UNIVERSITY OF SOUTHAMPTON

# Synthesis and Studies of Modified Nucleotides and Oligonucleotides

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ABSTRACT

FACULTY OF SCIENCE

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SYNTHESIS AND STUDIES OF MODIFIED NUCLEOSIDES AND OLIGONUCLEOTIDES

By Ruth McGuire

Large-scale genotyping programmes employ fluorescently-labelled nucleoside triphosphates as a means to facilitate detection. A novel protocol for the solid-phase synthesis of these compounds is described.

A series of nucleoside-5'-triphosphate analogues with structurally dissimilar linker arms is outlined. A variety of dyes were introduced to the terminus of the linker arms whilst attached to the solid support.

A series of PCR labelling experiments were performed to evaluate the incorporation of the labelled nucleoside triphosphate analogues by DNA polymerase enzymes. Good yields were obtained when replacing 40-90% of the unmodified dTTP with the modified dUTP analogues.

A simple, novel real-time PCR based assay, which is amenable to high throughput genotyping programmes is described. The assay requires a probe with a single fluorescent dye, two primers and triphosphate analogues labelled with a quencher molecule. Initially the system is fluorescent, however during PCR a decrease in fluorescence will be observed as the probe hybridises to the target and the fluorophore is quenched by the modified triphosphates incorporated into the nascent strand.

The synthesis of a Peptide Nucleic Acid monomer and a Carbocyclic nucleoside is outlined

**For Dad**

# Contents

# Abbreviations

| | |
|---|---|
| A | adenine |
| Å | angstrom |
| ABI | Applied Biosystems |
| Abs | absorbance |
| Ac | acetyl |
| Aq | aqueous |
| BSA | bovine serum albumin |
| *t*-Bu | tertiary butyl |
| Boc | *t*-butoxycarbonyl |
| C | cytosine |
| $^{13}C$ | carbon [NMR] |
| $CDCl_3$ | deuteriated chloroform |
| CPG | controlled pore glass |
| Cy | cyanine (dye) |
| δ | chemical shift (parts per million) |
| DCC | dicyclohexylcarbodiimide |
| DCM | dichloromethane |
| DIC | diisopropylcarbodiimide |
| DIG | digoxigenin |
| DIPEA | diisopropylethylamine |
| DMAP | 4-dimethylaminopyridine |
| DMF | *N*, *N*-dimethylformamide |
| DMSO | dimethylsulphoxide |
| DMT | 4, 4'-dimethoxytrityl |
| DNA | deoxyribonucleic acid |
| DNP | 2, 4-dinitrophenyl |
| DVB | divinylbenzene |
| dNTP | 2'-deoxynuceloside triphosphate |
| dU | 2'-deoxyuridine |

| | |
|---|---|
| ε | molar extinction coefficient |
| EDC | dimethylaminopropyl-3-ethylcarbodiimide |
| EDTA | ethylenediamine tetraacetic acid |
| ES | electrospray (mass spetrometry) |
| EtOAc | ethylacetate |
| EtOH | ethanol |
| F | fluorophore |
| FAM | 5(6) carboxyfluorescein |
| FISH | fluorescence *in situ* hybridisation |
| Fmoc | fluorenylmethoxycarbonyl |
| G | guanine |
| $^1$H | proton [NMR] |
| HEX | hexachlorinated fluorescein |
| HG | hypogel |
| HOBt | *N*-hydroxybenzotriazole |
| HPLC | high performance liquid chromatography |
| HRP | horseradish peroxidase |
| hr | hour |
| Hz | hertz |
| mer | nucleotides in length |
| MeCN | acetonitrile |
| MeOH | methanol |
| MeRed | methyl red (2-[4-dimethylamino-phenylazo]-benzoic acid) |
| Mp | melting point |
| NHS | *N*-hydroxysuccinimide |
| NMP | *N*-methyl pyrrolidone |
| NMR | nuclear magnetic resonance |
| OD | optical density |
| PAGE | polyacrylamide gel electrophoresis |
| PC | personal computer |
| PCR | polymerase chain reaction |

| | |
|---|---|
| PEG | polyethylene glycol |
| Ph | phenyl |
| PNA | peptide nucleic acid |
| ppm | parts per million |
| PS | polystyrene |
| Q | quencher |
| RNA | ribonucleic acid |
| RP | reverse phase |
| T | thymine |
| TBAF | tetrabutylammonium fluoride |
| TBDPS | *t*-butyldiphenylsilyl |
| TBS | *t*-butyldimethylsilyl |
| TCA | trichloroacetic acid |
| TET | tetrachlorinated fluorescein |
| TFA | trifluoroacetic acid |
| THF | tetrahydrofuran |
| TG | tentagel |
| Tlc | thin layer chromatography |
| Tm | melting temperature |
| Tris | tris(hydroxymethyl)aminomethane |
| U | uridine |
| UV | ultraviolet |
| Vis | visible |

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# Chapter 1

# Introduction to Nucleic Acids and their Detection

# 1.0 Introduction to Nucleic Acids and their Detection

## 1.1 Nucleic Acid Background

### 1.1.1 Primary Structure

The genes essential to all living organisms are comprised of DNA (deoxyribonucleic acids) or in the case of some viruses, RNA (ribonucleic acids). These nucleic acids are very long thread-like macromolecules made up of a linear array of either ribonucleotides (RNA) or 2'-deoxyribonucleotides (DNA). All nucleotides are constructed from three components: a nitrogen heterocyclic base, a D-ribose sugar and a phosphate residue (figure 1.1). The major bases are monocyclic pyrimidines, thymine (T), cytosine (C) and uracil (U) (where uracil replaces thymine in RNA) and bicyclic purines, adenine (A) and guanine (G). These are attached through either N1 (T/U or C) or N9 (A and G) of the base to the 1'-position of the sugar by a β-glycosidic linkage. Each nucleotide is linked to the next through the covalent linkage of the phosphate groups, attached to the 5'-hydroxyl of one ribose to the 3'-hydroxyl of the next. It is this 3'-5' phosphodiester bond which gives the polymer chain direction and at physiological pH, each phosphate exists as an anion.

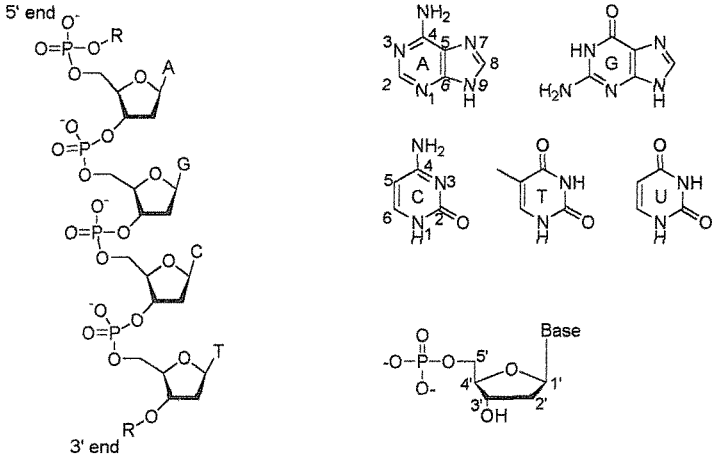



Figure 1.1 - The primary structure of DNA, the heterocyclic bases and a 2'-deoxynucleotide

### 1.1.2 Secondary Structure

In 1953, James Watson and Francis Crick elucidated the three-dimensional structure of DNA[1], known as the "double helix" and suggested structures for the two sets of base pairs. This research was based on the earlier x-ray diffraction data of Rosalind Franklin[2,3] and Maurice Wilkins[4,5] and information contained in Chargaff's rules. Chargaff[6] postulated that the ratio of adenine to thymine and guanine to cytosine was always unity and the number of purine bases was always equal to the number of pyrimidine bases. This gave rise to the classic Watson-Crick A-T, G-C hydrogen bonded base pairs. Therefore the sequence of one strand of DNA directly defines the sequence of another.

Watson and Crick described the secondary structure of DNA as two separate antiparallel chains of DNA coiled around an axis. This coiling produces a double helix that is right handed with the hydrophilic sugar-phosphate chain forming the external backbone and the hydrophobic heterocyclic bases stacking above one another to form the core of the helix. This α-helical conformation gives rise to a major and a minor groove. The two strands of the helix are held together by the hydrogen bonding of the individual base pairs and extra stability imparted on the duplex by π-π and other interactions between the stacked bases.

### 1.1.3 The Genetic Code

The mechanism through which the genetic information stored in DNA is relayed through RNA to a protein is termed the 'Central Dogma of Molecular Biology' (figure 1.2). The linear sequence of nucleotides in a gene specifies the sequence of amino acids in the resultant protein. This correspondence between the nucleotide sequence and the sequence of amino acids in a protein is termed the "genetic code". It is a triplet code in which three nucleotides (a codon) encode one amino acid. The genetic code is degenerate since eighteen out of the twenty amino acids are specified by more than one codon. This situation arises because there are sixty-four none

overlapping codons which comprise the genetic code, sixty-one coding for amino acids and three for stop codons.

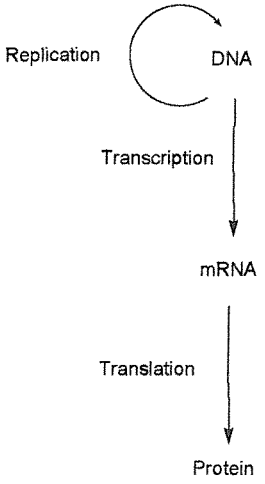



Figure 1.2 - The central dogma of Molecular Biology.

In order for the genetic code to be deciphered, double stranded DNA is transcribed into single stranded mRNA. The information carried by the mRNA is then used as a template during protein synthesis. The mechanism of protein synthesis, termed translation is a complex, three stage process, necessitating the co-ordinated interplay of mRNA, tRNA and ribosomes[7].

## 1.2 Detecting DNA

In order for DNA to be employed diagnostically, the nucleic acid sequence of the gene associated with a particular disease must be known. Secondly, a method for identifying the presence or absence of this nucleic acid sequence in an individual must be established. Nucleic acid hybridisation is the most powerful method for revealing and quantifying specific DNA or RNA and indeed this association of complementary strands has been widely exploited in the understanding of gene structure and function[8]. Used in conjunction with appropriate probe technology, nucleic acid hybridisation has emerged as the primary tool for applications in genetic

research, biomedical research and clinical diagnostics[9,10]. A probe is a short piece of DNA or RNA that is labelled to allow its detection subsequent to hybridisation.

## 1.2.1 DNA Diagnostics

Rapid developments in DNA technology, in particular the Polymerase Chain Reaction (section 1.4.3) have opened up the possibility of rapid, accurate, sensitive and economical diagnostic tests. They are theoretically capable of detecting a single organism in a clinical specimen and in practice are capable of sensitivities approaching that level[11,12,13,14,15].

Single nucleotide substitution, insertion or deletion mutations are permanent, heritable alterations in the base sequence of DNA. They arise either through spontaneous errors in DNA replication, meiotic recombination or as a consequence of the damaging effects of physical or chemical agents on the DNA. Such mutations are responsible for gene variants associated with genetic disease e.g. cystic fibrosis, sickle cell anaemia[16] and Alzheimer's disease[17]. These defects can be screened for directly or by probing for linked genetic markers coinherited with the mutant gene[18]. Analysis is important for pre-natal diagnosis, and for genetic counselling of affected families. Additionally, genetic information can be used to identify the likelihood of susceptibility of an individual to exogenous risks such as environmental factors or diet.

DNA probes are also used to clinically diagnose infectious diseases e.g. tuberculosis, measles, rubella, HIV[19] and hepatitis A,B and C, by allowing the detection of pathogenic organisms. Selection of a particular DNA sequence allows tailoring of a test to a particular disease i.e. HIV screening of donated blood samples.
The sensitivity and accuracy of forensic analyses have also greatly improved as a consequence of developments in gene probe technology. The identification of individuals at the DNA level is more accurate than traditional identification by blood

types, finger prints or physical characteristics. DNA can be extracted from the body fluids of an individual and used in paternity testing or criminal investigations where the highly variable microsatellite sequences of the human genome act as a 'barcode'[20] of an individual.

## 1.2.2 Classical Methods of DNA Detection – Radioactive labelling

In early work, DNA probes were labelled radioactively and this was achieved by the enzymatic incorporation of radioactively labelled nucleoside triphosphates[21, 22]. These enzymatic methods namely nick translation[23], random priming[24, 25] and PCR[26] (section 1.4.2) produce DNA or RNA molecules which are labelled uniformly or at the 3' end. DNA is labelled at the 5' end by means of bacteriophage T4 polynucleotide kinase which transfers the γ-phosphate of [γ-$^{32}$ P] ATP onto the 5' termini of DNA[27, 28].

The most common isotopes used are $^{125}$I, $^{32}$P, $^{35}$S and $^{3}$H. $^{32}$P and $^{35}$S are generally introduced into the phosphate ester or thioester respectively, of the nucleoside triphosphate, whereas other isotopes are more often introduced into the purine or pyrimidine bases[21, 22]. The choice of labelling strategy is determined by the nature of the probe (DNA/RNA, ds/ss) and the choice of the isotope depends mainly on the sensitivity, stability and resolution required.

| Isotope | $^{32}$P | $^{35}$S | $^{125}$I | $^{3}$H |
|---|---|---|---|---|
| *Half-life* | 14.3 days | 87.4 days | 60 days | 12.43 years |
| Achievable sensitivity | 2 pg target cm$^{-2}$ | 200 pg target cm$^{-2}$ | 100 pg target cm$^{-2}$ | 3000 pg target cm$^{-2}$ |

Figure 1.3 - The half-lives and achievable sensitivity of commonly employed isotopes[29] using nick translation.

An early example of the use of a radiolabelled DNA probe was described by Southern in 1975[30]. This technique known as Southern Blotting, allows the mapping of DNA fragments relative to restriction endonuclease sites. DNA is cut up into fragments by restriction enzymes; the restriction fragments are then separated, according to their size by agarose gel electrophoresis. The fragments are transferred to a nylon membrane and a $^{32}P$ labelled probe, complementary to the target DNA is introduced; after hybridisation and extensive washing, the bands complementary to the probe can be visualised by autoradiography.

An extension of the technique of Southern Blotting which allows the quantification of the amount of DNA or RNA in a sample, is known as dot blotting[31]. In this technique the labelled probe is hybridised to the target nucleic acid on the membrane as a dot. After careful washing, the intensity of the probe remaining on the membrane is measured and the reading directly corresponds to the amount of target sequence present in the sample.

Radiolabelling still remains one of the most sensitive methods of nucleic acid visualisation, achieving detection levels of down to 1.6 amol in hybridisation assays[21]. However despite the ease of incorporation of radiolabels there are numerous disadvantages associated with this technique:

i) hazardous handling: regulated by government and can be limited
ii) short half life of commonly used isotopes therefore short shelf life of probe
iii) expensive
iv) limited signal emissions preventing high detection
v) waste disposal issues

This has led to an increasing resistance to the use of radiolabelling which has resulted in the development of a wide variety of alternative methods.

## 1.2.3 Non – Isotopic Labelling of Nucleic Acids

Non-isotopic labelling involves the detection of either a colorimetric[32, 33], chemiluminescent[34], bioluminescent[35] or fluorescent[36, 37] signal. The signal is generated from the addition of the corresponding reporter group or label to the oligonucleotide probe. This provides the opportunity for multiple label incorporation per oligonucleotide[38] and also permits the simultaneous detection of several DNA targets within one experiment[17]. As a consequence of the progress made in non-isotopic labelling, significant developments in automated DNA sequence analysis have been made. Prober *et al*[39] used a different coloured fluorophore on each of the four dideoxynucleoside terminators thus allowing a whole sequencing reaction to be performed on a single lane of a polyacrylamide gel (section 1.5.3).

A label is a molecule capable of emitting a signal when attached to a protein, a nucleic acid or another molecule. Essentially it is comprised of three groups:

(i) the signal generating group (label)

(ii) the reactive group (anchor)

(iii) a spacer moiety

As highlighted in the schematic (figure 1 4).


Figure 1.4: The three components of a label

### *1.2.3.1 Requirements for a suitable label*[40]

In order for a compound to be suitable as a label for a DNA probe, it must satisfy the following criteria:

(i) coupling to the DNA probe must be simple and convenient

(ii) the signal generating properties of the compound should not be altered on attachment of the DNA probe

(iii) attachment of the compound should not affect the hybridisation properties of the oligonucleotide

(iv) detectable at low concentrations using simple instrumentation

(v) produce a hybridisation signal which is easily distinguishable from that of an unreacted signal.

(vi) allow simultaneous detection of several labels in one experiment

(vii) allow multiple addition of the compound to a single probe

(viii) stable to long term storage

(ix) easily disposed of

*1.2.3.2 The Signal Generating Group*[40]

The signal generating group or label must exhibit a sufficient quantum yield in aqueous solutions and must be stable to the hybridisation conditions employed. Additionally, it must be readily functionalised to incorporate the reactive group and to allow its physical properties to be modified for example, increase solubility in aqueous solutions or change emission characteristics such as wavelength and decay time. Such variability is a prerequisite for broad application of the label. Generally, the signal generating group is either a fluorescent dye for direct labelling (section 1.3.2) or an enzyme or hapten such as biotin for indirect labelling (section 1.3.1).

*1.2.3.3 The Reactive Group*[40]

The reactive or anchor group facilitates the covalent bonding of the label to the substance to be labelled, for example the oligonucleotide probe. There are several methods available for the covalent labelling of nucleic acids:

(i) Reaction with a free amino group on an oligonucleotide with *N*-hydroxysuccinimide esters, isothiocyanates or activated carboxyl groups of luminescent dyes to produce stable amide or thiourea bonds. The luminescent

dye can react with an intrinsic amino group of the heterocyclic bases or with an amino group which has been derivitised onto the 5' or 3' end of the oligonucleotide. This coupling reaction proceeds under mild conditions in aqueous solution (figure 1.5 entry 1,2)[41-45]

(ii) *Alternatively the oligonucleotide can be synthesised with a free thiol at either* the 3' or 5' terminus. This can react (Michael addition) with a α,β-unsaturated ketone attached to the luminescent dye. (figure 1.5 entry 3)[46]

(iii) *Also the luminescent label may be functionalised with an azido group which* upon activation can react with various functionalities within the oligonucleotide. As implied this results in non-specific labelling of the oligonucleotide[47, 48].

(iv) The luminescent label may be derivitised as a phosphoramidite which can be incorporated into the oligonucleotide during solid phase oligonucleotide synthesis. This method allows the oligonucleotide to be labelled internally or at the 3' or 5' termini (figure 1.5 entry 4)[33, 34, 49].

(v) Similar to method (i), the activated carboxyl group of the luminescent dye may react with functionality present on a nucleoside triphosphate. These labelled triphosphates are then incorporated into the oligonucleotide by enzymatic methods[50] (section 1.4.2).

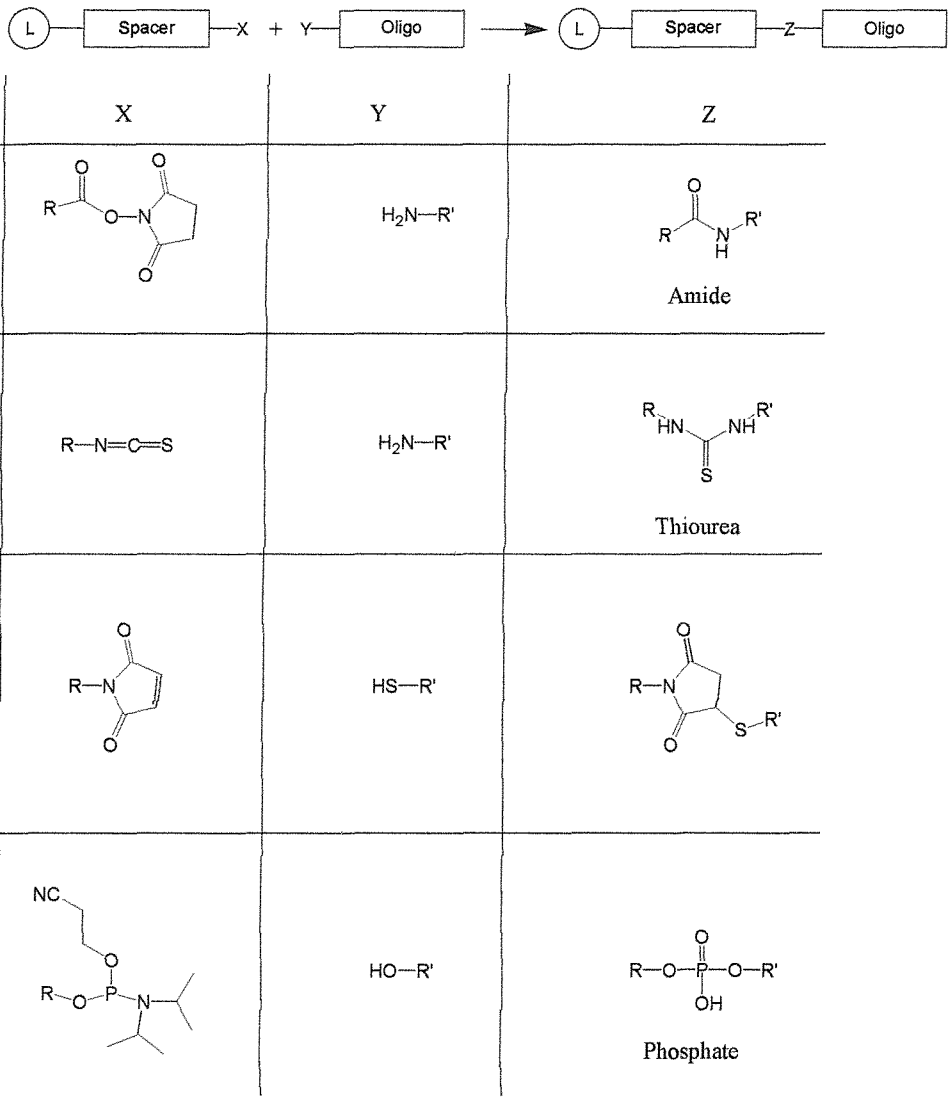



Figure 1.5 - Important methods of labelling oligonucleotides.

