	5697
TB1765	**ODN12**	GGATCCAAGTTTTTC6	5792	5798
TB1766	**ODN13**	MGAAAAAACTTGGATCC	5442	5143
TB1763	**ODN14**	BGAAAAAACTTGGATCC	5240	5254
TB1781	**ODN15**	BGGATCCAAGTTTTTC2	6093	5892
TB1778	**ODN16**	BGGATCCAAGTTTTTC3	6041	5669
TB1777	**ODN17**	BGGATCCAAGTTTTTC4	-	5942
TB1783	**ODN18**	BGGATCCAAGTTTTTC5	6055	6054
TB1793	**ODN19**	GAAAAAACTTGGATCC	4881	4897
RS0419	**ODN20**	BGCAGCCGTTTCCTCCACTGTTGCGCTGC2	-	-
RS0420	**ODN21**	MGCAGCCGTTTCCTCCACTGTTGCGCTGC2	9413	9491
RS0421	**ODN22**	BGCAGCCGTTTCCTCCACTGTTGCGCTGC5	9674	9675
RS0422	**ODN23**	MGCAGCCGTTTCCTCCACTGTTGCGCTGC5	9846	-
RS0423	**ODN24**	GCAACAGTGGAGGAAACG	5607	5609
RS0045	**ODN25**	4ATGCCCTCCCCCATGCCATCCTGCGTTB	9375	-
RS0046	**ODN26**	4ATGCCCTCCCCCATGCCATCCTGCGTTM	9255	9270
RS0047	**ODN27**	7ATGCCCTCCCCCATGCCATCCTGCGTTB	9479	9450
RS0048	**ODN28**	7ATGCCCTCCCCCATGCCATCCTGCGTTM	9360	9370
RS0049	**ODN29**	8ATGCCCTCCCCCATGCCATCCTGCGTTB	9455	-
RS0050	**ODN30**	8ATGCCCTCCCCCATGCCATCCTGCGTTM	9330	9340
RS0051	**ODN31**	2ATGCCCTCCCCCATGCCATCCTGCGTTB	9321	9320
RS0052	**ODN32**	2ATGCCCTCCCCCATGCCATCCTGCGTTM	9201	9210
RS0215	**ODN33**	2CCCGCGCCTTTCCTCCACTGTTGCGCGCGGGBHATGGT GTGTCTTGGGATTCA	-	-
RS	**ODN34**	2CCCGCGCCTTTCCTCCACTGTTGCGCGCGGGMHATGGT GTGTCTTGGGATTCA	-	-
RS	**ODN35**	2CTTTCCTCCACTGTTGCHATGGTGTGTCTTGGGATTCA	-	-
RS	**ODN36**	GCAACAGTGGAGGAAAGM	-	-
RS	**ODN37**	GCAACAGTGGAGGAAAGB	-	-
RS0203	**ODN38**	TCCAAGTTTTTBCTA	5049	5040
RS0205	**ODN39**	TCCAAGBTTTTTCTA	5049	5040
RS0206	**ODN40**	TCCAAGTTTTTTCBA	5049	5040
RS0236	**ODN41**	TCCAAGTTTTTTCTAB	5251	5250
RS0464	**ODN42**	BTCCAAGTTTTTTCTA	-	-
RS0465	**ODN43**	TAGAAAAAACTTGGA2	-	-
RS0209	**ODN44**	2TAGAAAAAACTTGGA	5185	5170
RS0207	**ODN45**	TCCAAGTTTTTTCTA	4505	4510
RS0235	**ODN46**	TAGAAAAAACTTGGA	5196	-
RS0237	**ODN47**	ATTAGAAAAAACTTGGA	-	-
TB1164	**ODN48**	CGCAPTACGC	3012	3012
TB1165	**ODN49**	GCGTAATGCG	3068	3066