Non-covalent labelling of oligonucleotides is a less versatile technique than covalent labelling and as such is a less popular method. The intercalation of ethidium bromide in ds DNA is one example of non-covalent labelling.

### *1.2.3.4 The Spacer*[40]

Usually simple aromatic or aliphatic molecules are employed to separate the signal generating group from the substance to be labelled. This spacer unit is added to minimise any undesirable steric interactions between the label and the probe,

preserving the hybridisation properties of the probe. In addition the spacer unit must not interfere with the luminescent properties of the label. However, by careful choice of the spacer molecule, the hydrophobic or hydrophilic nature of the probe together with its flexibility may be changed.

## 1.3 Labelling Strategies

### 1.3.3 Indirect Labelling

Indirect labelling is a non-isotopic method of labelling and detecting DNA. In its most simple case, indirect labelling involves the covalent attachment of a small molecule onto a nucleic acid probe which is then detectable by a highly specific binding protein. The simplest practical example involves the incorporation of a hapten onto the probe which then forms a tightly binding, specific interaction with an antibody. This antibody is either covalently linked to a luminescent molecule or conjugated to an enzyme (horseradish peroxidase, alkaline phosphotase or β-D-galactosidase) which is capable of generating a signal (section 1.3.2.3). Since a detectable label has subsequently been introduced, the presence of the probe can be identified (figure 1.6 (i)). In a more complex, yet sensitive system, the primary antibody which binds to the probe is unlabelled. This is then recognised by a second, labelled antibody thus allowing the system to be detected. Signal amplification can occur as numerous secondary antibodies may bind to the primary antibody. This results in the production of multiple signals per probe.

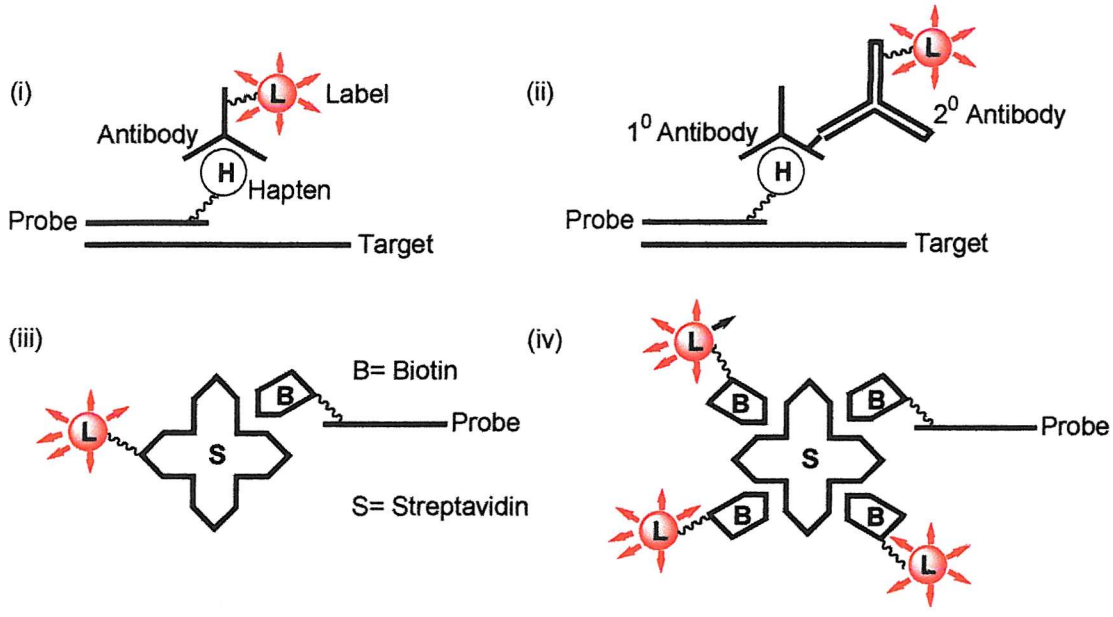



Figure 1.6 - Indirect labelling strategies.

The same concept can be realised using the most widely studied combination of biotin and avidin (or streptavidin)[52]. Biotin is introduced onto a nucleic acid probe by either chemical[53] or enzymatic[54] methods. It has a high binding affinity and specificity with the glycoprotein avidin or the non-glycosylated equivalent streptavidin which are again labelled with a luminescent group or enzyme (figure 1.6 (iii)). This interaction is stable over a range of pH and temperature conditions[52] and is exploited in the visualisation of nucleic acids. Signal amplification arises as avidin and streptavidin contain four biotin binding sites only one of which is occupied by biotin attached to the probe. Therefore multiple attachment of labelled biotin molecules would result in multiple signals per probe (figure 1.6 (iv)).

A drawback of using biotin as a label in nucleic acid hybridisation studies is that biotin is present at high levels in certain tissues, leading to high background signals. With such biological samples, an attractive alternative to biotin is the alkaloid digoxigenin[55] which can be detected using high affinity anti-digoxigenin antibodies[55, 56]. Another indirect detection system is the 2,4-Dinitrophenyl group (DNP) used with anti-DNP antibodies[57, 58]. This system benefits from the relatively small and chemically unreactive nature of the DNP group together with low background signals

since DNP is not a naturally occurring molecule. Other haptens used include *N*-acetoxy-*N*-2-acetylaminofluorene (AAAF) and its 7-iodo derivative (AAIF)[59, 60].

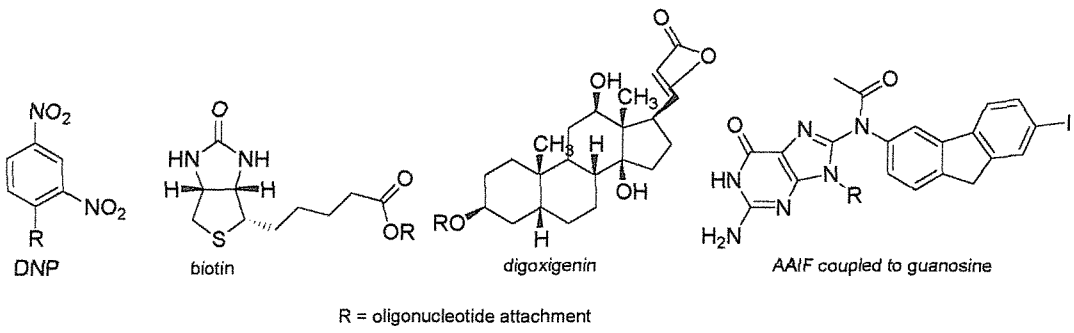



Figure 1.7 – Indirectly detectable primary labels.

## 1.3.4 Direct Labelling

In contrast to indirect labelling, direct labelling involves the covalent attachment of the label immediately to the nucleic acid probe (figure 1.8). Rapid and direct detection[61] occurs due to the specific molecular recognition of the probe annealing to its complementary target sequence. For this reason, the label must be stable to these hybridisation conditions and must not interfere with the hybridisation process. The nucleic acid probe can either be a cloned cDNA or genomic fragment, or a synthetic DNA oligonucleotide of defined sequence and length. The isolation and characterisation of cDNA probes is often time consuming and their use often depends upon practical considerations such as their availability and sequence homology to the target of interest[62]. Therefore, synthetic DNA probes are increasingly becoming one of the most used methods for analysing gene structure. Synthetic probes are advantageous as they can be easily prepared in large quantities and the probe length can be used to control the melting temperature of the probe-target duplex. Alteration of the hybridisation temperature can be used to favour formation of the desired duplex over mismatch hybrids, and can allow the assay to proceed at lower temperatures and with shorter annealing times.

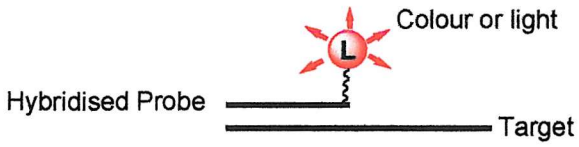



Figure 1.8 - Principle of Direct labelling.

There are various frequently used direct reporter groups such as fluorescent dyes or enzymes and the choice of a particular label to use for a gene probe is generally dependent on the sensitivity of detection required.

*1.3.4.1 Radioactive Labels*

For many applications radioactive labels are often employed as they offer the twin advantages of excellent sensitivity and compatibility with many labelling techniques. Especially high signal sensitivities are observed when the nucleic acid probe is multiply labelled. However, there are inherent disadvantages of radioisotope labelling (section 1.2.2).

*1.3.4.2 Chemiluminescent Labels*

Chemiluminescence is the emission of light by a substance as a result of a chemical reaction. More specifically, the enthalpy of the chemical reaction promotes an electron to a vibronically excited state. On decay of the electron from this excited state, a photon of light is emitted. Chemiluminescent molecules have been used to detect nucleic acids and offer the advantage of short exposure times, good sensitivity and resolution and the convenience of an X-ray film copy of the results. Practical examples of classics of chemiluminescent molecules employed in diagnostics include cyclic arylhydrazides, acridinium derivatives, stable dioxetanes and oxalic acid derivatives. Typical examples from each class are luminol used with horseradish peroxidase[63], lucigenin used with hydrogen peroxide and base[64], thermolysis of

adamantylideneadamantane-1,2-dioxetane[65] and oxalyl chloride used with hydrogen peroxide and a fluorescent dye[66] respectively.

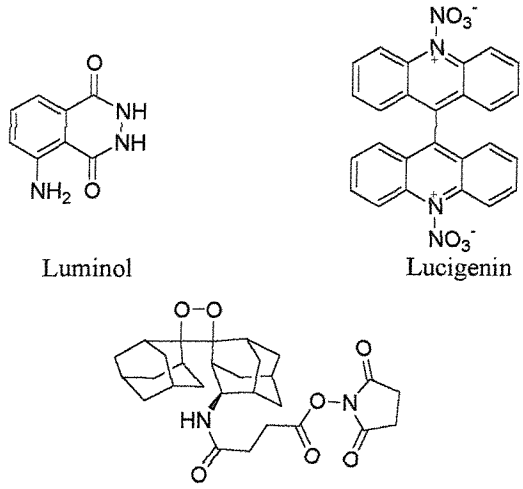



Figure 1.9 - Chemiluminescent molecules[48].

*1.3.4.3 Enzyme Labels*

The use of enzymes as labels presented the first alternative to radioactive labelling[67, 68]. Enzymes are covalently attached to nucleic acids and because enzymes produce multiple signal-generating species, an effective signal amplification mechanism is incorporated from the outset. The enzymes can be detected by the turnover of a substrate to produce colour or light (figure 1.10).

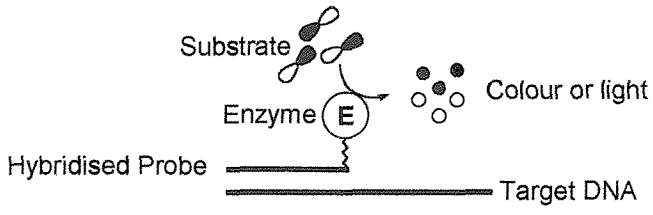



Figure 1.10 - Enzyme labelling and detection.

The three most important enzymes that are used as labels for DNA probes are horseradish peroxidase (HRP) (40kD), alkaline phosphotase (AP) (approx. 100kD) and β-D-galactosidase (GAL) (approx. 500kD). Alkaline phosphotase is used with either nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) or *p*-nitrophenyl phosphate to produce a coloured precipitate or solution respectively[69]. Horseradish peroxidase is typically used with diaminobenzidine (DAB) in the presence of hydrogen peroxide[40, 69], whereas β-D-galactosidase prefers 4-methylumberlliferyl compounds as substrates[40]. These enzymes have proven to be highly sensitive labels allowing rapid visualisation with low background signals. However, it is important that the activity of the enzyme is monitored to ensure that the conditions employed during hybridisation do not denature the enzyme.

*1.3.4.4 Lanthanide Labels.*

Time-resolved fluorometry with lanthanides, such as europium, as a label is a theoretically more sensitive labelling and detection method for DNA probes than radioisotopes[60]. Lanthanides can be bound to organic molecules by mediation of EDTA derivatives. These chelates are highly fluorescent with large Stokes shifts and exceptionally long lifetimes and this is the basis for time-resolved fluorometry[70,71]. Time-resolved fluorometry has been applied to the detection of hybridised DNA[60]. The method is based on the introduction of antigenic groups (i.e. AAIF) onto the DNA probe. After hybridisation with the target, visualisation occurs indirectly using a second antibody labelled with Europium (Eu) (figure 1.11). This type of immunochemical detection method is quantitative and the sensitivity is ten-fold compared to the corresponding enzymatic detection method.

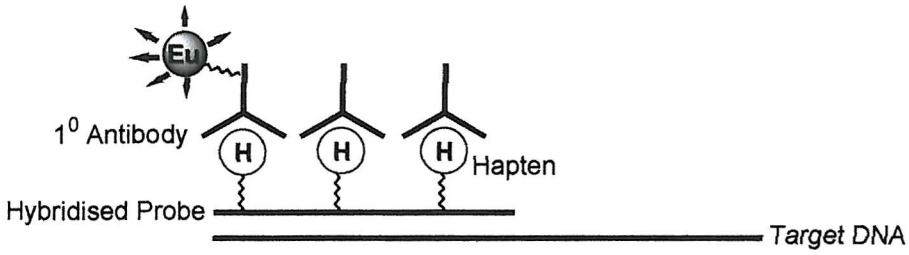



Figure 1.11 - Quantification of DNA hybrids by time-resolved fluorometry

Cytosine triphosphate has also been labelled with an europium carbonate chelate[91]. This process causes multiple labelling of the DNA, which can affect the efficiency of hybridisation by significantly altering the melting temperature of the probe-target duplex. It has been reported that the optimum system contains four to eight europium chelates per hundred bases. In principle the measurement of Eu-fluorescence is extremely sensitive detecting $10^{-16}$M solutions of Eu[72]. In practice, however, the sensitivity is dependent on signal to noise ratios.

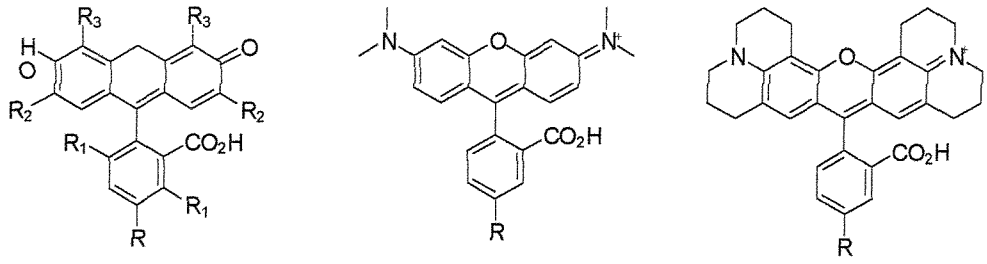
### *1.3.4.5 Fluorescent Labels*

Fluorescent labels have been used extensively for a wide range of applications in biology, biomedicine and analytical methods[73]. Fluorescent molecules absorb light of a specific wavelength and emit light of lower energy and longer wavelength. Nucleic acids have been labelled using direct and indirect strategies with a variety of different fluorescent molecules. Direct labelling with fluorescent dyes eliminates the additional steps required for antibody conjugation and, in principle, fluorescent measurements of the highest sensitivity are possible. However, in practice, the sensitivity can be limited by background fluorescence, light scattering and quenching effects[40]. In addition to these limitations, the potential sensitivity can be further compromised since there is no scope for signal amplification. There is a substantial range of commercially available fluorescent dyes and the excitation and emission wavelengths of a selection of dyes are represented in figure 1.12.

| Dye | Excitation Wavelength / nm | Emission Wavelength / nm |
|---|---|---|
| FAM | 500 | 525 |
| TET | 525 | 550 |
| HEX | 540 | 565 |
| TAMRA | 555 | 580 |
| ROX | 585 | 610 |
| BODIPY FL C3 | 502 | 510 |
| BODIPY FL-X | 503 | 510 |
| BODIPY TR-X C3 | 587 | 616 |
| Cy2 | 489 | 506 |
| Cy3 | 550 | 570 |
| Cy3.5 | 581 | 596 |
| Cy5 | 649 | 670 |
| Cy5.5 | 675 | 694 |
| Cy7 | 743 | 767 |
| R6G | 535 | 555 |
| Texas red | 584 | 603 |

Figure 1.12 - Excitation and emission maxima of a selection of fluorescent dyes.

The most popular fluorescent dyes include 6-carboxyfluorescein (FAM)[61, 74, 75] and its tetra and hexachlorinated analogues (TET and HEX). This is due to their high absorption and emission wavelengths. The use of fluorescein and its derivatives in various nucleic acid hybridisation and DNA sequencing applications has been extensively reviewed[21, 50]. However there are several disadvantages associated with the use of fluorescein derivatives. The broad nature of their emission peaks complicates the simultaneous detection of multiple sequences, they are sensitive to pH[76] and susceptible to photobleaching. The rhodamine dyes, carboxy-x-rhodamine (ROX)[77] and tetramethylrhodamine (TAMRA)[78] (figure 1.13) have reduced some of these disadvantages for example the fluoresence emission of rhodamine dyes is not strongly pH dependent. However the rhodamine dyes and the fluorescein based dyes are affected by autofluorescence of biological tissue yielding a low signal to noise ratio. The problems associated with the conventional fluorescent dyes have led to the development of alternatives such as the CyDyes™ and the BODIPY™ spectral range dyes[79].




$R_1 = R_2 = R_3 = H$, Fluorescein
$R_1 = R_2 = Cl$, $R_3 = H$, TET
$R_1 = R_2 = R_3 = Cl$, HEX

TAMRA

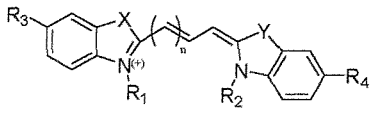
ROX

R = Oligonucleotide attachment point

Figure 1.13 - The conventional fluorescent dyes

Cyanine Dyes (CyDyes™)

These dyes were introduced by Amersham Life Science[80] and have the general structure shown in figure 1.14. They emit in the far red region of the visible spectrum, have high extinction coefficients, high quantum yields and excellent photostability making them ideal for use as fluorescent probes. The spectral properties (excitation and emission maxima 500-750nm) are selected by the appropriate choice of heterocyclic nuclei (X and Y) and the length of the polymethine chain (n). The groups $R_1$-$R_4$ are variable, providing the desired functionality, charge, reactivity and solubility and it is the addition of these groups that contribute to the high quantum yield. Cyanine Dyes[81, 82, 83, 84] are commercially available, pH insensitive and have proved to be popular for detecting proteins[85, 89], antibodies and nucleic acids, especially for *in situ* hybridisation applications (section 1.5.1).

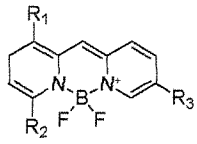



$R_1$-$R_4$ = variable substituent
n = 1,2,3.....
X and Y = S, O, NR, $C(CH_3)_2$

Figure 1.14 - Generic structure of a cyanine dye.

BODIPY™ Dyes

These dyes have been patented by Molecular Probes Inc. and have the general structure shown in Figure 1.15. Again the groups $R_1$ and $R_2$ are variable to either increase or decrease the solubility of the dye, or the level of conjugation[87] within the dye; this alters the spectral characteristics of the dye. Hence the BODIPY dyes are highly versatile and can potentially span the visible spectrum. The improved spectral characteristics and sensitivity have been exploited in DNA sequencing applications[88] and have found to be more economical.




$R_1$, $R_2$ = variable substituent
$R_3$ = linker

Figure 1.15 - Generic structure of a BODIPY dye.

However, some BODIPY dyes show instability under the conditions of PCR cycling.

Fluorescence Resonance Energy Transfer (FRET) Dyes

FRET requires the use of two dyes in conjunction: a donor and an acceptor. Dipole-dipole resonance between the dyes results in the transfer of excitation energy from one dye (donor) to the other (acceptor) and this leads to an overall improvement in the magnitude of the fluorescent signal emitted. Fluorescein and rhodamine dye derivatives are commonly used as donor and acceptor dyes respectively[89]. The use of FRET dyes[90-92] or 'big dyes' in DNA sequencing applications has led to improved results when compared to conventional dyes. In addition, FRET is an extremely powerful tool for probing nucleic acid structure[93] and hybridistaion[94].

However a major drawback of fluorescent dyes is their low fluorescence at high temperatures (> 50°C).

## 1.4 Label Incorporation

The methods available for the covalent labelling of nucleic acids has been summarised in section 1.2.3.3 however this will now be discussed in more detail.

### 1.4.1 Chemical Methods

Advances in solid phase synthesis techniques have resulted in the routine production of relatively large quantities (10-100nmol) of oligonucleotides that may possess a variety of modifications at the heterocyclic base, sugar or phosphate group[95]. The synthesis of labelled oligonucleotides by solid phase methods is advantageous as it enables the incorporation of a defined number of reporter groups at specific sites on the oligonucleotide. The label may be incorporated internally or at either the 5' or 3' end of the oligonucleotide (figure 1.16)

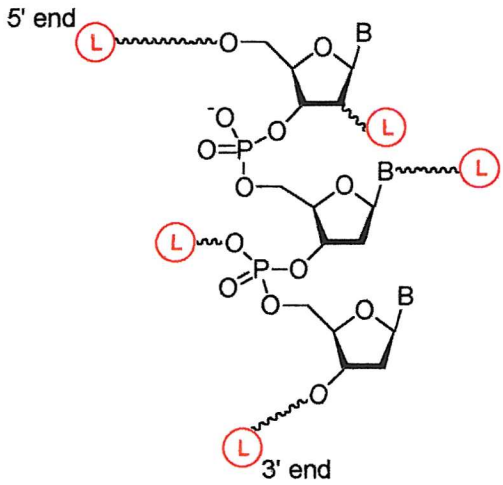



Figure 1.16 - Sites of label incorporation in an oligonucleotide.

However the main disadvantage associated with solid phase synthesis of labelled oligonucleotides is the lability of certain reporter groups to the conditions used during oligonucleotide synthesis and deprotection. In addition the poor solubility of some reporter groups in the solvents used during oligonucleotide synthesis can be problematic. In such cases, the reporter groups are often incorporated after oligonucleotide synthesis.