TB1175	**ODN50**	GCGTAGTGCG	3084	3083
TB1176	**ODN51**	GCGTACTGCG	3044	3042
TB1177	**ODN52**	CGCATTACGC	2988	2987
TB1277	**ODN53**	GCGTAAPGCG	3092	-
TB1278	**ODN54**	CGCGTTACGC	3004	3006
TB1346	**ODN55**	CGCCTTACGC	2964	2965
TB1299	**ODN56**	CGCDTTACGC	3003	3074
TB1300	**ODN57**	GCGTAAGGCG	3093	3094
TB1301	**ODN58**	GCGTAACGCG	3053	3054
TB1302	**ODN59**	GCGTDATGCG	3083	3153
TB1305	**ODN60**	CGCATGACGC	3013	3012
TB1306	**ODN61**	CGCATCACGC	2973	2974
TB1303	**ODN62**	GCGDATTGCG	3083	3154
TB1307	**ODN63**	CGCAATTCGC	2988	2990
TB1310	**ODN64**	GCGAATTGCG	3068	3070
TB1308	**ODN65**	CGCAATGCGC	3013	3015
TB1309	**ODN66**	CGCAATCCGC	2973	2975
TB1304	**ODN67**	CGCDTTACGC	3018	3159
RS0310	**ODN68**	GCGPAAPGCG	-	-
RS0311	**ODN69**	GCGPAAPGPG	-	-
RS0290	**ODN70**	GCGTAARGCG	3107	3107
RS0291	**ODN71**	GCGRAARGCG	3146	3145
RS0292	**ODN72**	GCGRAARGRG	3200	3198
RS0293	**ODN73**	GCGTAAEGCG	3127	3126
RS0294	**ODN74**	GCGEAAEGCG	3186	3184
RS0295	**ODN75**	GCGEAAEGEG	3260	3259
RS0312	**ODN76**	GCGTAATGTG	-	-
RS0298	**ODN77**	CACATTACGC	2972	2971
0064R	**ODN78**	GCGTAAJGCG	3097	3094
0065R	**ODN79**	GCGJAAJGCG	3126	3119

Modifications key to oligonucleotide sequences:

M = methyl red

Q = diaminoanthraquinone (DAQ)

B = disperse blue (DB)

X = C6 linker

H = hexaethylene glycol (HEG)

P = propyne-dU (p-dU)

R = propargylamino-dU (pa-dU)

E = 2'-aminoethoxy-dU (2'-aeo-dU)

D = diaminopurine phosphoramidite (DAP)

J = 2'-aminoethyl-dU (2'-ae-dU)

2 = FAM

3 = Cy3

4 = TAMRA

5 = Cy5

6 = Cy5.5

7 = ROX

8 = JOE

4.7 Fluorescence studies

All fluorimetry was performed on a Perkin Elmer LS 50B luminescence spectrometer. Oligonucleotides were diluted with 0.1M sodium phosphate buffer (pH 7, 0.1M NaCl, 1mM EDTA). Fluorescence cuvettes (3ml, quartz glass) were used in conjunction with a temperature probe and a Julabo waterbath heating unit. Slit widths of 5nm were used and temperature steps were made at 1°C intervals.

4.7.1 Fluorescence melting studies

An oligonucleotide containing a 3'-fluorescent moiety (0.1nmol) and a complementary oligonucleotide containing a 5'-quenching moiety (2 eq.) were added to a 3ml cuvette and diluted with sodium phosphate buffer. The cuvette was heated to 70°C and allowed to cool to 20°C during which time the oligonucleotides would anneal and fluorescence would be minimal. The cuvette was then heated from 20-70°C taking fluorescence readings at 1°C intervals. A curve of fluorescence vs temperature was created (figure 4.6.1)

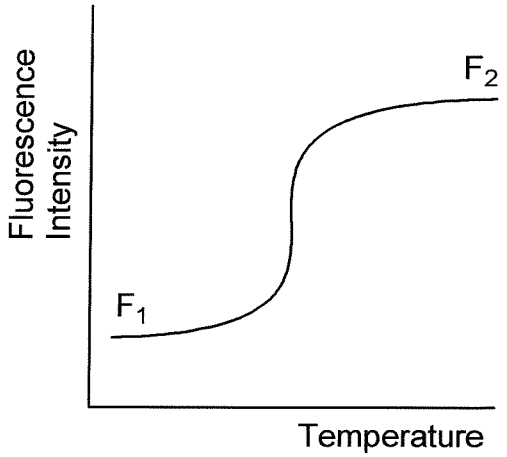


Figure 4.6.1: Schematic of fluorescence melt curve.

Signal-to-noise ratio could be calculated from this curve as F_2/F_1.