### *1.4.1.1 Post-synthetic labelling*

Post-synthetic derivatisation is the only method available for the modification of oligonucleotides that have been isolated from natural sources or have been synthesised enzymatically[96]. In this strategy, the reporter groups are coupled via the incorporation of a protected nucleophilic functionality during oligonucleotide synthesis. The nucleophile is generally introduced onto the oligonucleotide through a linker or spacer group (section 1.2.3.4). The linker serves to increase the accessibility of the nucleophile, and to increase the distance between the oligonucleotide and the reporter group[40] thus alleviating any potential steric problems.

A wide range of commercially available phosphoramidites are employed in this strategy but generally nucleosidic phosphoramidites, with nucleophile-linker modifications on the base are employed. These types of phosphoramidites cause minimal disruption to duplex formation and have the advantage of allowing multiple sites for label attachment[45]. Most commonly, the nucleophile attached to the linker is either an amine or a thiol.

Amino Functionalised Oligonucleotides

Initially, the most popular functionality used in post-synthetic labelling strategies was the primary amine. Labelling methods were originally developed from protein labelling protocols, which involved reaction at lysine residues. This allowed a versatile and efficient protecting group strategy to be employed. Use of the base labile

trifluoroacetyl[69], phthalimide[97] or Fmoc protecting groups allows the amino functionality to be unmasked during oligonucleotide deprotection. The amino group can then be reacted with reactive functional groups such as *N*-hydroxysuccinimide esters, or carboxylic acids used in conjunction with a carbodiimide coupling reagent[98]. This strategy has been employed to synthesise a cytidine phosphoramidite possessing a protected amino-alkyl linker at N4[69, 99] (figure 1.17 (i)). The resulting amino modified oligonucleotide was then labelled with biotin, a range of fluorescent dyes or by incorporation of a europium chelate.

More commonly, linkers are introduced at the C5 position of 2'-deoxyuridine as this site is not involved in hydrogen bonding in duplex DNA and therefore has least effect on hybridisation. There have been many 2'-deoxyuridine analogues synthesised containing a protected amino-alkyl linker[97], some of which are commercially available (figure 1.17 (ii)). These have been used to functionalise the resulting oligonucleotides which are then labelled with fluorescent dyes[83] and employed in DNA-enzyme interaction studies.

Amino-alkynyl linkers are also commonly used, for example Haralambidis[100] synthesised a 2'-deoxyuridine phosphoramidite (figure 1.17 (iii)) possessing a protected aminopropargyl linker at the C5 position. Again this was exercised in the synthesis of an amino-modified oligonucleotide that was labelled with the fluorescent dye fluorescein.

Both amino-alkyl and amino-alkynyl linkers have also been introduced at the N6 position of adenine and the N2 position of guanine[101]. However standard labelling protocols frequently employ modified pyrimindines. The use of labelled purines is less common as they are chemically less accessible.

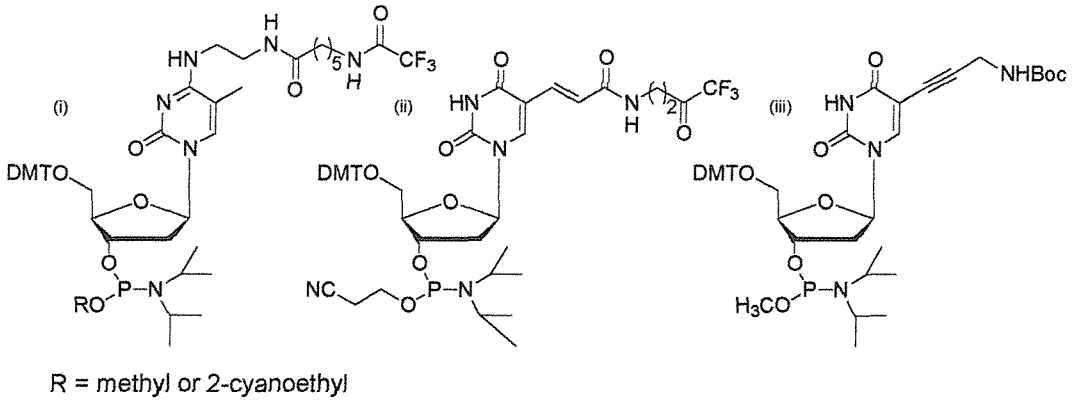



Figure 1.17: Amino modified phosphoramidites.

Thiol Modified Oligonucleotides

Thiols are more reactive than primary amines. However, an extra deprotection step is often required and the unmasked thiols are susceptible to oxidation to the disulfide. In a similar strategy to that employed for amino modified oligonucleotides, generally thiol modified pyrimidine phosphoramidites are used for oligonucleotide synthesis. The reporter groups are then incorporated into the oligonucleotides *via* derivatives bearing thiol-reactive functionalities such as maleimides, or bromo-, or iodo-acetamides. An example of a thiol modified phosphoramidite is 5-thiocyanato-2'-deoxyuridine phosphoramidite (figure 1.18). This was synthesised by Bradley and Hanna[102] and utilised in oligonucleotide synthesis. Treatment of the resulting oligonucleotide with dithiothreitol (DTT) yielded the unmasked thiol, which was then reacted with 5-iodoacetamidofluorescein.

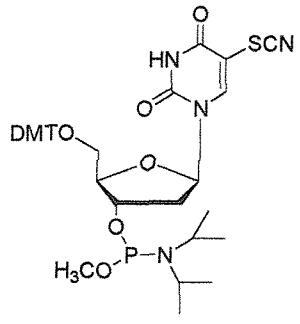



Figure 1.18 - Thiol modified phosphoramidite.

*1.4.1.2 End Labelling*

Oligonucleotides can be chemically modified with a hapten or fluorescent dye at the 5' or 3'-end in a two step procedure. In the first step, the oligonucleotide is synthesised with a functional group at either the 3' or 5'-end. This functional group must be inherently more reactive towards labelling reagents than the other reactive groups (phosphates, exocyclic amino groups) normally present in oligonucleotides. Generally, this functional group is a primary amine or thiol group that is introduced during oligonucleotide synthesis *via* an appropriately protected phosphoramidite. A variety of functionalised controlled pore glass (CPG) resins are commercially available for the introduction of an amine or thiol to the 3'-end of the oligonucleotide[101]. Non-nucleosidic phosphoramidites such as the commercially available 5'-amino modifiers[103] (figure 1.19), are commonly employed for 5'-modifications of oligonucleotides. In the second step, the reporter group is covalently attached to the oligonucleotide using a suitable derivatised label (section 1.4.1.1).

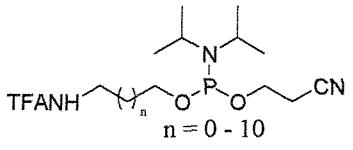



Figure 1.19 - Commercially available oligonucleotide 5' end modifiers.

Using this procedure Lenvenson and Chang[104] labelled oligonucleotides at the 5'-end with horseradish peroxidase (HRP). The method entailed the used of the hetero-bifunctional crosslinking reagent mal-sac-HNSA (maleimido-6-amino caproyl ester of 1-hydroxy-2-nitrobenzene-4-sulfonic acid sodium salt), which was used to introduce maleimidyl moieties to HRP. These could then be reacted with the thiol derivatised oligonucleotide (figure 1.20).

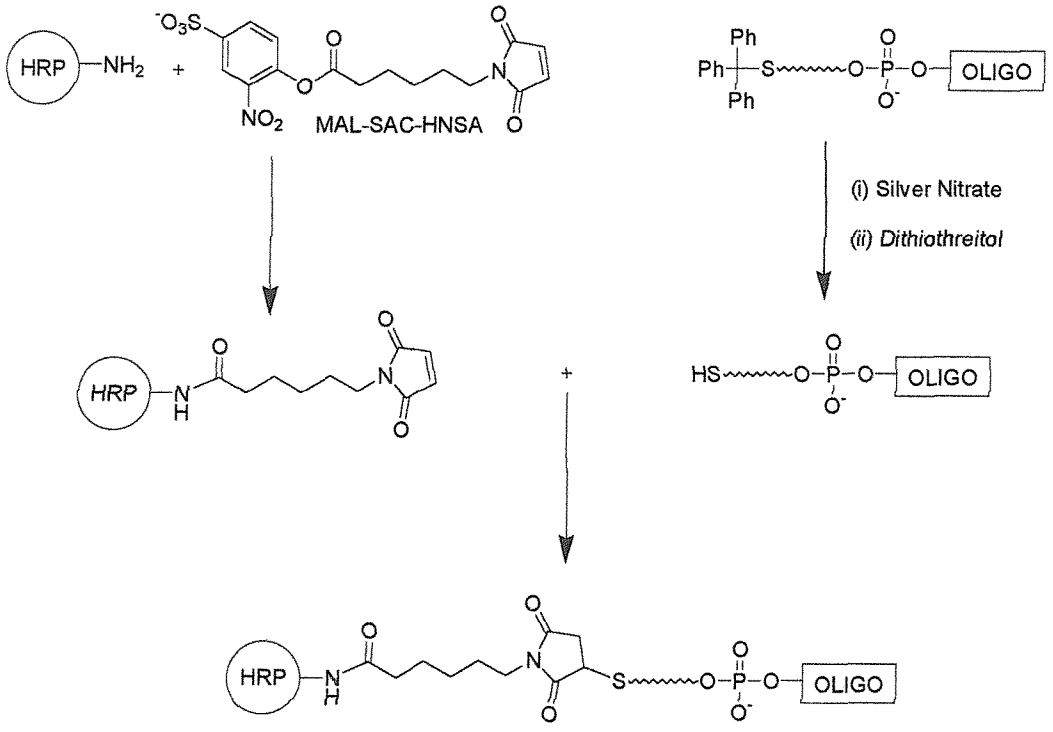



Figure 1.20 - 5'-End labelling of an oligonucleotide with HRP

Nucleic acid probes synthesised in this manner can be obtained in large quantities (100nmol per reaction); they have a high degree of specificity and good sensitivity. Another useful feature of 5' end-labelled oligonucleotides is that the 3'-end is available to act as a primer for DNA synthesis reactions[10]. Therefore, extension reactions such as PCR (section 1.4.3) can be conducted with labelled primers, allowing the non-radioactive detection of the reaction products.

### *1.4.1.3 Direct incorporation of a label*

The direct incorporation of a label during oligonucleotide synthesis obviates the need for post-synthetic labelling and subsequent purification steps. In general, the addition of the label proceeds more efficiently than by post synthetic methods[95], through the coupling of a labelled phosphoramidite monomer during oligonucleotide synthesis.

Various different reporter groups, in particular fluorescent dyes have been incorporated in this manner.

Most commonly the synthesis of the labelled phosphoramidite monomer, involves the introduction of a linker onto the heterocyclic base of the nucleoside in a similar fashion to that described in section 1.4.1. Likewise attachment of the label proceeds via reaction of a NHS ester or maleimide derivatised label. Nucleoside phosphoramidites possessing base modifications have been utilised in the incorporation of fluoresceinyl[106] labels either internally or at the 5'end. Non-nucleosidic functionalised CPG resins possessing a fluorescent label are commercially available[103] for 3'-end labelling of an oligonucleotide. Alternatively, a functionalised CPG resin derivatised with a fluoresceinated nucleoside analogue[107] may be used. Biotin labelled phosphoramidites have also been adopted to label oligonucleotides at the 5'-end and these have then been used in the affinity purification of PCR products[69].

## 1.4.2 Enzymatic Methods

The enzyme-mediated incorporation of labels into DNA is based on the enzyme-catalysed incorporation of modified 2'-deoxynucleoside-5'-triphosphates (dNTPs)[♦] into ss or ds DNA and synthetic oligonucleotides[50]. The modified triphosphates are either radiolabelled, or labelled with fluorescent dyes or haptens such as biotin or digoxigenin. With non-isotopic labelling, the reporter group is generally attached to the heterocyclic base of the nucleotide in a strategy similar to that described for nucleoside phosphoramidites (section 1.4.1.3). These reporter groups are relatively large and often possess a number of potentially reactive functionalities. The addition of these sterically bulky groups onto a dNTP can limit the utility of the nucleotide as an enzyme substrate. Therefore in some cases, for example when using an enzyme as

[♦] Specific dNTPs are referred to using the letters for each base (i.e. dATP). Modified dNTPs are referred to using the nomenclature 'reporter group'-'linker length'-dNTP (i.e. biotin-4-dUTP).

a label, enzymatic methods for label incorporation are not suitable. However enzymatic incorporation produces probes of the highest sensitivity[105] and so by including the naturally occurring dNTP in the labelling reaction, reasonable product yields[50] may be obtained when using non-isotopic labels. There is often a fine balance between product yield and labelling density and this is determined by the ratio of modified to unmodified (naturally occurring) nucleoside present in the labelling reaction[108].

A variety of approaches are possible for the enzymatic incorporation of reporter groups into DNA. The choice of approach is dependent on the type of labelling required: internal labelling or end-labelling.

### *1.4.2.1 Internal Labelling*

Internal or uniform labelling of DNA probes can be obtained by either random priming, nick translation or PCR (section 1.4.3) or by reverse-transcription from an RNA template (c-DNA synthesis).

Random Priming[50]

Random priming synthesis produces uniformly labelled DNA of high specific activity. The procedure initially developed by Feinberg and Vogelstein[26] uses random short oligonucleotide sequences, typically hexamers, as primers in a polymerisation reaction which is catalysed by *E.coli* DNA polymerase I (figure 1.21). With this straightforward method, superior levels of label incorporation are observed, generating DNA probes of extremely high specific activity from small amounts of template DNA.

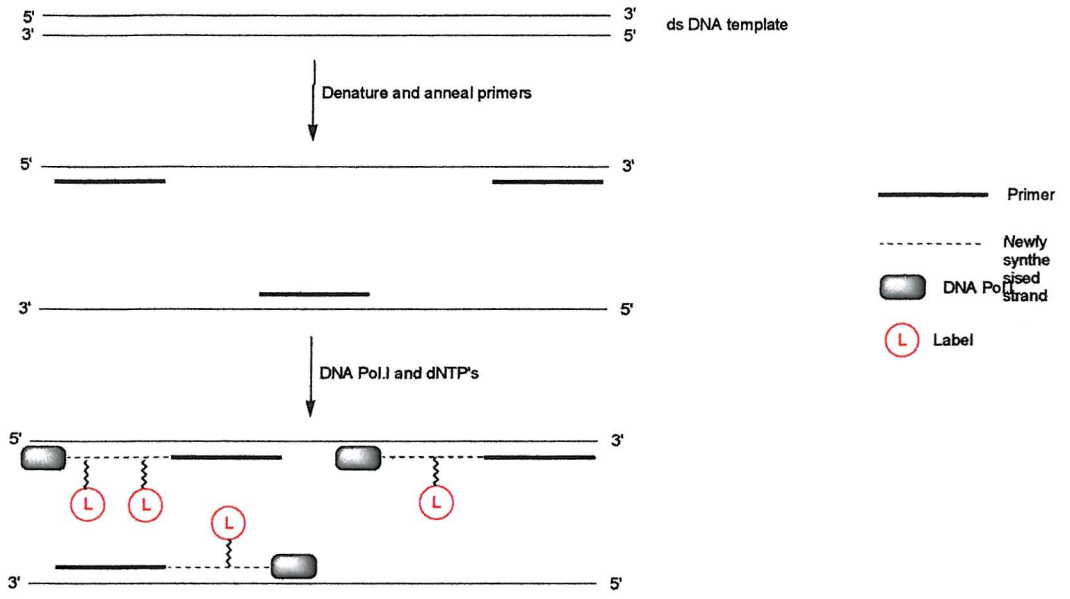



Figure 1.21 - DNA labelling by random priming.

The technique has been used to synthesise DNA labelled with biotin[9], digoxigenin[109] and fluorescein[60] *via* the incorporation of the respective modified dUTP analogue. There is also evidence to suggest that this technique is more tolerant to bulky dNTP analogues than other techniques. Waggoner *et al*[82] successfully synthesised a DNA probe in good yield with a high labelling density using only the modified dCTP analogue (no naturally occurring dCTP) in the random priming reaction mixture. Such an approach was not possible with this dCTP analogue in other labelling techniques.

Nick Translation[50]

*Traditionally, Nick Translation has been the most frequently employed technique to* uniformly label dsDNA[23] as it is rapid, straightforward and relatively inexpensive. The DNA template is nicked at a series of positions along the strand, usually by the nuclease enzyme DNase I. This is then incubated with *E.coli* DNA polymerase I and a mixture of labelled and unlabelled dNTPs. Beginning at a nicked site, the 5' to 3' exonuclease activity of the enzyme removes nucleotides from the 5' phosphoryl terminus whilst synchronously the 5' to 3' polymerase activity of the enzyme

introduces new nucleotides at the 3' hydroxyl terminus. The net effect is the replacement of unlabelled nucleotides. Since DNase I cuts the DNA template randomly, nicks are introduced into both strands, resulting in uniform labelling of the DNA (figure 1.22).

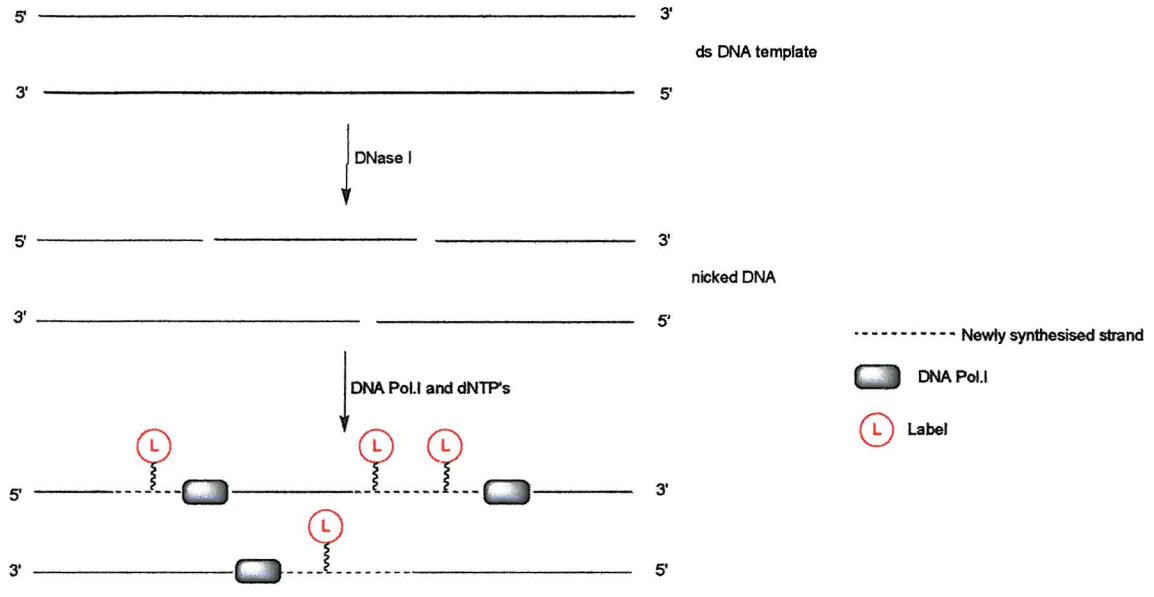



Figure 1.22 - DNA labelling by Nick Translation.

The specific activity of the nick-translated DNA is proportional to the amount of nucleotides replaced by labelled nucleotides. This is controlled by optimising the concentration of DNase I and the reaction time. In general, short incubation times or low concentrations of DNase I result in insufficient label incorporation. In practice, conditions are chosen that result in 30% to 60% incorporation of the modified nucleoside. This technique initially used by Langer *et al*[54], is frequently used to prepare sequence-specific probes for a wide variety of hybridisation techniques. Weigant *et al*[110] utilised fluorescein, tetramethylrhodamine and coumarin labelled dUTP analogues in the synthesis of the oligonucleotide probes employed in fluorescence *in situ* hybridisation (section 1.5.1). Cyanine dyes have also been introduced into DNA in this manner, using Cy3-dUTP and Cy5-dUTP, by Waggoner *et al*[81,82].

*1.4.2.2 Enzymic-End Labelling*

The 5' end of DNA is labelled using the enzyme T4 polynucleotide kinase to catalyse the transfer of the terminal γ-phosphate of ATP to the 5' hydroxyl termini of DNA, RNA and oligonucleotides. There are two different reactions employed to achieve the 5'-end labelling of DNA. In the forward reaction, the DNA is firstly dephosphorylated with a phosphatase enzyme (usually bacterial or calf-intestinal phosphatase). The T4 polynucleotide kinase then transfers the [γ-$^{32}$P] of ATP to this dephosphorylated 5' terminus. The second reaction, termed the exchange reaction, is carried out in the presence of excess ADP. This causes the polynucleotide kinase to transfer the terminal 5' phosphate from phosphorylated DNA to ADP. The DNA is then re-phosphorylated by the transfer of the [γ-$^{32}$P] of ATP by the polynucleotide kinase enzyme. The exchange reaction is less efficient than the forward reaction and is therefore less commonly used. Oligonucleotides that are unphosphorylated at the 5'-end can be labelled by this method.

There are three different enzymes used for the 3'-end labelling of DNA: terminal deoxynucleotidyl transferase, *E.coli* DNA polymerase I and T4 DNA polymerase. The choice of enzyme employed is dependent on the nature of the DNA to be labelled. Terminal deoxynucleotidyl transferase and T4 DNA polymerase react preferentially at the 3' hydroxyl termini and therefore require a 3' overhang or 5' recessed end of the DNA. Whereas *E.coli* DNA polymerase I reacts at the 5'-phosphoryl termini and therefore requires a 5' overhang or 3' recessed end.

Terminal deoxynucleotidyl transferase

This enzyme provides a unique method for labelling the 3'-end of DNA. Terminal deoxynucleotidyl transferase catalyses the non-template directed repetitive addition of deoxynucleotides to the 3'-hydroxyl termini of the DNA, preferably ss DNA or short oligonucleotides, accompanied by the release of inorganic phosphate[111]. In this way a short 'tail' of modified nucleotides can be attached to the strand to create a labelled probe. The action of the enzyme was first exploited by Moyzis *et al*[112] who

incorporated biotin-11-dCTP into DNA. The enzyme can also incorporate one single label by using a modified ddNTP such as a digoxigenin-labelled ddUTP which was employed by Kessler *et al*[56] for labelling DNA for use in hybridisation experiments.

*E.coli* DNA polymerase I

The Klenow fragment of *E.coli* DNA polymerase is the enzyme used in this method. This enzyme fragment possesses the 5' to 3' polymerase and the 3' to 5' exonuclease proof reading activity of the holoenzyme but lacks the 5' to 3' exonuclease activity. The DNA to be labelled is partially digested by the appropriate restriction enzyme, producing recessed 3'-ends. The recessed ends are then filled in by the Klenow fragment by catalysing the incorporation of labelled dNTPs. This procedure is commonly employed for synthesising labelled DNA fragments for use as size markers during gel electrophoresis. Langer, Waldrop and Ward[54] used this method to label DNA with biotin by the incorporation of bio-4-dUTP.

T4 DNA polymerase

The T4 DNA polymerase enzyme is a product of bacteriophage T4. This method of label incorporation utilises both the 5' to 3' polymerase and 3' to 5' exonuclease activity of the enzyme. Incubation of the DNA with T4 DNA polymerase in the absence of dNTPs degrades the 3'-ends of the DNA by the 3' to 5' exonuclease activity of the enzyme. Introduction of the dNTPs at this stage results in extension from the 3'-end by the polymerase activity of the enzyme. Hence, both 3'-ends of a linear duplex DNA molecule can be selectively and extensively labelled using modified dNTPs (figure 1.23). Indeed, dUTP analogues have been successfully incorporated by T4 DNA polymerase[54].

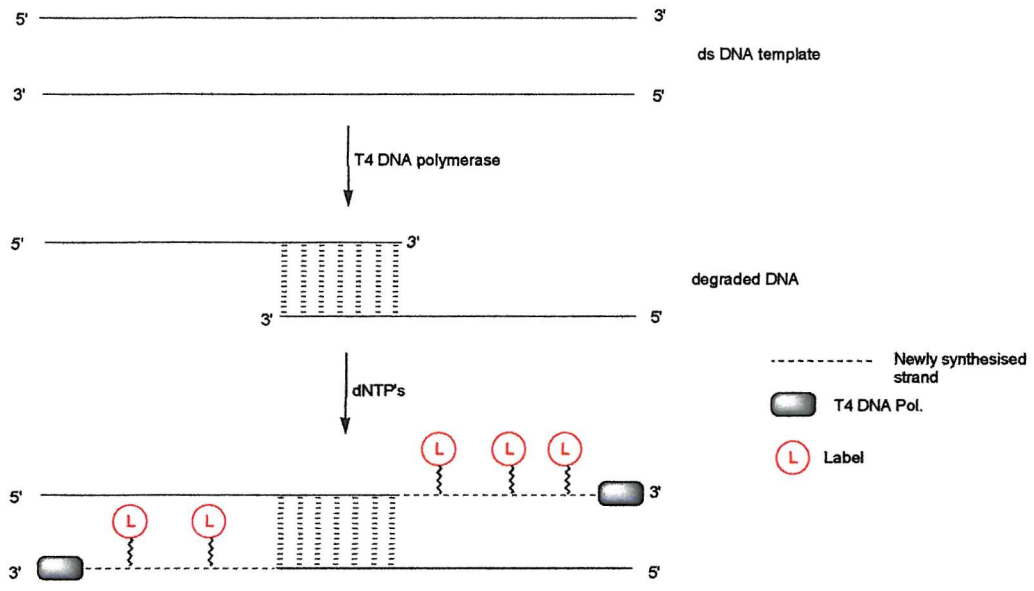



Figure 1.23 - Synthesis of labelled DNA by T4 DNA polymerase.

Subsequent digestion of the labelled DNA produced, with an appropriate restriction enzyme, allows the selective removal of the label from one of the 3'-ends.

## 1.4.3 PCR

The Polymerase Chain Reaction (PCR) is an *in vitro* method of nucleic acid synthesis by which a particular segment of DNA (the target) can be specifically replicated. The technique was first introduced by Saiki *et al*[113] in 1985 and has become an essential tool in molecular biology as an aid to cloning and gene analysis[114]. In order to amplify a section of the target DNA during PCR, sequence information must be known of the areas that flank the DNA fragment to be amplified. Two short oligonucleotide primers, typically 18-30 nucleotides in length each of which is complementary to one end of the target strand are prepared (usually synthetically). These primers are designed to have similar G and C content so that they anneal to their complementary sequences at similar temperatures. They are also designed to anneal on opposite strands of the target sequence so that they will be extended

towards each other by addition of nucleotides to their 3'-ends. The region of the template bound by the primers is amplified by a series of cycles (figure 1.24). In the first cycle, the duplex target is heated to 95°C to denature the strands. Subsequent cooling to approximately 55°C allows the primers to anneal to the template strands. The actual temperature depends on the primer lengths and sequences[114]. The temperature is then slightly elevated to the optimum temperature for activity of the DNA polymerase enzyme, for example 72°C for the thermostable *Taq* polymerase. The DNA polymerase, in the presence of $Mg^{2+}$ ions, then extends the primers along the length of the target by incorporating dNTPs to their 3' ends until the beginning of the second cycle. This produces two new ds sections of DNA, which are denatured in the second cycle by heating to 95°C. Each single strand then acts as a template for primer annealing and extension. During the second cycle, polymerisation can only occur as far as the end of the first primer. This affords newly synthesised molecules of the correct length. During subsequent temperature cycles only products of the correct length are amplified which soon outnumber the original target molecule and increase two-fold with each cycle. Theoretically, after n cycles, one target sequence would be amplified $2^n$ times. In practice, generally 20-40 cycles are used.

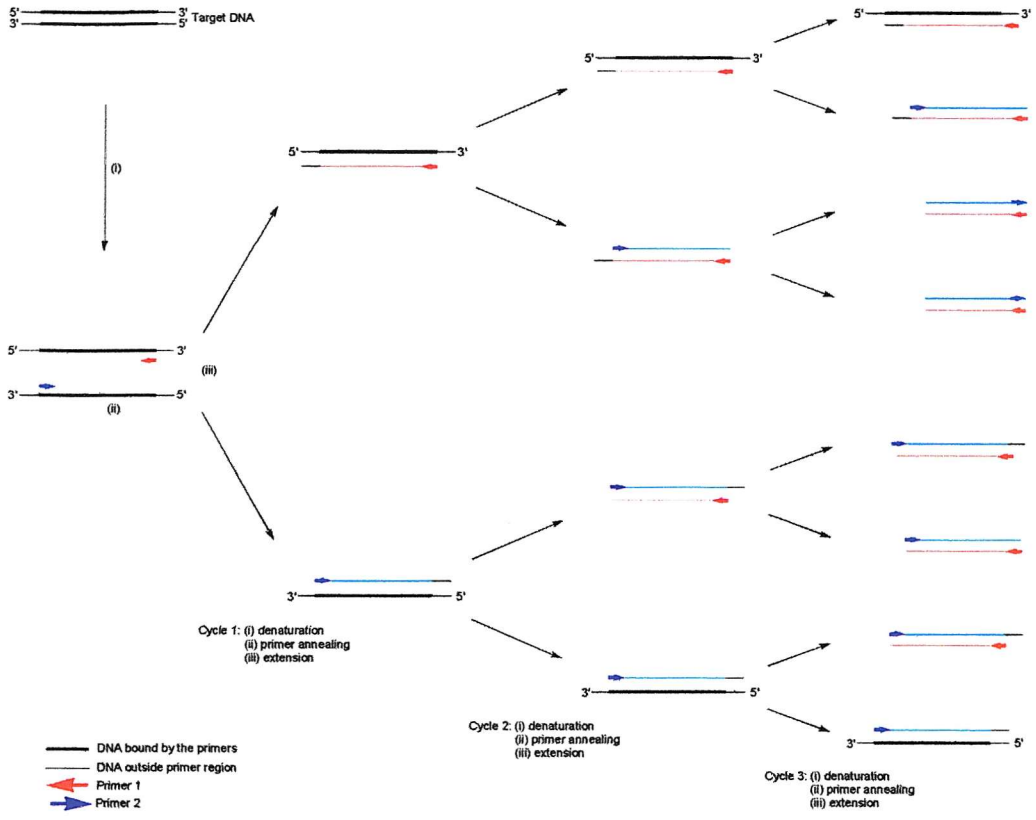



Figure 1.24 - The first three cycles of a PCR reaction[123].

The initial PCR protocols[113, 115] used the Klenow fragment of the mesophilic DNA polymerase I to catalyse polymerisation. This necessitated multiple additions of an aliquot of the enzyme during each cycle of the PCR. Being labour intensive and expensive limited the application of the technique. The discovery of thermostable polymerases obtained from thermophilic organisms such as *Taq* DNA polymerase isolated from *Thermus aquaticus*[116], greatly simplified the technique. The use of thermostable polymerase facilitated automation of PCR through the use of thermal cyclers. A wide variety of DNA polymerases are commercially available for use in PCR[117] which provide variation in properties such as processivity, replication fidelity and associated activities. This has widened the potential of PCR which has found wide spread use in many fields including forensic analysis[118], clinical diagnosis[119], oncology[119] and various research areas[120]. Such popularity has led to significant

modifications to the original protocol[119-122]. These included quantitative PCR[123], *reverse transcriptase PCR (RT PCR)* [124, 125] *and label incorporation by PCR.*

*1.4.3.1 Label incorporation by PCR*

Label incorporation during PCR provides an alternative to traditional methods for the synthesis of labelled probes for use in hybridisation assays. Additionally, the incorporation of labels into PCR products has facilitated the development of reverse probe hybridisation assays[119](figure 1.25) and the direct detection of PCR products without the need for probe hybridisation.

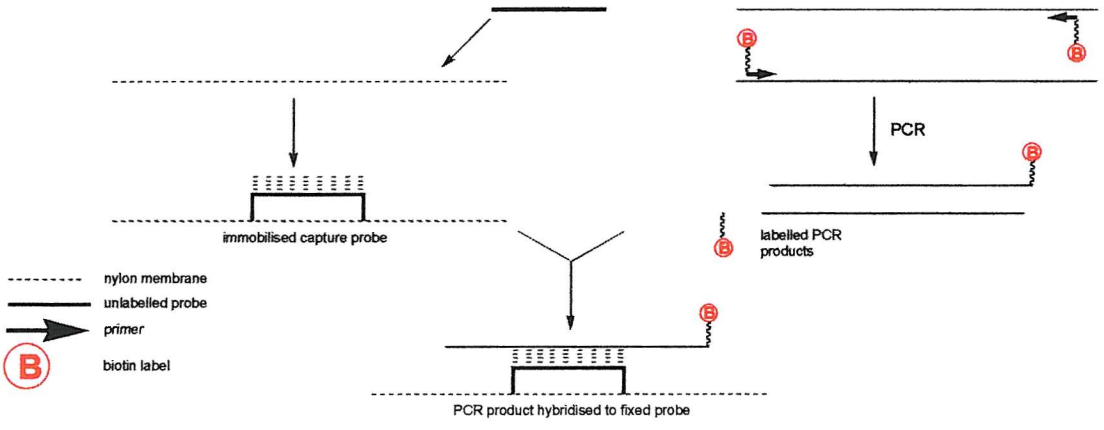



Figure 1.25 - Reverse probe hybridisation assay

PCR products may be labelled during synthesis by either incorporation of labelled dNTPs during the amplification reaction or by introduction of a label onto the PCR primer. However, the addition of modified substrates into the PCR mixture can result in decreased yields and a reduction in reaction specificity[126].

Incorporation of labelled dNTPs

The principle of PCR labelling by the incorporation of modified dNTPs involves the total or partial replacement of a naturally occurring dNTP with a labelled analogue during PCR (figure 1.26). The use of modified dNTPs obviates the need to synthesise

labelled primers and has the potential to produce PCR products with a greater labelling density than when using modified primers. However, the synthesis of labelled PCR products by this method often results in a greater reduction in PCR efficiency[126].

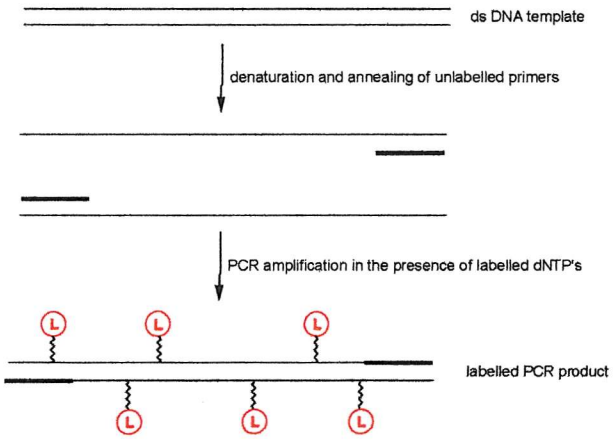



Figure 1.26 - Incorporation of labelled dNTPs during PCR amplification.

Woolford and Dale[127] described a protocol for the synthesis of fluorescently labelled PCR products, by performing the PCR reaction in the presence of a mixture of dTTP and fl-12-dUTP. This enabled the direct detection of the PCR product by the use of a UV transilluminator, without the need for ethidium bromide staining. This labelling strategy has also been employed to incorporate rhodamine[89] and cyanine[108] labelled dUTP analogues. These labelled PCR products have been used for various research and diagnostic purposes.

In some cases the labelling reaction may be performed without inclusion of the natural dNTP in the reaction mixture. PCR products have successfully been labelled with 5-BrdUTP[128] and subsequently detected using an anti-BrdU antibody conjugated to peroxidase. This analogue which has a similar size to dTTP is incorporated by *Taq* DNA polymerase as efficiently as the natural substrate.

An alternative strategy involves incorporation of a modified dNTP into a crude PCR reaction after amplification[126]. Incubation of the reaction mixture at room temperature

for approximately 16 hours produced a labelled product. However inferior results have been obtained using this strategy compared to incorporation of dNTP analogues during PCR.

Labelling of PCR primers

The introduction of reporter groups into PCR products through the use of labelled primers has the least detrimental effect on the efficiency of the PCR reaction[126]. PCR primers can be labelled internally, at the 5'-end or more unusually at the 3'-end. Internal labelling offers the potential of multiple label incorporation, however 5'-end labelling is more convenient and is thought to have least effect on annealing of the primers to the template[60] (figure 1.27).

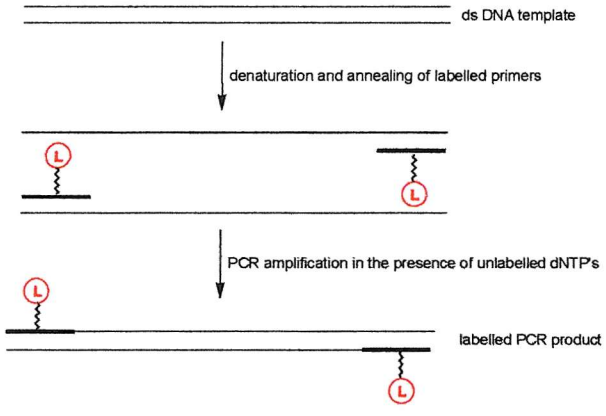



Figure 1.27 - Labelling of PCR products through use of labelled primers.

Various different reporter groups have been introduced into the PCR primer internally during solid phase synthesis. Ganesh *et al*[106] incorporated various different fluoresceinated 2'-deoxyuridine phosphoramidites into the primer to introduce 2,4 or 5 fluorescent labels into the PCR product. However, it was found that a maximum of 4 fluorescent labels, (2 per primer) could be introduced before non-specific amplification occurred. PCR primers have also been labelled post-synthetically with fluorescent dyes by reaction with the corresponding aryl azide derivative[129]. This places the fluorescent label at random positions along the oligonucleotide primer.

Both cyanine dyes[82] and biotin[127] have been used to label PCR products *via* 5'-end labelled primers. Biotin-labelled primers have been employed in PCR reactions where they have subsequently been used for affinity purification of the product[130]. Since this application does not require multiple labelling, incorporation of a single label at the 5'-end is the most effective method to use.

## 1.5 Practical Applications of Fluorescent Probe Systems

### 1.5.1 Fluorescence *In Situ* Hybridisation (FISH)

Fluorescence *In Situ* Hybridisation is a microbial method that allows the detection of whole-bacterial cells and the analysis of chromosomes *via* the labelling of specific nucleic acids with fluorescently labelled oligonucleotide probes. Clinical and fundamental research have both benefited from the application of FISH techniques, which have become an indispensable cytogenetic tool. FISH is the most powerful method for gene mapping and has significantly enhanced the field of cytogenetic diagnosis[140]. The principle of the technique is simple and is based on the annealing of a labelled probe to its complementary strand in fixed cells or tissues followed by detection of the label. FISH is different to other probe systems as the target is embedded in a complex matrix that can hinder probe access and destabilise the probe-target hybrids formed. In addition, it is important that the target is accessible to the probe and must be retained *in situ*, not degraded by nucleases. The labelled probe (DNA or RNA) is usually prepared by either chemical synthesis, nick translation, random priming or PCR (sections 1.4.1, 1.4.2 and 1.4.3). The length of the probe is dependent on the particular application however the typical length is between 200bp and 1kb. Shorter probes decrease the efficiency of detection of a hybridisation site however longer probes increase non-specific background signals. High probe concentrations decrease the signal : noise ratio.

There are three different types of FISH probes routinely employed, each of which has a different application.

### *1.5.1.1 Locus specific probes*

Locus specific probes hybridise to a particular region of a chromosome. This type of probe is used to determine the location of a gene on a chromosome when a small portion of that particular gene has been isolated. A labelled probe is prepared from the piece of the gene allowing the visualisation of the chromosome to which the probe hybridises.

### *1.5.1.2 Alphoid or centromeric repeat probes*

Alphoid or centromeric repeat probes are generated from repetitive sequences found at the centromeres of chromosomes. These probes are ideal for the detection of aneuploidy (abnormality of chromosome number) by FISH and probes exist for almost all human chromosomes. They produce intense zones of fluorescence on the chromosomal target region and provide an efficient method for detecting aneuploidies because the signals can be easily evaluated by conventional microscopy. Alphoid probes have been used successfully to detect aneuploidies in amniotic cell fluids[141] and specific tumours such as gliomas, medulloblastomas and carcinomas of the breast and bladder[141, 142, 143].

### *1.5.1.3 Whole chromosome probes*

Whole chromosome probes are collections of smaller probes, each of which hybridises to a different sequence along the length of the same chromosome. Using these libraries of probes, entire chromosomes may be 'painted' or stained generating a spectral karyotype. Structural changes in defined chromosomes such as microdeletions, inversions and translocations may be analysed by FISH using these

specific probes[140]. The applications of FISH using human chromosome-specific libraries have significantly increased in recent years. This is due to the convenience, efficiency and the growing availability of these 'painting' probes. Chromosome painting[145, 146] by combinatorial or ratio labelling of specific probes has led to the staining of each of the twenty four different human chromosomes with distinct colours which can provide a general screening test for chromosome abnormalities.

## 1.5.2 DNA Chip Technology

DNA chip technology utilises high-density microscopic arrays of nucleic acids immobilised on solid support for biochemical analysis. The technique originated from the automation and miniaturisation of 'dot-blotting' (section 1.2.2) that showed how nucleic acid hybridisation could be used on a large scale to exploit the data emerging from genome programmes[147]. DNA microarrays are distinguished from dot blots by the use of an impermeable, rigid substrate, such as glass, which has a number of practical advantages over porous membranes and gel pads[148]. Glass is a durable material that sustains high temperatures and washes of high ionic strength and due to its low intrinsic fluorescence, it does not significantly contribute to background noise. In addition, the surface of glass is easily derivatised allowing the covalent attachment of oligonucleotides. High density DNA probe arrays are prepared by combining the techniques of photolithography and solid-phase DNA synthesis (figure 1.28) Synthetic linkers modified with a photochemically removable protecting group are attached to the glass support. Light is then directed through a photolithographic mask to specific areas on the surface to produce localised photodeprotection. These sites are then coupled with a series of protected deoxynucleosides. Next, light is directed to a different region of the support by a new mask and the chemical cycle is repeated. In this manner thousands of oligonucleotides can be fixed to a small grid producing a high-density DNA probe array.

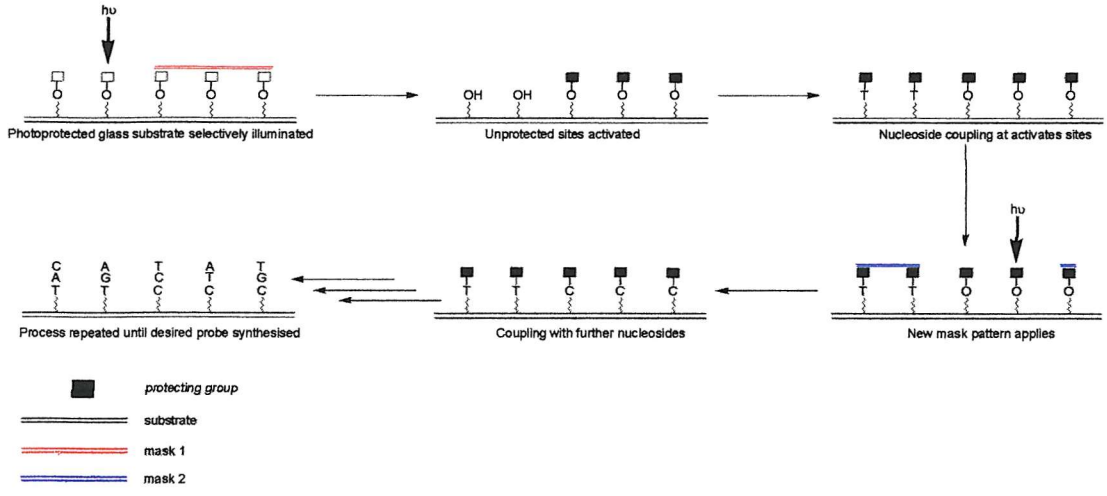



Figure 1.28 - Preparation of a DNA probe chip[161].

Such gene chips are extremely beneficial in the area of diagnosis. A genetic sample, either DNA or RNA samples from biological sources are labelled with a fluorescent dye during PCR amplification (section 1.4.3). Hybridisation of these targets to the DNA microarray and subsequent detection can be used for DNA sequencing, genotyping and polymorphism detection. Analysis of the CFTR gene was performed by the preparation of a microplate loaded with 428 features to identify mutations in exon 11 and a microarray containing 96,600 20mers was used to identify mutations over the entire 3.45kb of exon 11[150].