4.7.2 Molecular beacon studies

Molecular beacons (0.1nmol) were studied with and without the presence of target oligonucleotide (5 eq.). Molecular beacons were studied at a single temperature (in the case of DAQ beacons, section 2.3.5) and also over a range of temperature (DB beacons, section 2.5.3). All molecular beacons studied were of the same common sequence,

differing only by the fluorophores and quenchers incorporated. These beacons were designed to hybridise at relatively low temperatures, so they could be studied effectively at a single temperature of 30°C, or over a range of temperature from 20°C to 70°C. In all cases the signal-to noise ratios were calculated for comparison: (Fluorescence with target)/ (fluorescence without target) at a particular temperature.

4.7.3 Distance dependence of quenching studies

To study the effect of distance on the level of quenching, complementary oligonucleotide strands were designed with FAM on one oligonucleotide and DB at a number of positions on the other. Initially the fluorescent oligonucleotide (0.1nmol) was diluted with sodium phosphate buffer and studied by fluorescence. A quencher containing oligonucleotide (5 eq.) was then added and the fluorescence was studied again (figure 4.6.2).

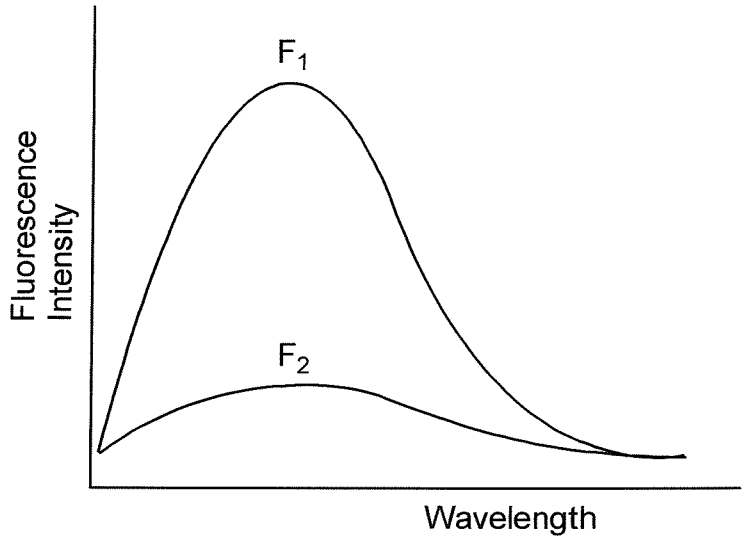


Figure 4.6.2: Schematic of fluorescent traces produced from study of quenching vs distance. F_1 = fluorescence without quencher oligonucleotide; F_2 = fluorescence with complementary quencher oligonucleotide present.

A value for the level of fluorescence quenching was calculated from the maximum values of these two fluorescence traces (F_2/F_1). This was calculated for each possible pairing of oligonucleotides and a graph displaying the fluorescence quenching vs base pair distance was plotted.

NB. 5 equivalents of quencher oligonucleotide was experimentally determined as being the most suitable quantity of quencher oligonucleotide to add.

4.8 TaqMan® real-time PCR

TaqMan® PCR reactions were carried out on an ABI Prism™ 7700 Sequence Detector. Total reaction volume was 50µl (all reagents supplied by Oswel Ltd.). The reaction mix contained 1x reaction buffer with ROX passive reference, used by the machine for normalisation of data. The buffer used for the probe that was labelled with ROX did not contain the passive reference, as this would normalise the probe signal. The reaction mix also included dNTP mix (200µM, including dUTP), 4mM $MgCl_2$, 1 unit of HOTGoldstar™ *Taq* polymerase and sterile water. Primers and probes were used at a concentration of 0.5µM and were designed to detect human β-actin. Human genomic DNA (30ng, Roche) were added to each reaction and this was replaced with sterile water for negative controls, run with each set of samples. Cycling conditions were standard TaqMan® conditions of: 95°C for 10 minutes (initial denaturation), then 40 cycles of 95°C for 15 seconds; 60°C for 1 minute.