## 1.5.3 DNA Sequencing

The use of fluorescent dyes in automated DNA sequencing provides another important application of enzymatic label incorporation (section 1.4.2). The Sanger[151] method of DNA sequencing is the most commonly used method for DNA sequencing, particularly in large scale genomic sequencing. It involves the template directed linear primer extension with a DNA polymerase enzyme in the presence of a mixture of deoxy and dideoxy nucleoside triphosphates. Termination of the primer extension results whenever ddNTPs are incorporated due to the absence of a 3'

hydroxyl group. Hence incubation with a mixture of dNTPs and ddNTPs results in a series of truncated primer extension products corresponding to chain termination occurring at each base. These products are then analysed by polyacrylamide gel electrophoresis.

Parallel with label incorporation during PCR (section 1.4.3), fluorescent reporter groups may be incorporated into the sequencing products *via* labelled primers, or *via* labelled dNTPs during primer extension or labelled ddNTPs during chain termination. The incorporation of labels into the sequencing products via a labelled ddNTP terminator as demonstrated by Prober *et al*[39] (figure 1.29) has a number of advantageous over the other methods. The major advantage is convenience. Synthesis of a labelled primer is not necessary hence fewer synthetic steps and reagents are required. Also since a different dye can be used to distinguish each base terminator, only one primer extension reaction is required and each sequencing reaction can be performed on a single lane of a gel. To fully exploit this more convenient approach, protocols have been established using a variety of dye labelled terminators and polymerase enzymes that produce favourable results in sequencing reactions. Dye terminators have been labelled with various different fluorescein and rhodamine[152] *derivatives and* BODIPY[165] *dyes. Energy-transfer dye terminators*[152, 154] *have also* been developed which have been incorporated into sequencing products producing improved results when compared with conventional dye terminators.

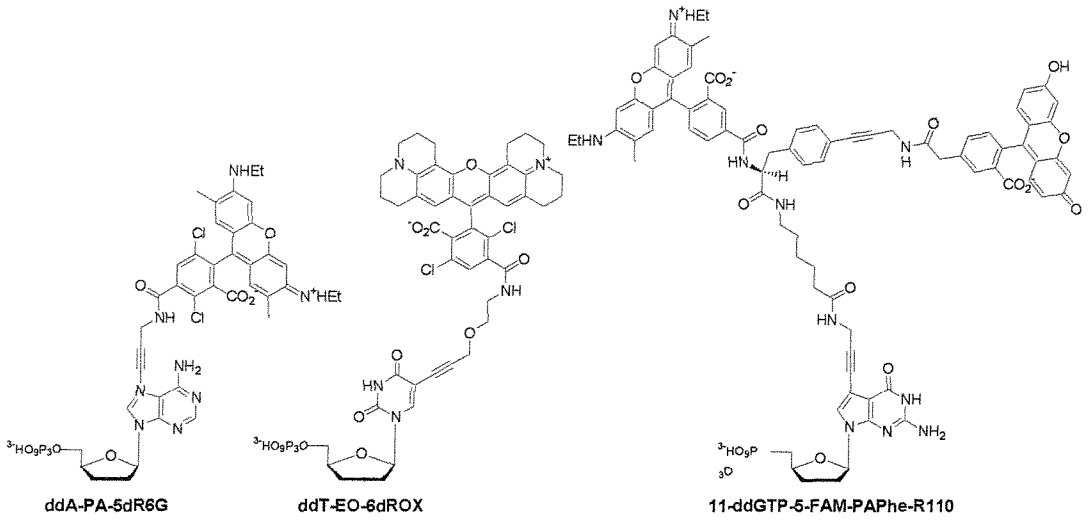



Figure 1.29 - Examples of fluorescent base terminators

The use of modified deoxy and dideoxy-nucleoside triphosphates in automated DNA sequencing has, in turn, led to developments in the number of polymerase enzymes available for this, and other, applications. There are now a number of engineered DNA polymerases used in sequencing protocols[117], such as Taq FS[155], and the *thermosequenase*[156]. *Use of these mutated polymerases has resulted in an improved* incorporation of dideoxynucleoside triphosphates.

Dye-labelled primer sequencing has benefited from the development of DNA polymerases which do not discriminate between deoxy and dideoxynucleosides[157]. Traditionally primers have been labelled at the 5'-end with fluorescent reporter groups such as fluorescein[39], rhodamine[158], BODIPY[158] and cyanine[159] dyes. *Fluorescent resonance energy transfer dyes (section 1.3.2.5) have also given* favourable results in sequencing experiments[160] compared to primers containing a single dye. DNA sequencing has also been performed using primers labelled at the 3'-end. CPG resins derivatised with a protected fluorescent moiety have been employed in the solid phase synthesis of 3'-end labelled primers. Alternatively primers may be labelled internally during a primer extension reaction in the presence of a labelled dNTP. The major disadvantage of the dye labelled primer method is the requirement

for four separate extension reactions and four dye-labelled primers for each template[152].

## 1.5.4 Taqman™ Assay

The Taqman™ assay is extensively exploited for the detection of accumulation of PCR specific products[131,132]. It requires the synthesis of a linear oligonucleotide probe which is labelled with a fluorogenic donor (5' FAM) and acceptor (internal or 3' TAMRA) molecule. Irradiation of this intact probe stimulates emission of a photon by the donor, which is quenched by the acceptor through the process of FRET (section 1.3.2.5). Hence no fluorescence is observed. However, the probe can hybridise to a complementary target and during the polymerisation stage of PCR, the probe will be cleaved. Cleavage of the probe arises due to the inherent 5' to 3' nucleolytic activity of Taq DNA polymerase. This cleavage causes separation of the donor and acceptor molecules that results in an increase in fluorescence intensity due to loss of quenching. Measurement of this increase in fluorescence intensity directly indicates the generation of probe specific amplicons (figure 1.30).

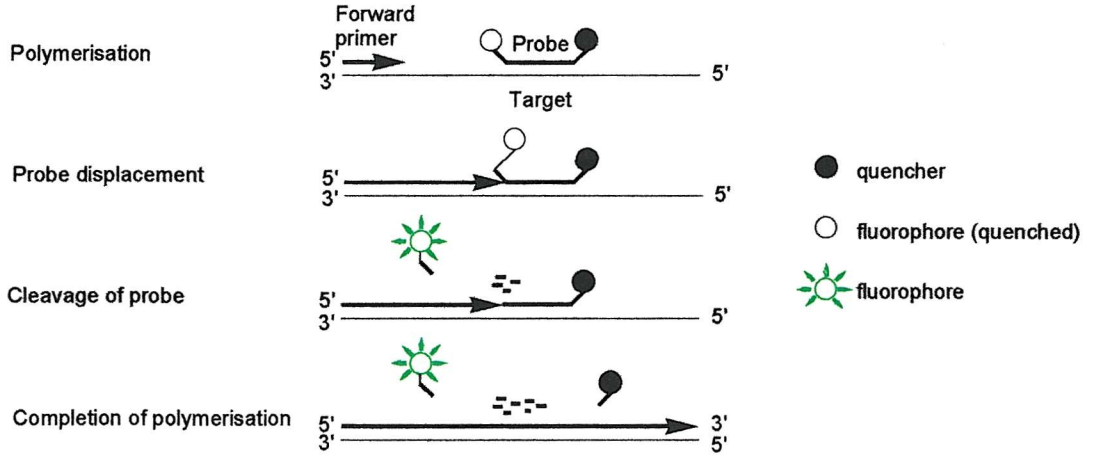



Figure 1.30 - The Taqman™ assay

The Taqman™ assay has been successfully employed for the rapid and accurate *detection of the hepatitis C virus in patient samples*[133] *and the detection of salmonella* in food samples[134].

## 1.5.5 Molecular Beacons

Molecular Beacons are probes described by Tyagi and Kramer, for the detection of specific nucleic acids in homogeneous solution[135]. These probes fluoresce only upon hybridisation to their complementary target producing sensitive, real-time signals that are indicative of the degree of hybridisation of the probe to the target oligonucleotide. The probe has a stem and loop structure (figure 1.31), which is the key feature of this probe technology. The stem portion consists of two short oligodeoxynucleotide arms either side of the loop, one of which is terminally labelled with a fluorophore, the other with a quencher (ideally the quencher is non-fluorescent). Annealing of the arms causes energy transfer from the fluorophore to the quencher with the energy being dissipated as heat. Therefore, in this confirmation, the probe is non-fluorescent. The loop portion consists of an oligodeoxynucleotide sequence that probes for its complementary target oligonucleotide in solution. On hybridisation of the loop to its target, a confirmation change occurs causing the stem arms to dissociate. Since the fluorophore and quencher are no longer in close proximity, fluorescence is restored.

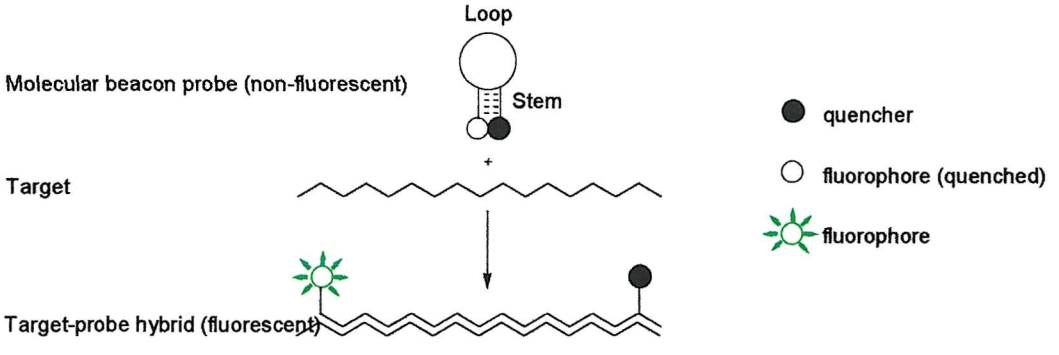



Figure 1.31: Principle of molecular beacons

Molecular Beacons are designed such that the sequence of the oligodeoxynucleotide arms are unrelated to the target sequence. The arms must be long enough to form a stem (>4bp) upon annealing but not so long that dissociation is difficult (<12bp). It is essential that hybridisation of the oligodeoxynucleotide arms produces a weaker interaction than probe to target annealing.

PCR amplicon concentration is detected and quantified using molecular beacons by measuring the intensity of fluorescence at the annealing stage in each cycle. Molecular beacons are well equipped for this application since they exhibit fast hybridisation kinetics, which allows for sealed tube experiments. The probes have the ability to recognise single base mutations and exhibit allelic discrimination, allowing the use of multicoloured probes in a single experiment.

In practice, molecular beacons have been employed in the detection of the single point mutation of cytosine to thymidine in the methylenetetrahydrofolate reductase (MTHFR) gene. This point mutation has been related to an increased risk of cardiovascular disease and neural tube defects. Molecular beacons specific for the wild type or mutant sequence demonstrated high levels of specificity for their target[138].

### 1.5.6 Scorpion Primers

Scorpion primers technology combines a highly specific probing region with a primer for a PCR reaction[75]. The probe region is chemically attached to the 5' end of one of the PCR primers and is blocked against copying by a hexaethylene glycol spacer. The primer binds to the target and is extended during polymerisation. This newly synthesised strand contains a sequence complementary to the probe sequence. This promotes annealing of the probing region to the newly synthesised strand resulting in an increase in fluorescence intensity (figure 1.32).

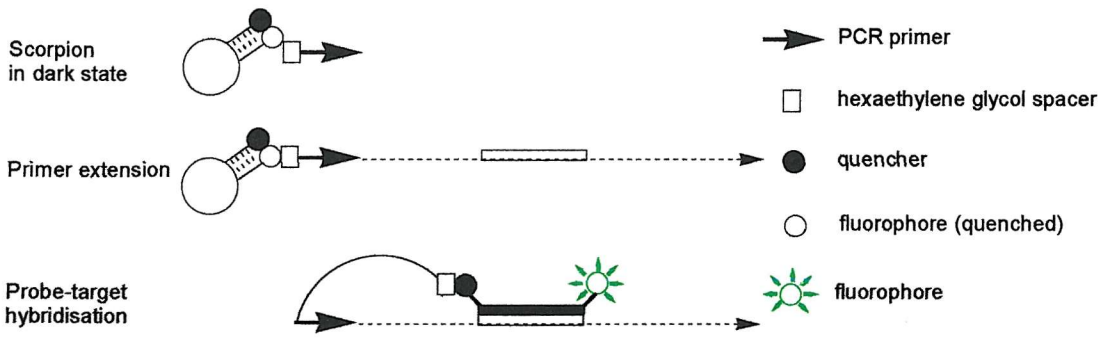



*Figure 1.32 - Principle of a Scorpion detection assay*

The assay is unimolecular and therefore kinetically and thermodynamically favoured over intermolecular assays such as Taqman™ [131] and molecular beacons[139]. The unimolecular nature of the assay increases the efficiency of the probing process since the probe is held in close proximity to its target, thereby reducing the time-scale of hybridisation. Intermolecular systems are limited by the formation of alternative intra-strand secondary structures and the competition of re-annealing complementary PCR amplicons. Scorpions have shown high specificity in single tube genotyping experiments of the two main variants of the hereditary Haemochromatasis gene: C282Y, the major disease causing mutation and H63D, a prevalent polymorphism of uncertain clinical relevance.

# Chapter 2

# Development of a Novel Protocol for the Synthesis of Labelled Nucleoside 5'-Triphosphates

# 2.0 Development of Novel Protocol for Synthesis of Labelled Nucleoside Triphosphates

The previous chapter introduced the reagents, methods and strategies employed in the design of systems used to probe genetic sequences. Large-scale genotyping programmes employ fluorescently labelled nucleoside triphosphates as a means to facilitate detection[154]. This chapter aims to record the work performed in the design and synthesis of a novel protocol for the synthesis of these compounds.

## 2.1 Introduction

The aim of this project was to develop a general and high yielding route to fluorescently labelled nucleoside-5'-triphosphates as this is an as yet unsolved problem[161]. Traditional methods of synthesis of these phosphorylated nucleosides involved transformation of mononucleotides to reactive intermediates such as morpholidates[166], imidazolites[167]or phosphoramidites[168] followed by displacement of the leaving group by pyrophosphate. Ludwig and Eckstein[169] demonstrated a new method of synthesis of dNTP's utilising salicylphosphochloridite and this method did not require protection of the heterocyclic bases. However, there are several disadvantages associated with the traditional solution phase synthesis of labelled nucleoside triphosphates. Firstly, preparation of the triphosphate itself involves combinations of ionic reagents and more lipophilic substrates making choice of an appropriate reaction media difficult. Consequently purification procedures are lengthy, the reactions are often low yielding and the nucleoside triphosphates are not particularly robust[170]. Also, the method of triphosphate synthesis is substrate-dependent, and only one fluorescent label may be incorporated per synthesis.

## 2.2 Solid-Phase Approach

### 2.2.1 Introduction

The substantial increase in the amount of literature describing the use of polymeric supports in organic synthesis over the past decade is a vivid demonstration of its impact in the chemical community[171]. The advantages gained by this methodology are striking, with four main factors contributing to the popularity of the technique: -

*i)* *Convenience* – reactions can be accomplished in only three steps: addition of reagents, filtering and washing the resin thus allowing many simple automated procedures to be developed.

*ii)* *The elimination of purification steps en route* – for each step of a multiple-step synthesis, the only purification needed is a resin-washing step. Only the final product of cleavage needs to be purified.

*iii)* *High concentrations of reagents can be used*- thus driving reactions to completion.

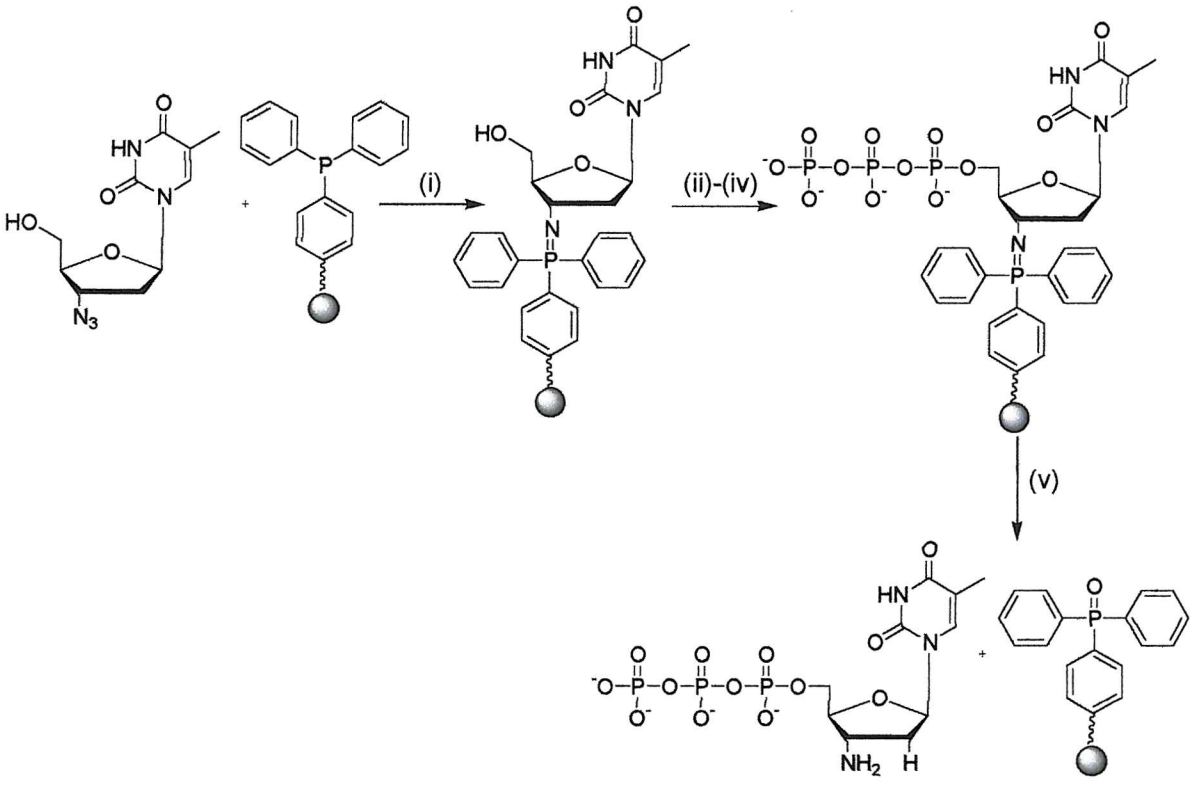
*iv)* *The straightforward nature of parallel solid-phase synthesis.*

However, for a solid-phase synthesis to be practical, several important issues need to be addressed, including the correct choice of solid support and the mode of attachment and cleavage of the material from the resin matrix.

#### *2.2.1.1 Triphosphate synthesis on solid-phase*

The method of triphosphate synthesis described by Ludwig and Eckstein[169] was further developed by Sproat *et al*[172] who performed the procedure on solid-phase thus eliminating the formation of major side products such as branched triphosphate and tripolyphosphate. This method was adapted towards the synthesis of a 2'-O-methyl-ribonucleoside 5'-triphosphate and its α-thio analogue. The nucleoside was attached to an amino functionalised solid support (CPG) via a succinate linkage, which served the dual purpose of anchor and protecting group. Again the method of Ludwig and

Eckstein[169] was employed by Engels *et al*[173] for the synthesis of modified triphosphates containing a 3'-amino group. These proved to be potent and base-specific chain terminators in DNA-sequencing reactions with the fluorescent dye being directly attached to the amino group. Furthermore, a rapid preparation of amino nucleoside-5'triphosphates based on a solid-phase approach was reported by Cech *et al*[174] in 1996. This synthetic method utilises polymer-bound triphenylphosphine for the reduction of 2' or 3'-azidonucleosides. The azido-nucleosides are fixed to the polymer-support via a stable phosphinimine linkage thus allowing chemical manipulations to be performed whilst the nucleoside is attached to the polymer (Figure 2.1). This technique afforded triphosphates in overall yields of 70-75% and represents an improvement both in time–efficiency and simplicity over the synthesis previously described for these compounds.



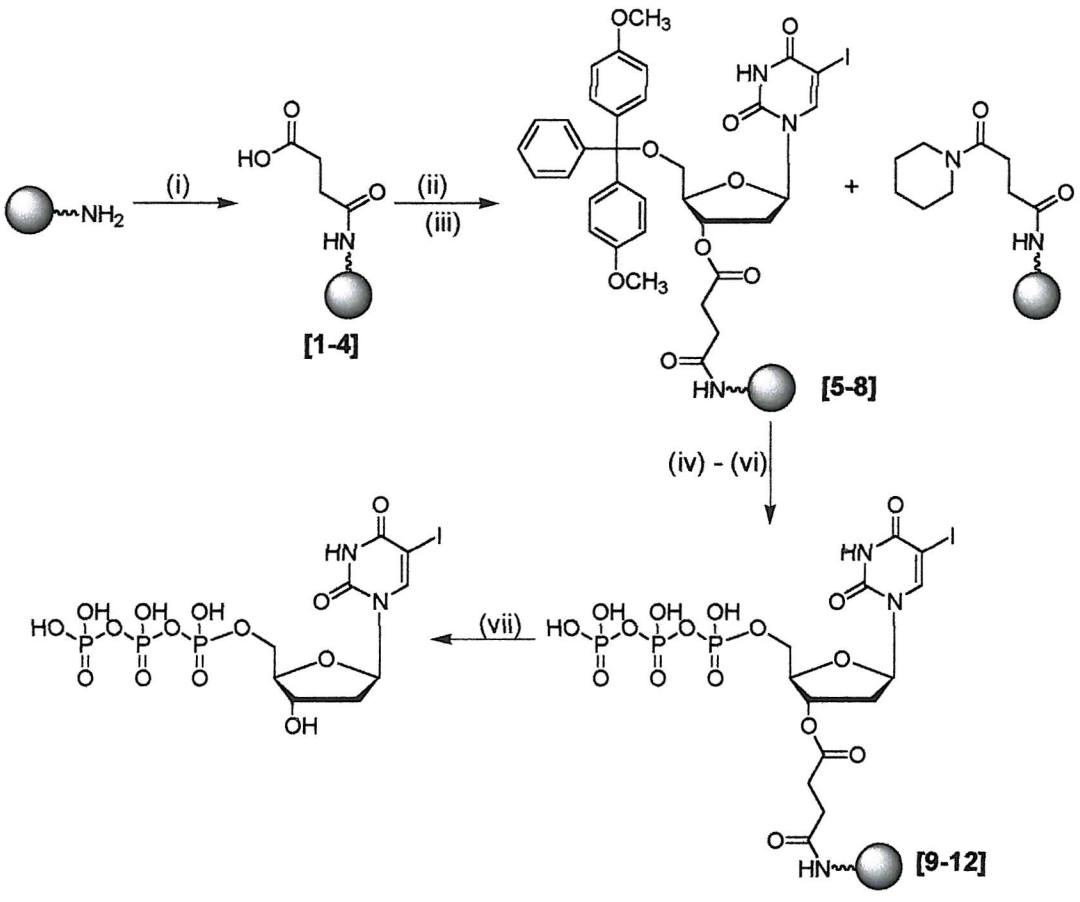

*Reagents and conditions*: i, dioxan, r.t., 2 hrs ii, salicyl phosphochloridite, dioxan, r.t. 25 mins iii, bis(tributylammonium) pyrophosphate, tributylamine, DMF, r.t. 30 mins iv, 2% solution of iodine in pyridine-water (98/2 v/v), v, conc. ammonia, 50°C, 2 hrs, 70%

Figure 2.1 - Solid-phase synthesis of 3'amino-ddTTP by Cech *et al*[15b].