4.9 Scorpion primer PCR

PCR reactions using scorpion primers were carried out on an ABI Prism™ 7700 Sequence Detector in the Oswel Sequencing laboratory. Methods, PCR cycling conditions and DNA loci are all described in the articles by Thelwell *et al*[102] and Solinas *et al.*[103]

4.10 UV-Melting

UV melting was performed on a PE UV/vis λ 2 spectrometer with PTP-1 temperature programmer. The two single stranded oligonucleotides to be melted were mixed (1:1, total 0.5 OD) and diluted with buffer (freshly made:100mM sodium phosphate, 1mM EDTA, 1M sodium chloride, pH7, which was filtered before use and was stored below 5°C). The solution was taken in a UV cuvette (pathlength 10mm) and heated to 80°C followed by slow cooling to 15°C, allowing efficient strand annealing. The sample was melted by heating at a rate of 1°C per minute and the cell monitored at 264nm at 10 second intervals. The data curves were smoothed and the first derivatives obtained using Perkin Elmer PECSS2 software. Melting temperatures (T_m) were calculated as follows:

$$T_m = [\{(T_{finish} - T_{start})/t_{finish}\} \times t_m] + T_{start}$$

where: T_m= melting temp. (°C t_m= time of melting (s)

T_{start}= start temp. (°C) t_{start}= start time (s)

T_{finish}= finish temp. (°C) t_{finish}= finish time (s)

All results were repeated, at least in triplicate (Appendix I) and averages were taken for comparison.

5. APPENDIX I - UV melting raw data

This appendix contains the readings taken during UV-melting studies, which lead to the average melting temperature values given in chapter 3. Further details of the oligonucleotides studied are given, which were used to calculate the volumes and concentrations necessary for thermal melting studies.

5.1 Data collected for p-dU (ODN48-52)

Oligonucleotide	Base	OD/ml	Conc. (nmol/μl)	Volume (μl)	No. moles (nmol)
ODN48 (TB1164)	p-dU	15.1	0.182	17	3.09
ODN49 (TB1165)	A	24.5	0.248	12	2.98
ODN50 (TB1175)	G	26.8	0.281	11	3.09
ODN51 (TB1176)	C	25.0	0.273	11	3.00
ODN52 (TB1177)	T	20.4	0.224	13	2.92

Oligonucleotide	**ODN48/ODN49**			**ODN48/ODN50**			**ODN48/ODN51**		
Temp. start (°C)	15.0	15.0	15.0	5.3	5.3	15.0	15.0	15.0	15.0
Abs. start	0.52	0.58	0.58	0.50	0.49	0.42	0.46	0.46	0.46
Temp. finish (°C)	75.0	74.9	75.0	66.7	66.6	75.0	75.0	74.9	74.9
Abs. finish	0.63	0.69	0.69	0.60	0.59	0.53	0.56	0.56	0.56
T_m (°C)	54.3	54.4	54.3	39.2	38.8	38.8	35.0	34.8	34.8

Oligonucleotide	**ODN52/ODN49**			**ODN52/ODN50**			**ODN52/ODN51**		
Temp. start (°C)	15.0	15.0	15.0	15.0	15.0	14.9	15.0	14.6	14.9
Abs. start	0.47	0.46	0.46	0.49	0.50	0.50	0.44	0.48	0.49
Temp. finish (°C)	75.0	75.0	75.0	74.9	75.0	74.9	74.9	74.9	74.9
Abs. finish	0.56	0.56	0.56	0.60	0.60	0.61	0.55	0.59	0.59
T_m (°C)	53.0	53.1	53.0	40.6	40.5	40.8	36.1	34.0	33.9

Figure 5.1: Oligonucleotide details and raw p-dU melting results for **ODN48-52**.

5.2 Data collected for p-dU (ODN49,52-55)

Oligonucleotide	Base	OD/ml	Conc.(nmol/μl)	Volume (μl)	No. moles (nmol)
ODN53 (TB1277)	p-dU	14.01	0.168	18	3.02
ODN54 (TB1278)	G	25.13	0.263	12	3.16
ODN52 (TB1177)	A	20.4	0.224	14	3.14
ODN49 (TB1165)	T	24.5	0.248	12	2.98
ODN55 (TB1346)	C	20.9	0.250	12	3.00