## 2.2.2 Resin Derivatisation

When selecting a resin for solid-phase organic synthesis and combinatorial chemistry, it is important to consider the efficiency and kinetics of the intended reaction on the resin support[162]. In solid-phase organic synthesis, molecules are built up on the end of a spacer group, which serves to attach the reactant molecule to the resin bead. The stereotypical view of a resin bead is a rigid, non-solution like matrix in which organic reagents have to overcome a steric barrier in order to diffuse into the resin bead. In order to permit reactions, generally resins require swelling by the solvent and this is highly solvent and temperature dependent. Despite swollen resins being heavily solvated, reactions are undoubtedly affected by the proximity of the attached reactant to the spacer and the polymer matrix. Controlled pore glass (CPG), as a solid support does not require swelling and therefore has greater versatility in terms of use of solvents. Polystyrene-based (PS) resins are highly hydrophobic, being made up of mainly 1% DVB cross-linked polystyrene. For PS resin, the spacer separating the reactant from the resin is usually short. Therefore reactions on PS resin tend to be affected by the hydrophobic PS matrix. A major step forward in this context has been the polystyrene-poly(ethylene glycol) (PEG) composite resin beads developed by Bayer and Rapp[164] which form the basis of the TentaGel® range of supports. The architecture of TG resin is based on 1-2% cross-linked styrene-DVB resin backbone that is grafted extensively with long PEG spacers (50-60 ethylene oxide units). The PEG content is up to 70% of the resin weight. Therefore, the properties of the PEG chains determine the mechanical, physicochemical behaviour of the resin. However, such high levels of PEG which are needed to generate these advantageous properties, unfortunately bring some limitations, for example, the composite can be very sticky and difficult to dry[165]. HypoGel®is a hydrophilic polystyrene gel-type resin that is based on a 1% cross-linked polystyrene-DVB matrix grafted with long, flexible PEG spacers, to form a high loaded hydrophilic resin. HG resins have swelling properties between PS and TG resins[175].

The purpose of the initial experiments was to establish the most suitable resin for the solid-phase synthesis of labelled triphosphates. Four reins were examined, each with different properties: long chain aminoalkyl-controlled pore glass (LCAA-CPG, Link Technologies), aminoalkylpolystyrene (Glen Research), Novasyn TentaGel amino resin (NovaBiochem) and aminoHypoGel resin (Rapp Polymere).




*Reagents and conditions*: i, succinic anhydride, DMAP, pyridine, r.t., 1 hr, loading = 68-1280 μmolg$^{-1}$, ii, compound **[13]**, DIC, DMAP, 16 hrs, loading = 20-410 μmolg$^{-1}$, iii, pentachlorophenol, DIC, DMAP, pyridine, r.t., 16 hrs, piperidine, r.t., 15 mins, iv, 1M salicylphosphochloridite in dioxan, pyridine : DMF (1:3), r.t. 15 mins, v, 0.5M *bis*-(tributylammonium)pyrophosphate in DMF, tributylamine, r.t., 20 mins, vi, iodine : pyridine : water (3:1:1), r.t., 30 mins, vii, conc. ammonia r.t., 30mins, 7-73%.

Figure 2.2 – Generic scheme for resin derivatisation

Each resin was derivatised with a succinyl linker as shown in figure 2.2. Firstly succinic anhydride was coupled to the free amino groups on the resin using DMAP in pyridine creating a stable amide bond between the linker and the resin. The loading of the linker was determined by employing the ninhydrin assay[176]. This determined the amount of unreacted amino groups remaining on the resin, which could then be subtracted from the initial resin loading to determine the loading of the linker. Condensation with nucleoside **[13]** using DIC and DMAP in pyridine provided intermediate resin **[5-8]** that was capped with pentachlorophenol and piperidine. The loading of the nucleoside was determined by acid-catalysed detritylation, followed by quantitation of the DMT cation released, from the resin, at 495nm. Following detritylation of the resin with either TCA : DCM (1:20) or hydrochloric acid : ethanol (3:2), solid phase phosphorylation was achieved using a 1M solution of salicylphosphochloridite in anhydrous dioxan for 15 minutes. Subsequent treatment with a 0.5M solution of *bis*-(tributylammonium) pyrophosphate in anhydrous DMF and tri-*n*-butylamine for 20 minutes; followed by oxidation with a solution of iodine : water : pyridine in THF (3:1:1) for 30 minutes; provided the desired resin-bound triphosphate **[9-12]**. This was confirmed by treating an aliquot of the resin with conc. aqueous ammonia at room temperature for 30 minutes, releasing the triphosphate into solution which was then purified by anion exchange HPLC and the purified peak identified by mass spectrometry, producing triphosphates in 7-73% yield.

The polystyrene (PS) amino resin gave poor synthesis leading to an extremely low overall yield of triphosphate (yield obtained with PS = 7%, compared to CPG = 73%). Such low yields may be attributed to the hydrophobic PS matrix, disfavouring reactions involving polar and charged species. However more significantly, treatment of the resin bound nucleoside with acid was expected to release the 4, 4'-dimethoxytrityl cation. Normally, the highly visible bright orange cation is carried away by solvent flow. Acid treatment of the nucleoside turned the resin orange, indicating release of the cation. However, all attempts to separate the cation from the resin by repeated washing or addition of known efficient trityl cation scavengers (triethylsilane, pyrole[177]) failed. As it was believed to be a physical interaction ($\pi$-$\pi$

interactions between the aromatic rings of DMT and the polystyrene) as opposed to a chemical interaction that was responsible for the observed effect, numerous aromatic solvents were used, to hopefully compete with the polystyrene for the DMT. Again, this had limited success.

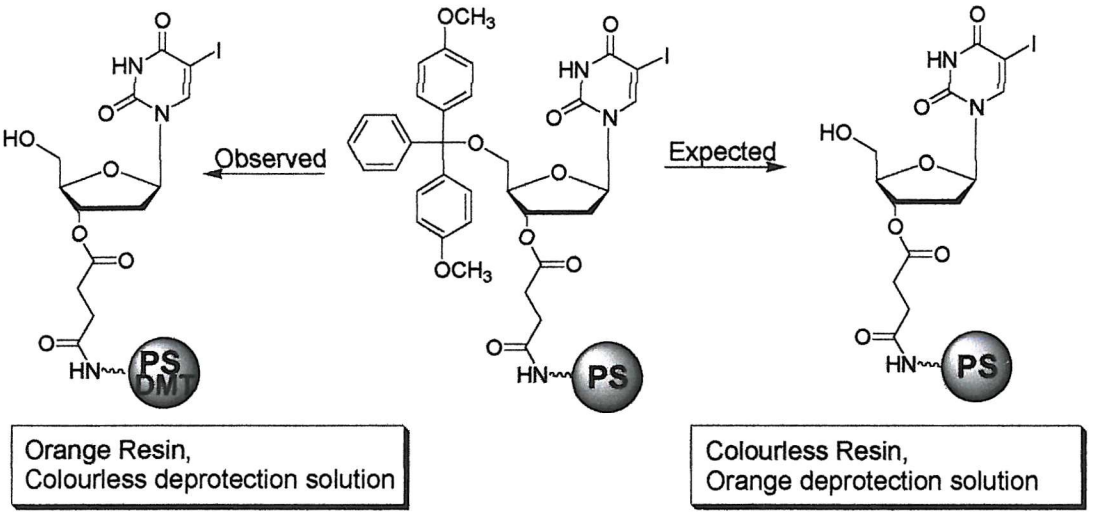



Figure 2.3 – Expected and observed results: acidic deprotection of resin-bound nucleoside.

The Novasyn TentaGel amino resin gave improved results over the PS resin with an overall yield of triphosphate of 17%. This improvement in synthesis presumably arises since the reactive sites in TG resins are located at the end of long, flexible PEG spacers and are therefore well separated from the polystyrene backbone and hence are less affected by the hydrophobic polystyrene matrix. However, despite the resin beads being uniform and medium in size, they were sticky and difficult to dry, making manipulations difficult.

The HypoGel amino resin again has the reactive centres located at the terminus of PEG spacers and a 30% yield of triphosphate was obtained with this support. The more hydrophilic nature of this resin perhaps accounts for this improvement in yield over the PS and TG resins. Unfortunately, again the resin beads were sticky and difficult to dry compared to CPG.

Controlled-Pore Glass is a rigid, microporous borosilicate glass support that is extensively employed in oligonucleotide synthesis[163]. CPG is hydrophilic and can be dried or frozen allowing long-term storage of immobilised biomolecules, it is also heat stable. Triphosphate assembly on LCAA-CPG proceeded cleanly (Figure 2.4) and in high yield (73%). The resin beads were easy to handle and extremely suitable for automated use in packed columns. Therefore despite the lower loading capacity of CPG resins compared to PS resins, greater quantities of triphosphate have been obtained using this support, due to the significant difference in yield. Hence CPG was used extensively in future studies.

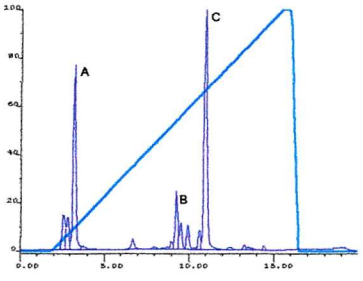



Figure 2.4 – Anion exchange HPLC of triphosphate **[14]** synthesised on CPG (A = unreacted nucleoside, B = diphosphate, C = triphosphate)

## 2.2.3 Optimisation of Reaction Conditions

In order to optimise the solid-phase triphosphorylation reaction, alterations to the initial reaction conditions were investigated. To improve the yield of triphosphate synthesised, the following parameters were believed to be important:

*i)* *reaction time*

*ii)* *solvent*

iii) *cleavage conditions*

To establish if the initial substitution reaction was occurring slower than anticipated, extended reaction times were investigated (Figure 2.5). These were found to have

little effect on the final yield of triphosphate. Since it was deemed possible that the salicylphosphochloridite reagent was hydrolysing before the initial substitution reaction was complete, two additions of the reagent were made. Again this had little effect on the final yield.

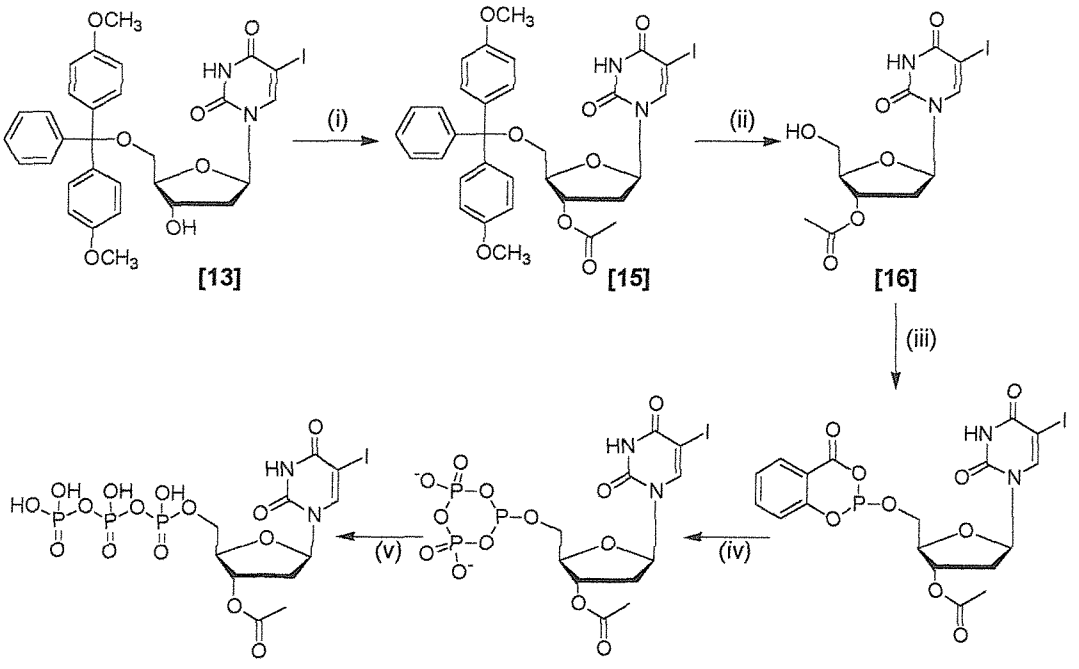
| Reaction Time (mins) | % Yield of triphosphate **[14]** |
|---|---|
| 15 | 73 |
| 20 | 70 |
| 30 | 71 |
| 45 | 68 |
| 2 x 15 | 70 |

Figure 2.5 – Reaction times employed in optimisation reactions.

Since acetonitrile is routinely used in oligonucleotide synthesis, a change of solvent from dioxan to acetonitrile was investigated. Unfortunately, this was found to have a deleterious effect on the final yield of triphosphate synthesised.

In order to monitor the efficiency of each stage of the reaction (substitution, displacement by pyrophosphate and oxidation), the reaction was performed in solution under identical conditions and monitored by TLC using compound **[16]** (Figure 2.6).

Results from this showed that each step of the reaction was working efficiently and required no further optimisation.




*Reagents and conditions*: i, acetic anhydride, pyridine, r.t., 3 hrs, 64%, ii, TCA : DCM (1:20), r.t., 1 hr, 94%, iii, 1M salicylphosphochloridite in dioxan, r.t., 15 mins, iv, 0.5M *bis*-(tributylammonium)pyrophosphate in DMF, tributylamine, r.t., 20 mins, v, iodine : pyridine : water in THF (3:1:1), r.t., 30 mins

Figure 2.6 – Solution-phase synthesis of triphosphate

The remaining area, which required consideration, was the mode of cleavage of the triphosphate from the resin matrix. Alternative cleavage conditions from resins **[9-12]** were investigated (figure 2.7). The results revealed that for this model system, ammonia treatment at room temperature for 30 minutes was the most suitable method of cleavage, producing a clean, compound in high yield (73%). Longer treatment with ammonia or heat resulted in slight decomposition of the triphosphate (59-67% yield).

| Cleavage Conditions | Time (hrs) | Temp. °C | Yield |
|---|---|---|---|
| Ammonia | 0.5 | r.t. | 73 |
| Ammonia | 0.5 | 40 | 64 |
| Ammonia | 0.5 | 60 | 59 |
| Ammonia | 3 | r.t. | 67 |
| Ammonia : ethanol (3:1) | 3 | 60 | 50 |
| 2M methanolic methylamine | 2 | r.t. | 55 |

Figure 2.7 – Conditions investigated for cleavage of triphosphate from resin **[9-12]**.

## 2.2.4 Synthesis of Modified Nucleoside

With the optimum conditions for solid-phase triphosphate synthesis established, the next stage requires the design and synthesis of a modified nucleoside from which the fluorescent label will be attached.

The incorporation of a linker and/or a label into DNA during PCR requires the insertion of a modified nucleotide into the nascent DNA strand by a DNA polymerase. This process is facilitated if the modification does not interfere with the base pairing properties of the nucleoside[178] and for this reason C5 substituted 2'-deoxyuridine analogues have proved a popular choice in this strategy. Positioning the linker at C5 not only minimises any disruption to the base pairing properties of the base[178, 21] but once incorporated, the linker and/or label will be situated within the major groove of the DNA duplex[178, 101]. This positioning helps to minimise steric repulsions between the label and the DNA duplex and maximises the accessibility of the linker or label for further derivatisation such as antibody-mediated detection (section 1.3.1). In addition to its position on the nucleoside, the steric and electronic properties of the linker will influence the base pairing properties of the base analogue and the stability of the DNA duplex[179]. It has been demonstrated that the presence of propynyl groups at the C5 position of 2'-deoxyuridine results in an increase in DNA/RNA duplex stability[180]. The stabilising effect was attributed to:

i) $\pi$-$\pi$ bond base stacking interactions between the propyne group and the surrounding bases

ii) displacement of water molecules by the hydrophobic propyne linker, resulting in an increased entropy of binding.

The use of propargylamino linkers that have a DNP group attached have also been shown to increase duplex stability[179]. In UV melting experiments, the replacement of a single, internal dT with the propargylamino-modified dU analogue resulted in an increase in $T_m$ of 4.4K for an 8-mer duplex. Similar experiments carried out using a propylamino linker did not result in a corresponding duplex stabilisation. Therefore, the use of an alkynyl linker positioned at C5 of 2'-deoxyuridine seemed a suitable

choice for a modified nucleoside. In addition incorporation of a terminal amino group would ensure compatibility with commercially available labelling reagents. Thus the propargylamino linker-modified 2'-deoxyuridine analogue shown in figure 2.8 was synthesised.

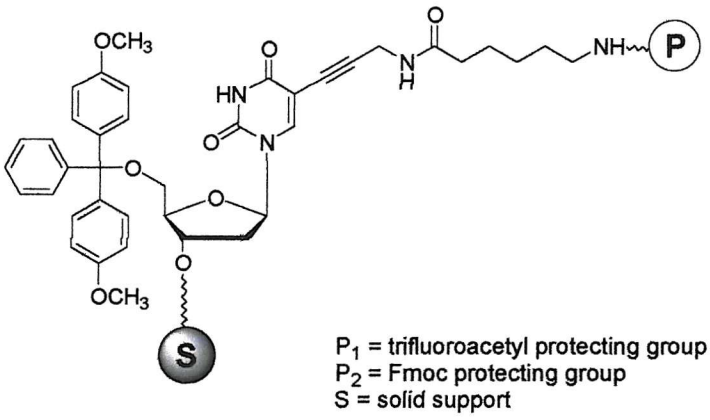



Figure 2.8 – Generic structure of modified nucleoside analogue

Three individual strategies encompassing both solution-phase and solid-phase methodologies were embraced in the synthesis of this target and are described in the following sections. Each method relies on a palladium catalysed coupling reaction, using Sonogashira[182] conditions, as a relatively simple and robust method of introducing the propynyl linker.

### *2.2.4.1 Solid-phase strategy*

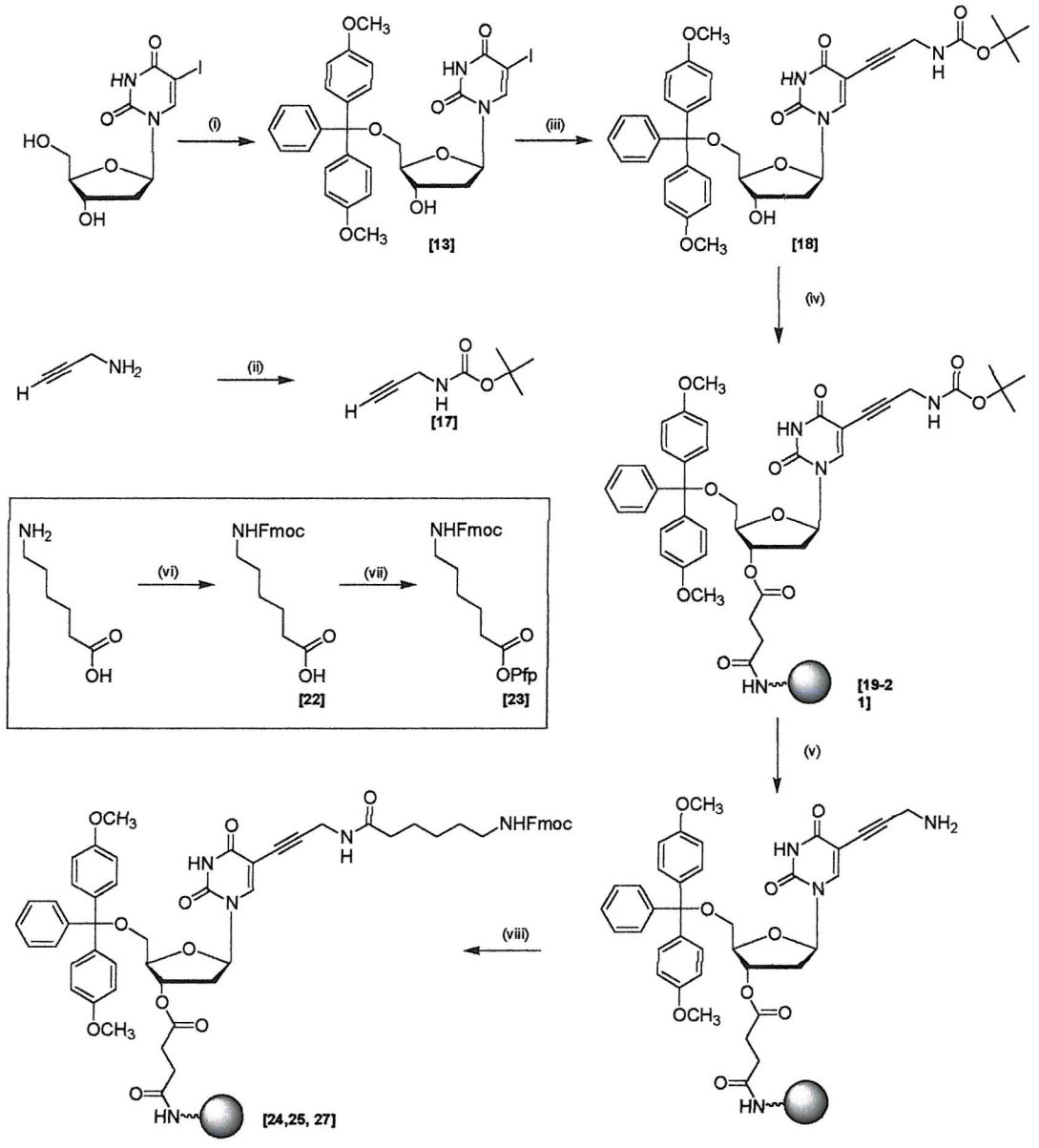
This approach involved synthesising the fully protected nucleoside analogue predominantly on solid-phase. This procedure was considered to be more convenient since it obviates additional solution-phase manipulations of the nucleoside derivatives and eliminates the synthesis of nucleoside-3'-O-succinates which is lengthy. Hence, assembly of the modified molecules whilst attached to the solid-support would simplify both synthesis and purification. Furthermore it has been reported that the rate

of coupling of nucleoside-3'-O-succinate esters to CPG resins is slow and generally yields moderate loading of support-bound nucleosides [181].

Robins and Barr[184, 185] successfully applied Sonogashira conditions to nucleoside chemistry and coupled a variety of terminal alkynes to 5-iodouracil derivatives. Since then the merits of different *N*-protected propargylamines under Sonogashira conditions[182] have been reported with the Boc protecting group being optimum[183]. Despite the acid lability of the Boc group, this protecting group was employed for the synthesis of the modified nucleoside (figure 2.9). The method is based on the large difference in nucleophilicity between primary amines and primary alcohols thereby allowing the selective acylation of an amine when a mild acylating agent such as a pentafluorophenyl ester is employed. This serves to extend the length of the linker arm.

Tritylation of the 5'-hydroxyl group of the commercially available 5-iodo-2'-deoxyuridine proceeded smoothly in 96% yield and protection of propargylamine with *di-tert*-butyldicarbonate proceeded as expected to give compound **[17]**. Subsequent Sonogashira coupling of compounds **[13]** and **[17]** was achieved in reasonable yield producing **[18]** which was then coupled to resin **[1, 2, 4]**. Removal of the Boc group was effected by treating the nucleoside on-resin with 90% TFA in ethanedithiol. The compound was then neutralised by treatment with 20% triethylamine in DCM. Acylation of the pendant amino group with compound **[23]** occurred with a high degree of selectivity. Comparison of the trityl analysis of the nucleoside on-resin prior to and following the acylation reaction confirmed the reaction had occurred almost exclusively at the amino group. The mild acylating agent **[23]** was synthesised from commercially available 6-amino-1-hexanoic acid. The amine functionality was initially protected with a Fmoc group. This protecting group was chosen as it is orthogonal to the acid-labile protecting group on the nucleoside and can be readily removed under mild conditions that do not cleave the succinyl linker. Both protection of the amine with Fmoc succinimide in dioxan and

esterification of the carboxylic acid with pentafluorophenol, and DCC in DMF proceeded as anticipated in good yield.




*Reagents and conditions*: i, DMT-chloride, pyridine, r.t., 12 hrs, 96%, ii, Boc anhydride, DCM, r.t., 3 hrs, 57%, iii, copper (I) iodide, $Pd(PPh_3)_4$, triethylamine, compound **[17]**, DMF, r.t., 24 hrs, 51%, iv, resin **[1, 2, 4]**, DIC, DMAP, r.t., 16 hrs, v, 90% TFA in ethanedithiol, 20% triethylamine in DCM, vi, Fmoc-succinimide, dioxan, r.t., 5 mins, 87%, vii, pentafluorophenol, DCC, DMF, r.t., 24 hrs, 85%, viii, compound **[23]**, HOBt, DMF, r.t., 45 mins

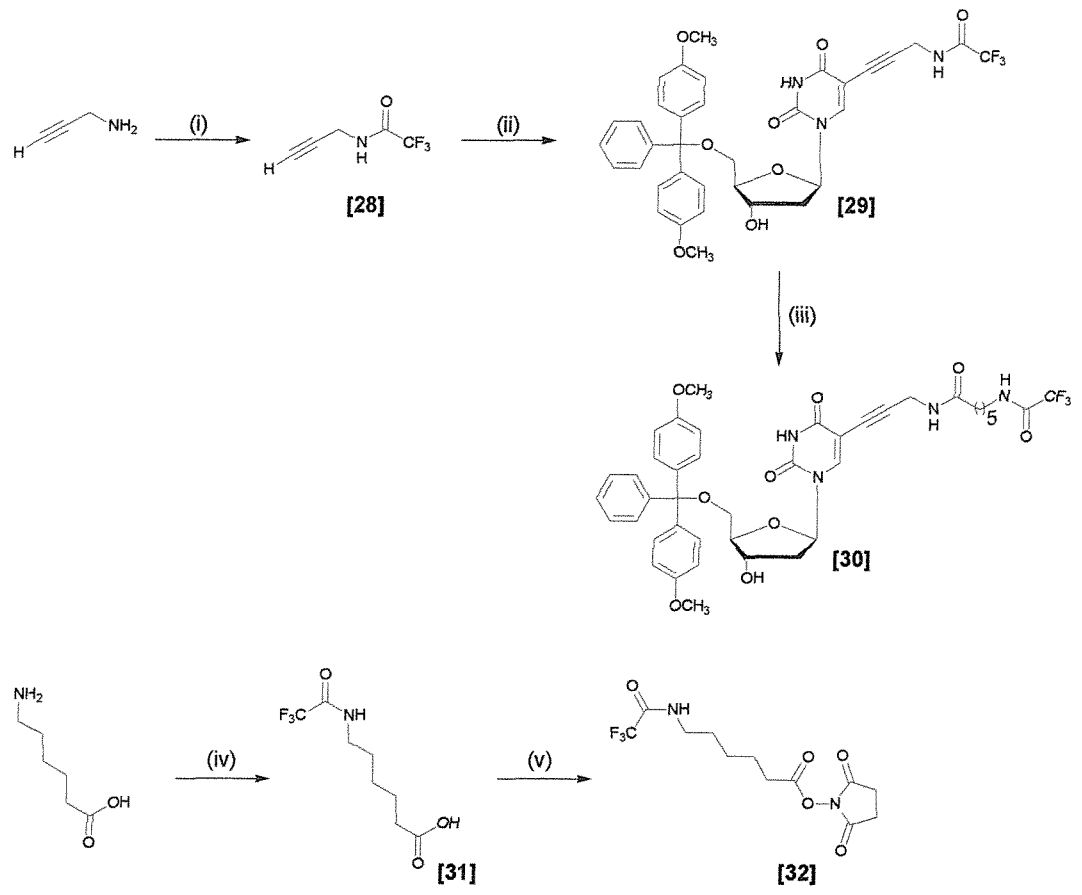
Figure 2.9 – Synthesis of the modified nucleoside using a solid-phase approach

*2.2.4.2 Solution-phase strategy*

This approach was developed principally for use with more complicated nucleoside analogues where a diverse range of functionalities may be present. Manipulations of nucleosides on solid support are limited by the stability of the linker and the degree of orthogonality to the conditions employed in synthesis. Therefore a strategy whereby the nucleoside was completely functionalised in solution prior to attachment to the support was investigated.

Research in the Oswel laboratory had previously completed the synthesis of a trifluoracetyl protected modified nucleoside (**[30]**, figure 2.10) by a stepwise route. Since this compound was compatible with the triphosphorylation chemistry previously established, this route was followed for the synthesis of nucleoside **[30]**.

Commercially available propargylamine was protected as a trifluoroacetamide and coupled to nucleoside **[13]** using Sonogashira conditions as before. In order to extend the length of the linker arm on the nucleoside, the commercially available 6-aminohexanoic acid was firstly protected as a trifluoroacetamide **[31]** using ethyltrifluoroacetate and converted to its NHS ester **[32]** by reaction with *N*-hydroxysuccinimide. Removal of the protecting group from **[29]** under basic conditions was expected to proceed routinely. Indeed treatment of **[29]** with methanolic methylamine cleaved the trifluoracetyl protecting group allowing **[32]** to be coupled in reasonable yield. However this reaction was not reproducible on a regular basis. Alternative basic conditions were investigated (figure 2.11) but unfortunately routine removal of the trifluoracetyl group was consistently problematic. For this reason, another more suitable synthetic pathway was investigated.



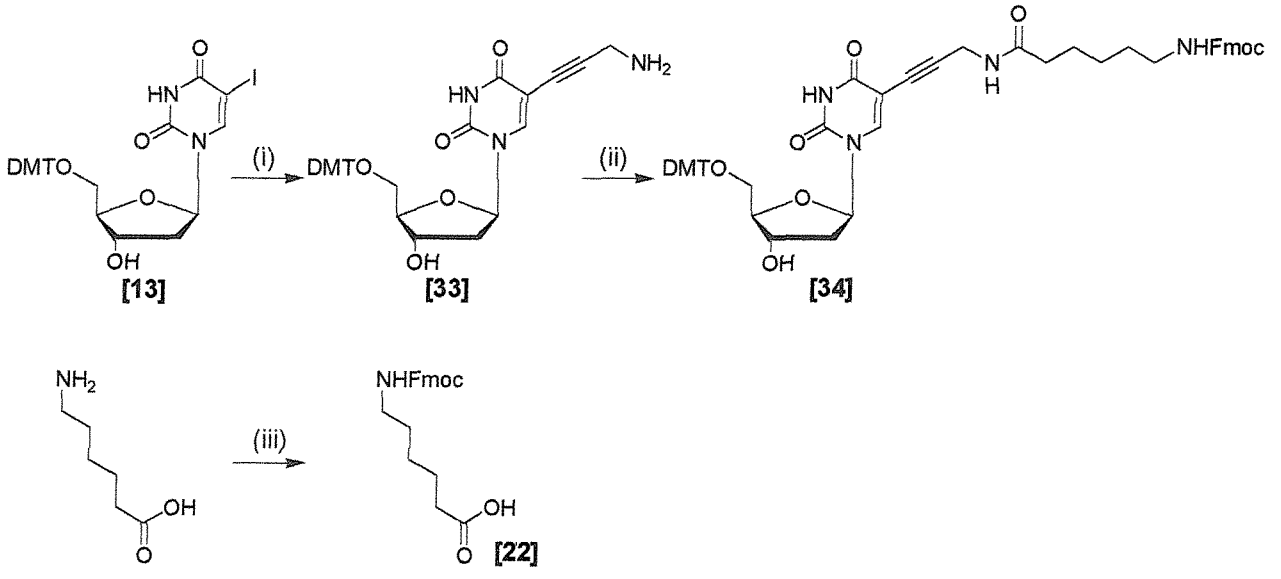

*Reagents and conditions*: i, ethyltrifluoroacetate, methanol, 0°C-r.t., 12 hrs, 77%, ii, compound **[13]**, copper (1) iodide, triethylamine, $Pd(PPh_3)_4$, DMF, 24 hrs, 77%, iii, methanolic methylamine, r.t., 1 hr, compound **[32]**, triethylamine, DMF, r.t., 2 hrs, 53%, iv, 6-aminohexanoic acid, ethyltrifluoroacetate, triethylamine, methanol, r.t., 45 mins, Dowex $H^+$, r.t., 30 mins, 94%, v, *N*-hydroxysuccinimide, EDC, r.t, 12 hrs, 84%

Figure 2.10 – Synthesis of trifluoracetyl protected uridine analogue **[30]**

| Deprotection conditions | Deprotection time (hrs) | % Yield |
|---|---|---|
| methanolic methylamine | 1 | 65 |
| methanolic methylamine | 6 | 67 |
| conc. aq ammonia : methanol (1:1) | 3 | 52 |
| conc. aq ammonia : methanol (1:1) | 8 | 56 |
| sodium hyrdoxide (XS) in methanol | 12 | 77 |

Figure 2.11 – Conditions for trifluoroacetyl removal from nucleoside **[29]**

A search of the literature, revealed conditions for coupling propargylamine[186] directly with nucleoside analogues thereby eliminating the need for additional protection and deprotection steps. Also the use of an alternative protecting group on amino-hexanoic acid would be beneficial. Therefore in order to establish a more efficient route to the modified nucleoside, the following synthetic pathway was undertaken (figure 2.12).




*Reagents and conditions*: i, propargylamine, copper (I) iodide, triethylamine, $Pd(PPh_3)_4$, DMF, 24 hrs, r.t., 89%, ii, compound **[22]**, EDC, pyridine, 12 hrs, r.t., 54%, iii, Fmoc-succinimide, DMF, r.t.20 mins, 93%

Figure 2.12 – Synthesis of modified nucleoside **[34]**

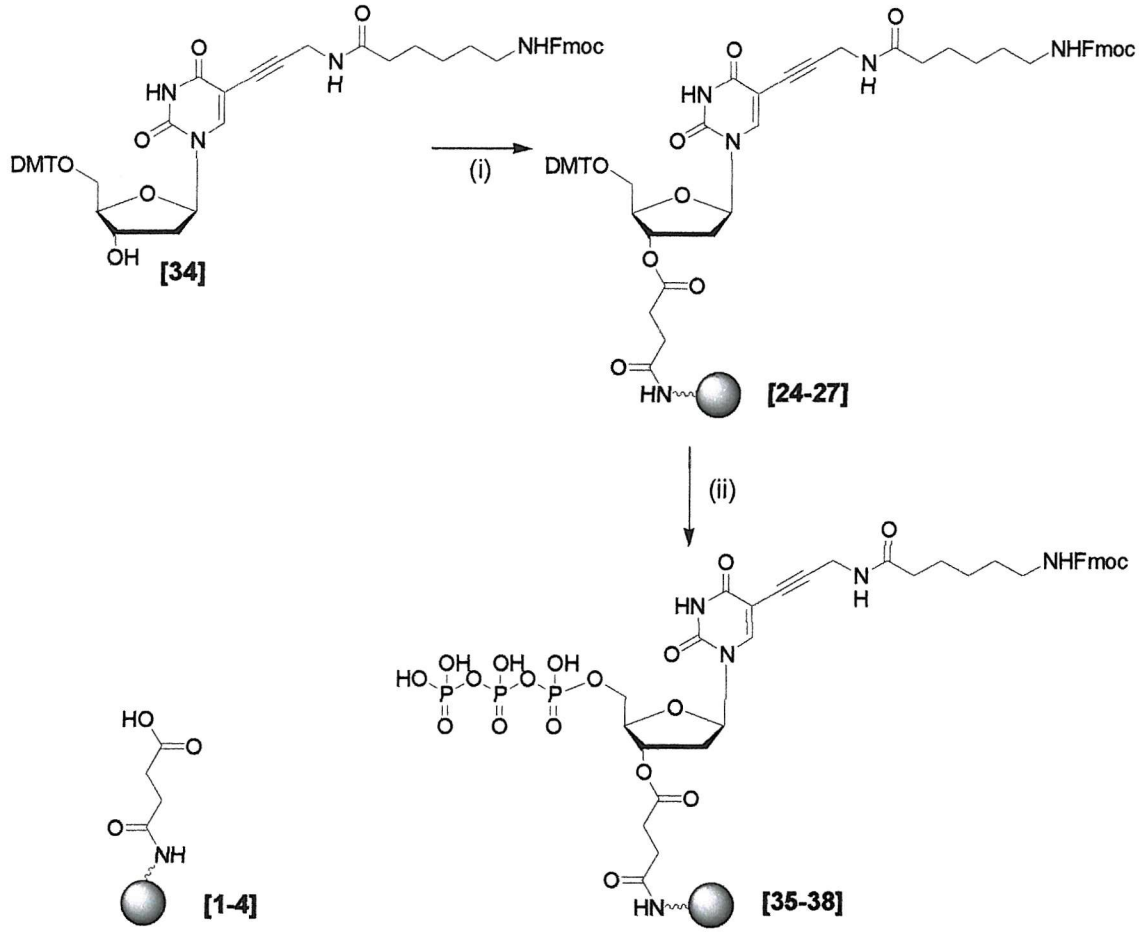
Assembly of the modified nucleoside **[34]** was straightforward and proceeded by Sonogashira coupling of **[13]** with propargylamine[186] to give the intermediate **[33]** in 89% yield. To extend the length of the linker arm an alkyl chain was introduced by condensation of **[33]** with the carboxylic acid **[22]**. This provided the required modified nucleoside ready for attachment to the solid support and conversion to the triphosphate.

## 2.2.5 Triphosphate Synthesis

The initial experiments on a model system had established the optimum conditions for the triphosphorylation reaction. Therefore all that was required was attachment of the modified nucleoside to the resin bead. Two different strategies for doing this were investigated.

### *2.2.5.1 Reaction with a carboxyl derivatised resin*

The model studies were performed on a nucleoside that had been reacted with a resin that had been derivatised with succinic acid **[1-4]**. This method eliminated the need for the synthesis of nucleoside-3'-O-succinates, which, can be time consuming. Therefore this method was repeated with the modified nucleoside **[34]** (figure 2.13). Using DIC together with DMAP in anhydrous pyridine, nucleoside **[34]** was coupled to resin **[1-4]** giving good loading of the nucleoside. Resin loading was calculated by acid-catalysed detritylation, DMT cation release was monitored at 495nm. After washing and drying of the resin, the resin-bound nucleoside was converted to its 5'-triphosphate using the conditions previously established. Cleavage of an aliquot of the resin with ammonia, purification by anion-exchange HPLC and mass spectral analysis of the purified compound confirmed the success of the reaction. However, somewhat disappointingly, the HPLC trace indicated that the reaction was not as clean as was anticipated (figure 2.15 i). The reason for this is believed to be due to the fact that with the use of a Fmoc protecting group on the linker arm of the nucleoside, no compatible capping reagent could be found for any unreacted carboxyl sites on resin **[1-4]** as this is the only difference between this system and the model system. Previously this resin had been capped by treatment with pentachlorophenol and piperidine. This produced the pentachlorophenol ester, which was then converted to the succinyl piperidine to prevent the slow release of pentachlorophenol. With the Fmoc protecting group present, this capping procedure could not be used.



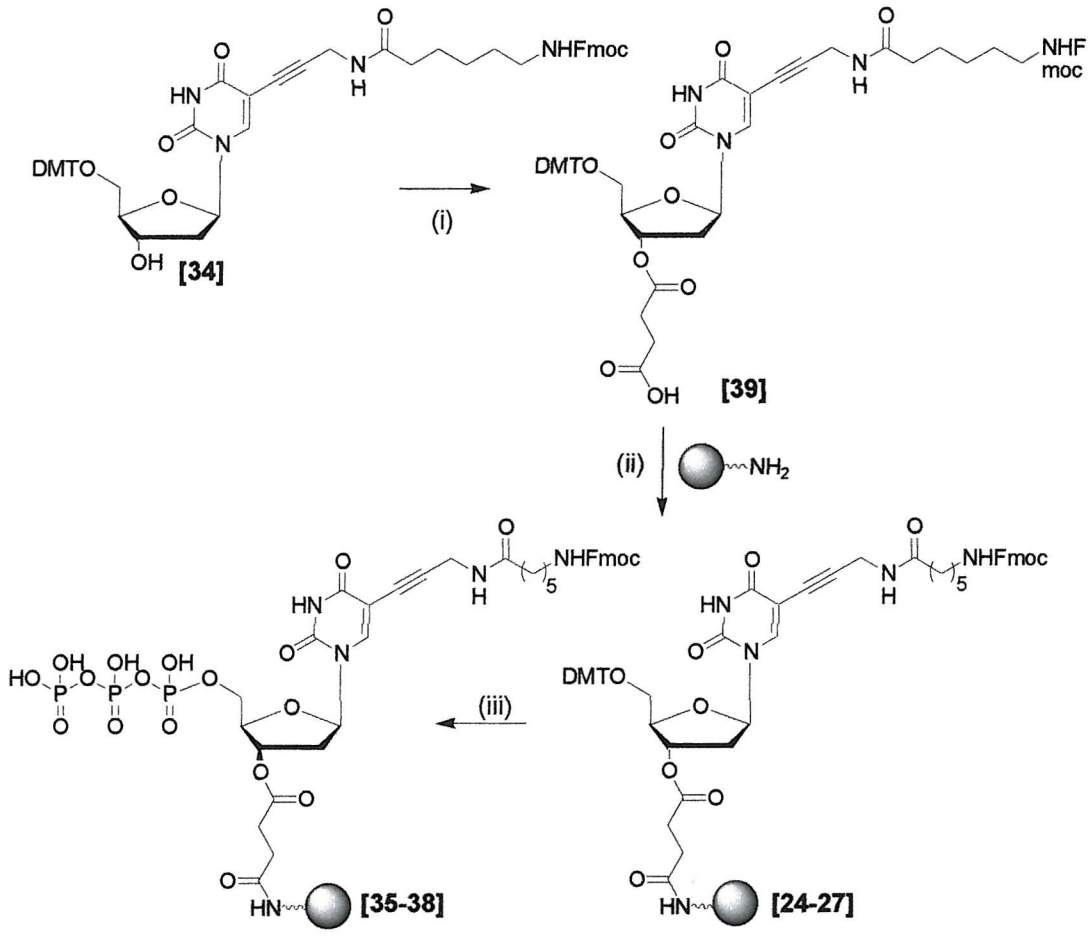

*Reagents and conditions*: i, succinyl resin **[1-4]**, DIC, DMAP, pyridine, loading = 49-260 mmolg$^{-1}$, ii, 1M salicylphosphochloridite in dioxan, pyridine : DMF (1:3), r.t. 15 mins, 0.5M *bis*-(tributylammonium)pyrophosphate in DMF, tributylamine, r.t., 20 mins, iodine : pyridine : water (3:1:1), r.t., 30 mins

Figure 2.13 – Synthesis of resin-bound triphosphate **[91-94]** by reaction of nucleoside **[34]** with a carboxylated resin **[1-4]**

### *2.2.5.2 Reaction with a nucleoside 3'-O-succinate*

Despite a publication stating that the rate of coupling of nucleoside-3'-O-succinate esters to CPG resins is slow and generally yields moderate loading of support-bound nucleosides [181], it was believed that this approach would result in a cleaner triphosphorylation reaction. By converting nucleoside **[34]** to its 3'-O-succinate and

coupling this to the commercially available amino-resins, after completion of the reaction, any unreacted amino sites on the resin may be capped with acetic anhydride. Conversion to the triphosphate using the conditions previously established should then proceed with fewer by-products (figure 2.14).