Oligonucleotide	**ODN53/ODN52**			**ODN53/ODN54**					
Temp. start (°C)	15.0	15.0	14.9	15.0	15.0	15.0	15.0	15.0	15.0
Abs. start	0.51	0.54	0.58	0.537	0.546	0.56	0.55	0.55	0.54
Temp. finish (°C)	74.9	75.0	75.1	75.2	75.1	75.1	75.2	75.1	75.2
Abs. finish	0.62	0.66	0.71	0.64	0.64	0.65	0.65	0.64	0.65
T_m (°C)	55.1	55.0	55.4	39.1	37.9	38.0	38.1	37.5	37.7

Oligonucleotide	**ODN52/ODN49**			**ODN54/ODN49**					
Temp. start (°C)	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0
Abs. start	0.64	0.67	0.51	0.56	0.57	0.57	0.60	0.60	0.61
Temp. finish (°C)	74.9	75.1	75.1	75.1	75.2	75.2	75.1	75.2	75.2
Abs. finish	0.77	0.79	0.62	0.66	0.67	0.67	0.71	0.71	0.71
T_m (°C)	53.5	53.4	53.4	39.0	38.9	38.8	40.0	39.1	39.6

Oligonucleotide	**ODN53/ODN55**				**ODN49/ODN55**				
Temp. start (°C)	15.0	15.0	15.0	15.0	14.9	14.9	15.0	14.9	15.0
Abs. start	0.51	0.52	0.52	0.52	0.56	0.56	0.56	0.56	0.58
Temp. finish (°C)	75.0	74.8	74.8	75.0	74.8	74.9	75.0	74.8	74.8
Abs. finish	0.61	0.61	0.61	0.60	0.64	0.65	0.64	0.65	0.65
T_m (°C)	34.0	34.3	33.9	33.5	31.9	30.9	32.5	30.2	31.3

Figure 5.2: Oligonucleotide details and raw p-dU melting results for **ODN49,52-55.**

5.3 Data collected for DAP (ODN49,52,56-58)

Oligonucleotide	Base	OD/ml	Conc. (nmol/μl)	Volume (μl)	No. moles (nmol)
ODN56 (TB1299)	DAP	9.2	0.119	27	3.22
ODN57 (TB1300)	G	41.3	0.407	8	3.26
ODN58 (TB1301)	C	40.9	0.420	8	3.36
ODN52 (TB1177)	A	20.4	0.224	14	3.14
ODN49 (TB1165)	T	24.5	0.248	12	2.98

Oligonucleotide	**ODN56/ODN49**			**ODN56/ODN57**			**ODN56/ODN58**		
Temp. start (°C)	15.0	14.9	15.0	15.0	14.9	14.9	15.1	14.9	14.9
Abs. start	0.55	0.58	0.59	0.51	0.50	0.54	0.55	0.56	0.54
Temp. finish (°C)	74.8	74.8	74.7	74.6	74.7	74.7	74.9	75.0	75.0
Abs. finish	0.69	0.70	0.71	0.60	0.60	0.64	0.643	0.65	0.63
T_m (°C)	56.0	56.2	56.1	38.2	38.7	38.0	24.9	25.4	26.9

Oligonucleotide	**ODN52/ODN49**			**ODN52/ODN57**			**ODN52/ODN58**		
Temp. start (°C)	15.0	14.9	14.9	15.0	15.0	14.9	14.9	15.0	15.0
Abs. start	0.61	0.62	0.62	0.60	0.61	0.61	0.56	0.52	0.52
Temp. finish (°C)	74.8	74.7	74.6	75.0	74.8	75.0	74.9	74.8	74.9
Abs. finish	0.71	0.72	0.72	0.71	0.72	0.74	0.65	0.61	0.62
T_m (°C)	53.5	53.4	53.4	43.2	42.7	42.8	26.9	26.6	25.7

Figure 5.3: Oligonucleotide details and raw DAP melting results for **ODN49,52,56-58.**

5.4 Data collected for DAP (ODN ODN49,52,59-61)

Oligonucleotide	Base	OD/ml	Conc. (nmol/µl)	Volume (µl)	No. moles (nmol)
ODN59 (TB1302)	DAP	35.3	0.42	7	2.94
ODN60 (TB1305)	G	38.4	0.41	7	2.87
ODN61 (TB1306)	C	41.1	0.46	6	2.76
ODN52 (TB1177)	T	20.4	0.22	13	2.86
ODN49 (TB1165)	A	24.5	0.25	12	2.98

Oligonucleotide	**ODN59/ODN52**			**ODN59/ODN60**			**ODN59/ODN61**		
Temp. start (°C)	15.0	15.0	14.9	15.0	14.9	15.0	15.0	15.0	15.0
Abs. start	0.36	0.36	0.33	0.37	0.35	0.31	0.573	0.672	0.51
Temp. finish (°C)	75.0	75.0	75.0	74.9	74.8	74.9	75.0	75.0	75.0
Abs. finish	0.46	0.46	0.43	0.46	0.44	0.39	0.68	0.780	0.57
T_m (°C)	54.8	55.0	55.0	37.0	37.5	37.5	38.0	38.2	38.2

Oligonucleotide	**ODN49/ODN52**	**ODN49/ODN60**			**ODN49/ODN61**		
Temp. start (°C)	see figure 5.3	15.0	15.0	15.0	15.2	15.0	15.0
Abs. start		0.43	0.44	0.45	0.59	0.59	0.63
Temp. finish (°C)		74.9	74.9	75.0	76.0	75.9	75.0
Abs. finish		0.51	0.52	0.54	0.66	0.66	0.71
T_m (°C)		38.6	39.6	40.3	31.4	31.2	30.5

Figure 5.4: Oligonucleotide details and raw DAP melting results for **ODN49,52,59-61**.

5.5 Data collected for DAP (ODN62-66)

Oligonucleotide	Base	OD/ml	Conc. (nmol/µl)	Volume (µl)	No. moles (nmol)
ODN62 (TB1303)	DAP	24.1	0.28	10.4	2.90
ODN65 (TB1308)	G	41.3	0.44	6.6	2.93
ODN66 (TB1309)	C	41.7	0.47	6.2	2.90
ODN63 (TB1307)	T	40.6	0.45	6.5	2.91
ODN64 (TB1310)	A	42.1	0.43	6.7	2.88

Oligonucleotide	**ODN62/ODN63**			**ODN62/ODN65**			**ODN62/ODN66**		
Temp. start (°C)	14.9	14.9	14.9	14.9	14.9	14.9	14.9	15.0	14.9
Abs. start	0.40	0.43	0.44	0.49	0.50	0.51	0.59	0.59	0.60
Temp. finish (°C)	74.8	74.9	74.9	74.8	74.8	75.0	75.0	74.9	74.9
Abs. finish	0.53	0.54	0.54	0.59	0.59	0.60	0.70	0.70	0.70
T_m (°C)	58.7	58.9	58.9	35.2	35.2	36.1	43.0	42.0	42.2

Oligonucleotide	**ODN64/ODN63**			**ODN64/ODN65**			**ODN64/ODN66**		
Temp. start (°C)	14.9	14.9	14.9	14.9	14.9	14.9	14.9	14.9	14.9
Abs. start	0.48	0.48	0.49	0.53	0.52	0.52	0.47	0.48	0.48
Temp. finish (°C)	74.9	75.0	75.0	74.9	75.0	75.0	74.9	75.0	75.0
Abs. finish	0.60	0.60	0.61	0.61	0.61	0.62	0.58	0.58	0.58
T_m (°C)	57.1	57.4	57.4	38.7	38.3	38.4	39.7	39.9	39.3

Figure 5.5: Oligonucleotide details and raw DAP melting results for **ODN62-67**.

5.6 Data collected for DAP (ODN49,67)

Oligonucleotide	Base	OD/ml	Conc. (nmol/µl)	Volume (µl)	No. moles (nmol)
ODN67 (TB1304)	di-DAP	23.7	0.38	10.0	4.00
ODN49 (TB1165)	T	24.5	0.25	16.0	4.00

Oligonucleotide	**ODN67/ODN49**			
Temp. start (°C)	14.9	14.9	14.9	14.9
Abs. start	0.57	0.58	0.59	0.56
Temp. finish (°C)	74.6	74.5	74.8	74.9
Abs. finish	0.70	0.71	0.70	0.71
T_m (°C)	57.9	58.1	58.2	58.5

Figure 5.6: Oligonucleotide details and raw DAP melting results for **ODN67,49**.

5.7 Data collected for amino group study (ODN49,52,68-77)

For an average absorbance of 0.5 OD for each duplex, approximately 2.3nmol of each oligonucleotide was used. Concentrations and volumes are given in the figure below. All T_m values were averaged for each particular duplex and comparison with the relevant unmodified duplex gave the change in T_m for each modified oligonucleotide duplex.

Oligonucleotide	Description	OD/ml	Conc. (nmol/µl)	Volume (µl)
ODN49 (TB1165)	Unmod. control 1	5.23	0.048	48
ODN52 (TB1177)	Complement 1	1.52	0.014	165
ODN53 (TB1277)	p-dU (single)	5.28	0.048	48
ODN68 (RS0310)	p-dU (double)	7.36	0.067	35
ODN69 (RS0311)	p-dU (triple)	5.62	0.051	45
ODN70 (RS0290)	pa-dU (single)	1.72	0.016	144
ODN71 (RS0291)	pa-dU (double)	2.19	0.020	116
ODN72 (RS0292)	pa-dU (triple)	0.99	0.009	257
ODN73 (RS0293)	2'-aeo-dU (single)	2.02	0.019	122
ODN74 (RS0294)	2'-aeo-dU (double)	3.24	0.030	77
ODN75 (RS0295)	2'-aeo-dU (triple)	1.60	0.015	154
ODN76 (RS0312)	Unmod. control 2	6.84	0.062	37
ODN77 (RS0298)	Complement 2	4.02	0.037	62

Figure 5.7: Oligonucleotide details for **ODN49,52,53,68-77**.

Unmodified control duplexes

Oligonucleotide	**ODN49/ODN52**			**ODN76/ODN77**		
Temp. start (°C)	20.0	20.0	20.0	20.0	20.0	20.0
Abs. start	0.46	0.46	0.46	0.71	0.72	0.73
Temp. finish (°C)	79.5	79.5	79.5	79.4	79.4	79.7
Abs. finish	0.56	0.56	0.57	0.83	0.84	0.86
T_m (°C)	48.4	48.6	48.6	54.5	54.3	54.7

p-dU

Oligonucleotide	**ODN53/ODN52**			**ODN68/ODN52**			**ODN69/ODN77**		
Temp. start (°C)	20.0	20.0	19.9	20.0	20.0	20.0	20.0	20.0	20.0
Abs. start	0.47	0.48	0.50	0.71	0.72	0.73	0.50	0.50	0.51
Temp. finish (°C)	79.3	79.3	79.7	79.4	79.4	79.7	79.1	79.2	79.3
Abs. finish	0.59	0.59	0.62	0.83	0.84	0.86	0.61	0.62	0.62
T_m (°C)	51.0	51.1	50.6	54.5	54.3	54.7	49.9	50.4	50.3

pa-dU

Oligonucleotide	**ODN70/ODN52**			**ODN71/ODN52**			**ODN72/ODN77**		
Temp. start (°C)	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
Abs. Start	0.47	0.47	0.48	0.48	0.48	0.49	0.48	0.48	0.49
Temp. finish (°C)	79.4	79.2	79.4	79.3	79.3	79.4	79.3	79.4	79.4
Abs. Finish	0.57	0.57	0.57	0.57	0.58	0.58	0.58	0.58	0.59
T_m (°C)	51.2	51.1	50.9	55.3	55.3	55.2	52.5	52.8	52.5

2'-aeo-dU

Oligonucleotide	**ODN73/ODN52**			**ODN74/ODN52**			**ODN75/ODN77**		
Temp. start (°C)	19.9	20.0	20.0	14.9	19.9	19.9	19.9	19.9	19.9
Abs. start	0.47	0.48	0.49	0.48	0.49	0.50	0.41	0.41	0.41
Temp. finish (°C)	79.3	79.4	79.3	74.1	79.9	79.9	79.3	79.3	79.3
Abs. finish	0.57	0.58	0.59	0.58	0.61	0.62	0.52	0.53	0.53
T_m (°C)	45.6	45.7	46.0	43.2	42.1	43.9	45.0	44.7	44.8

Figure 5.7: Melting results for study of oligonucleotides containing one, two or three modifications of either p-dU **(33)**, pa-dU **(39)** or 2'-aeo-dU.

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