*Reagents and conditions*: i, succinic anhydride, DMAP, pyridine, r.t., 16 hrs, 92%, ii, DIC, HOBt, pyridine, r.t., 16 hrs, loading = 52-350mmolg$^{-1}$, iii, 1M salicylphosphochloridite in dioxan, pyridine : DMF (1:3), r.t. 15 mins, 0.5M *bis*-(tributylammonium)pyrophosphate in DMF, tributylamine, r.t., 20 mins, iodine : pyridine : water (3:1:1), r.t., 30 mins

Figure 2.14 – Synthesis of resin-bound triphosphate **[35-38]** by reaction of nucleoside 3'-O-succinate **[39]** with commercial amino resin

The synthesis of the nucleoside 3'-O-succinate proceeded smoothly, producing **[39]** in excellent yield. Coupling to the amino resin was indeed slow but gave satisfactory loading of the nucleoside that, was then converted to the triphosphate. Again, an aliquot of the resin was treated with ammonia releasing the triphosphate into solution. Results from anion-exchange HPLC of this solution were very satisfying, providing confirmation that this strategy did indeed proceed with fewer by products (figure 2.15 ii).

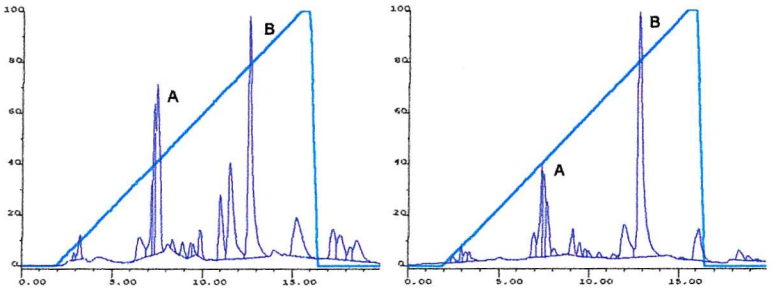



Figure 2.15 – Comparison of the anion exchange HPLC chromatograms obtained from i) reaction with a carboxylated resin and ii) reaction with nucleoside 3'-O-succinate (A = diphosphate, B = triphosphate)

## 2.2.6 Conclusions

A model system was employed to study the optimum conditions for the solid-phase synthesis of modified nucleoside triphosphates. This involved obtaining the most suitable solid-support, optimising the reaction conditions and determining the most effective yet mild method of cleaving the product from the support.

A range of structurally dissimilar resins have been investigated for this purpose and results indicated that Controlled-Pore Glass is the most suitable for the reasons described. Reaction conditions were optimised and for the triphosphates synthesised, ammonia treatment at room temperature was found to be the most effective method of cleavage.

A generic structure of a suitable modified nucleoside was designed. Two approaches were investigated for the synthesis of this common intermediate using both solid and solution-phase methodologies. Despite the advantages of solid-phase chemistry, in this instance large-scale assembly of the nucleoside with the protected side chain on the 5-position of dU in solution proved to be more beneficial. Of the nucleoside analogues **[30]** and **[34]** (figure 2.16) synthesised by this manner, nucleoside **[34]** was most suitable for the reasons described.

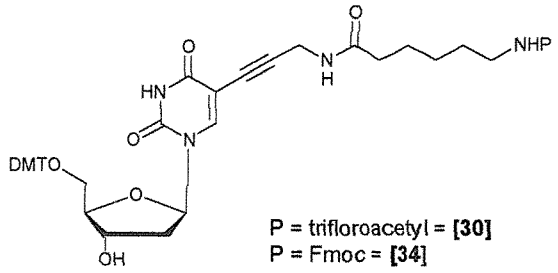



Figure 2.16 – Modified nucleoside analogues **[30]** and **[34]** prior to triphosphorylation

Two strategies were employed for attaching the modified nucleoside to the solid-support. Of these, conversion of **[34]** to its 3'-O-succinate **[39]** followed by conversion to its triphosphate was the most efficient. The potential for undesirable side-reactions is removed by capping the resin prior to triphosphorylation.

## 2.3 Label Incorporation

### 2.3.1 Introduction

The introduction of labels onto nucleosides and nucleoside triphosphates modified with an aminoalkyl linker or synthetic oligonucleotides derivatised at the 3' or 5' terminus with an amino function (aminolink) is routinely performed in solution by reaction with *N*-hydroxysuccinimide esters of dyes, in a simple labelling process[39]. The methods available for the covalent labelling of nucleic acids has been discussed

in detail in section 1.4. Such labelling reactions have not been performed to label modified nucleosides on solid-phase.

The idea was to synthesis fluorescently labelled nucleoside triphosphates by introducing fluorescent dyes to the terminus of the linker arm on the nucleoside whilst attached to the solid support. Theoretically, the fluorescent dye may be introduced either prior to or subsequent to conversion to the triphosphate however literature precedent suggests that conversion to the triphosphate before introduction of the dye is more beneficial[39, 187, 188]. An additional advantage of following the latter sequence is that an array of fluorescent dyes may be added to the triphosphate in a combinatorial fashion thus generating a greater number of labelled triphosphates (figure 2.17).

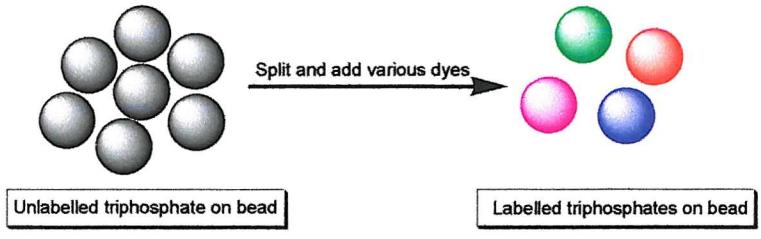



Figure 2.17 – Proposed addition of multiple dyes to resin-bound triphosphate

## 2.3.2 Amine Reactive Functionalities

Once deprotected, the amino group at the terminus of the linker arm of resin **[35-38]** can be reacted with reactive functional groups such as *N*-hydroxysuccinimide esters or carboxylic acids used in conjunction with a carbodiimide coupling reagent, thereby allowing the incorporation of fluorescent dyes on solid-phase.

Fluorescein isothiocyanate is a popular, commercially available reactive fluorescent dye that is used extensively to label oligonucleotides and peptides under mild conditions. Therefore this dye was employed for the initial labelling reactions.

Removal of the Fmoc group from the nucleoside on-resin TG triphosphate resin **[38]** and CPG triphosphate resin **[38]** proceeded smoothly using 20% piperidine in DMF for 10 minutes. The fluorescent group was then introduced by soaking the resin in a solution of fluorescein isothiocyanate in pyridine for 2 hours. After extensive washing the resin remained fluorescent (figure 2.18).

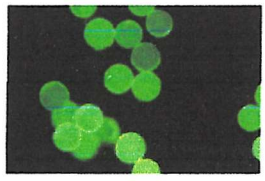


Figure 2.18 – Photograph of fluorescently labelled triphosphate resin beads

The labelled triphosphate was cleaved from the resin with conc. aq. ammonia at room temperature for 30 minutes and converted to its lithium salt by treatment with Dowex $Li^+$ ion-exchange resin. Having lithium as the counter ion ensures the triphosphate is in its most stable form. Purification of the triphosphate by anion-exchange HPLC proceeded as anticipated giving the final labelled triphosphate **[40]** in 69% yield (figure 2.19). The identification of this triphosphate was confirmed by NMR and mass spectrometry.

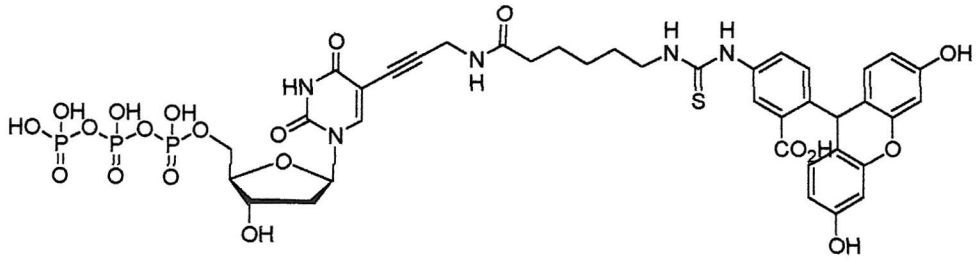
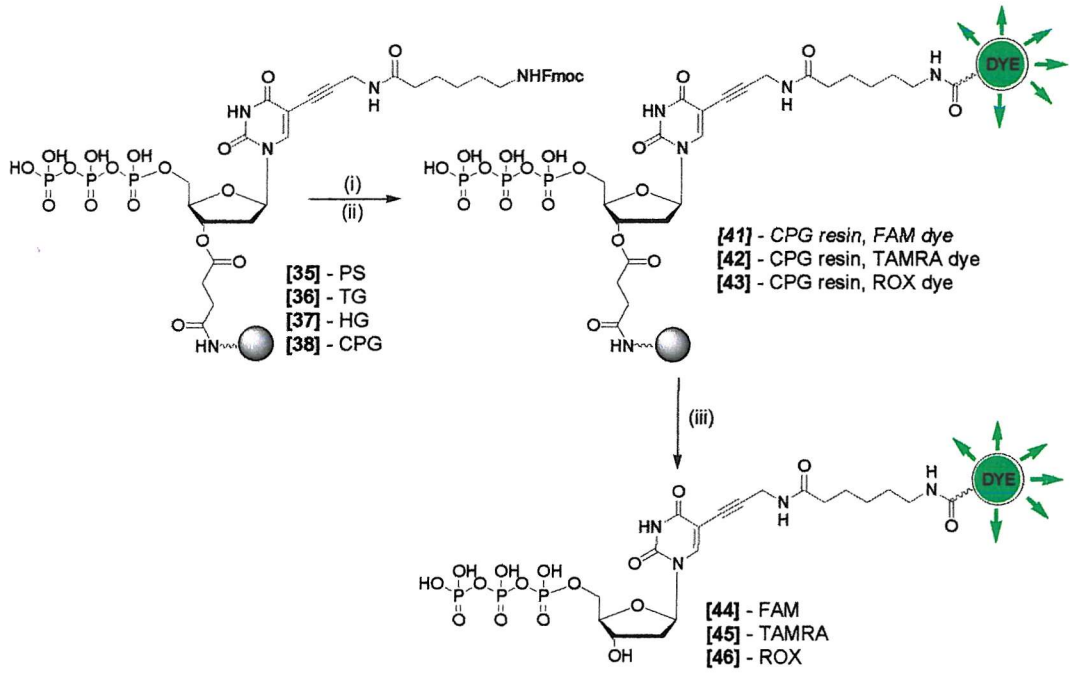


Figure 2.19 – FAM labelled triphosphate **[40]**

There are several disadvantages associated with the use of fluorescein derivatives. The broad nature of their emission peaks complicates the simultaneous detection of multiple sequences, they are sensitive to pH[76] and susceptible to photobleaching however rhodamine dyes, carboxy-x-rhodamine (ROX)[77] and tetramethylrhodamine

(TAMRA)[78] have reduced some of these disadvantages. These dyes were available from the Oswel laboratory and it was believed that standard carbodiimide coupling techniques should be efficient in coupling the dyes to the resin-bound nucleotide triphosphate. Due to the expense of these dyes, initial investigations were performed with 6-carboxyfluorescein. Figure 2.20 outlines the final steps in the synthesis of these labelled triphosphates.




*Reagents and conditions: i*, 20% piperidine in DMF, r.t., 10 mins, *ii*, reactive dye, DIC, HOBt, pyridine, r.t., 1.5 hrs, iii, conc.aq. ammonia, r.t., 20 mins, or water : methanol : tributylamine (2:1:1), 60°C, 1.5 hrs, 71% (FAM), 41% (TAMRA), 46% (ROX)

Figure 2.20 – Synthesis of labelled triphosphates **[44-46]**

The CPG triphosphate resin **[38]** was again treated with 20% piperidine in DMF to remove the Fmoc protecting group. This was then treated with a solution of 5(6)-carboxy fluorescein activated with HOBt in pyridine and DIC coupling agent was added. In order to monitor the extent of the reaction, after 1.5 hours the resin was washed and dried. The resin was observed to be highly fluorescent. An aliquot of this resin was treated with conc. aq. ammonia at room temperature for 20 minutes, producing a fluorescent solution. Analytical analysis of a sample of this solution by

anion-exchange HPLC indicated complete consumption of the unlabelled triphosphate and that the reaction had gone to completion. The entire resin was therefore treated and purified in this manner. The appropriate fraction from HPLC was lyophilised repeatedly to remove excess salt, giving the final product as a yellow lyophilised solid.

This method of synthesis was repeated to introduce the rhodamine dyes TAMRA and ROX to the triphosphate. A good yield was observed for the triphosphate labelled in this manner with fluorescein (71%) however poorer yields were obtained with the rhodamine dyes (27% for TAMRA and 30% for ROX). This may be due to the limited solubility of these dyes in pyridine and future optimisation of this reaction may improve the yield of labelled triphosphate obtained, for example by performing the reaction in either DMF or DMSO. Also, the coupling with TAMRA was performed using a mixture of regioisomers as a sample of a pure isomer was not available. However, it was observed that with the rhodamine dyes, the resin remained coloured after treatment with ammonia suggesting that material still remained attached to the resin. Therefore alternative cleavage conditions were investigated and it was found that a mixture of water : methanol : tributylamine (2:1:1) at 60°C for 1.5 hours was more suitable giving better yields of labelled triphosphates (41% for TAMRA and 46% for ROX) however due to the need for heat, decomposition of the triphosphate may have occurred. The UV/Vis spectrum of the purified products (figure 2.21) gave $\lambda_{max}$ values corresponding to the fluorescent label and the nucleoside. This was further confirmed by mass spectrometry.

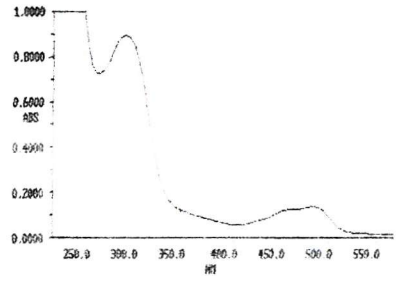
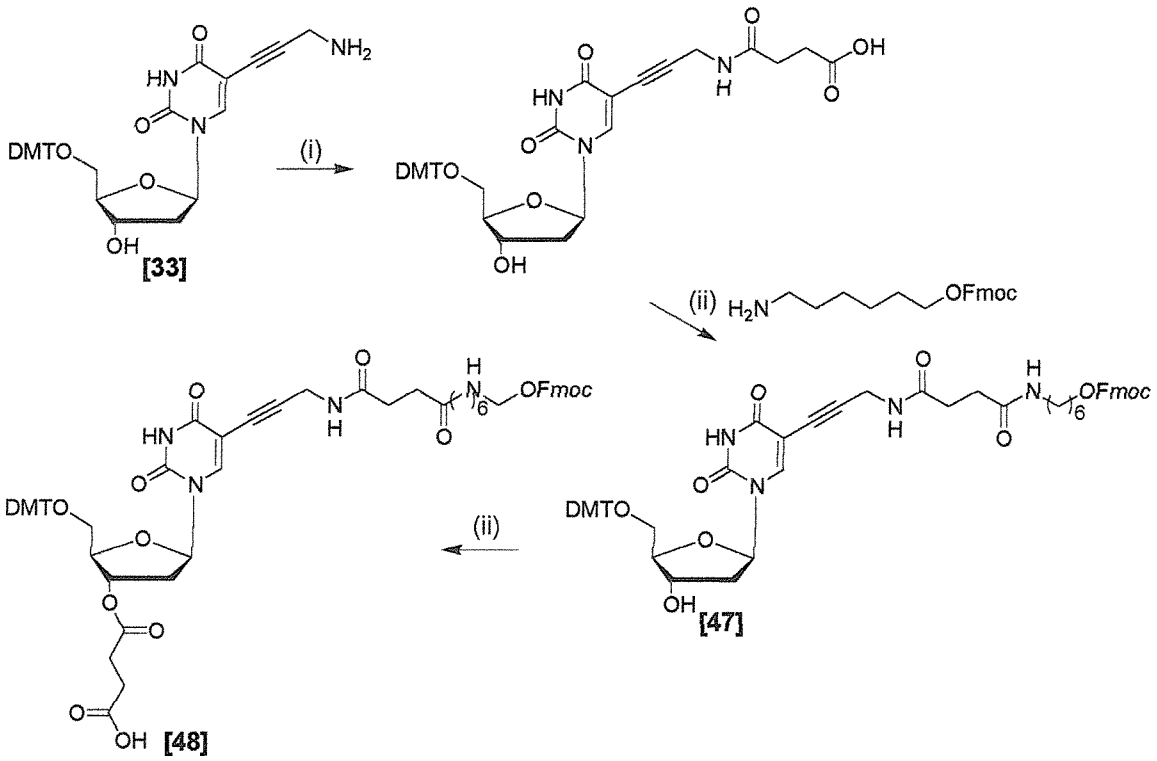



Figure 2.21 – UV/Vis spectra of fluorescein-15-dUTP **[44]** showing $\lambda_{max}$ values corresponding to fluorescein (483nm) and the nucleoside (286nm)

## 2.3.3 Synthesis of a Further Modified Nucleoside

Developments in probe technology have increased the demand for modified oligonucleotides and hence modified nucleosides. To fully exploit the range of commercially available fluorescent dyes and hence generate a library of labelled triphosphates, the modified nucleoside **[47]** was synthesised (figure 2.22).




*Reagents and conditions*: i, succinic anhydride, pyridine, r.t., 1 hr, ii, EDC, pyridine, r.t., 12 hrs, 41%, iii, succinic anhydride, DMAP, pyridine, r.t., 12 hrs, 81%

Figure 2.22 – Synthesis of modified nucleoside **[48]** with a linker containing a protectected hydroxyl terminus

Deprotection of the Fmoc protected hydroxyl terminus from the pendant linker arm of **[48]** allows the automated addition of commercially available non-nucleosidic fluorescent phosphoramidites such as the Cyanine Dyes™ (CyDye). This labelling methodology has been well established within our laboratory for labelling

oligonucleotides[189] and has been proven to be extremely effective on a CPG support. The nucleoside **[48]** was prepared by treating product **[33]** with succinic anhydride in pyridine and subsequent coupling of the Fmoc-hydroxyamine [190] with EDC gave nucleoside **[47]** in 41% yield over the two steps. This was again reacted with succinic anhydride and DMAP in pyridine affording nucleoside **[48]** in 81% yield that was then coupled with the commercial amino-CPG resin and converted to the resin-bound triphosphate **[49]** using the conditions previously described.

## 2.3.4 Automated Addition of Non-Nucleosidic Phosphoramidites

There is a wide range of commercially available fluorescent dye phosphoramidites (figure 2.23) that are routinely used in the synthesis of labelled oligonucleotides[189].

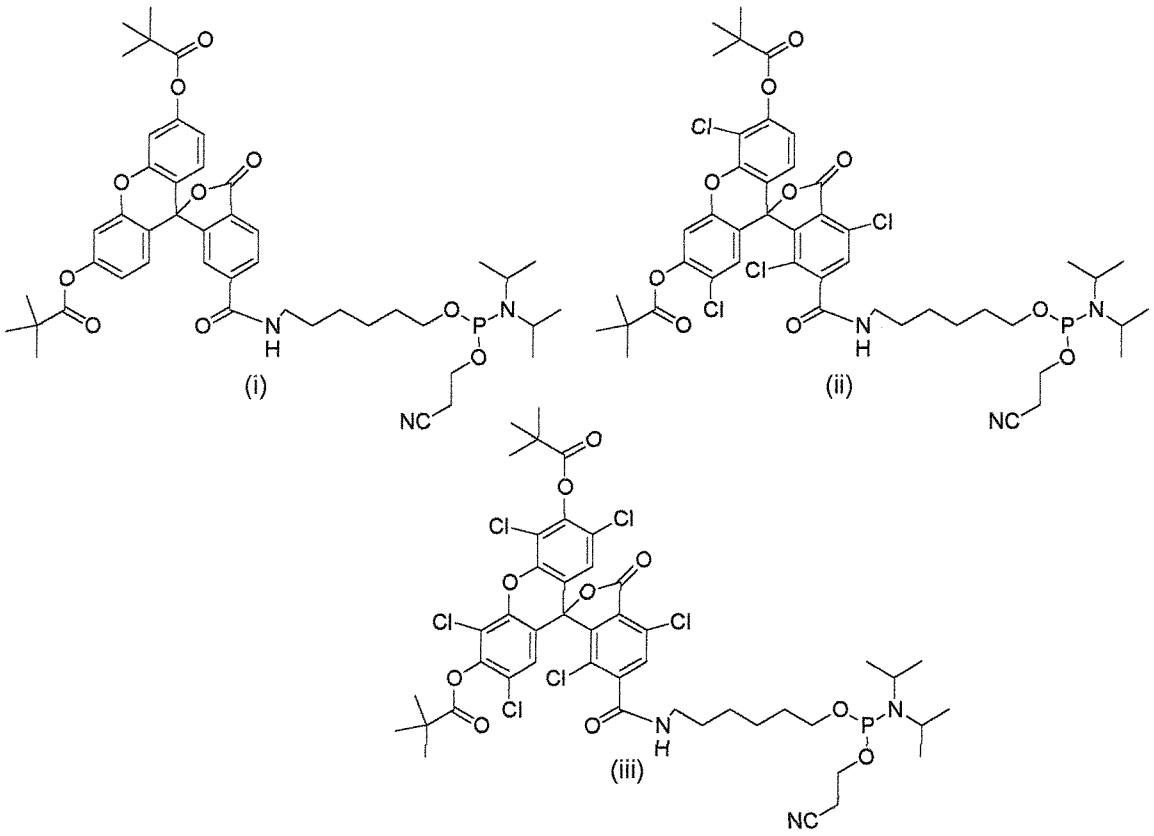
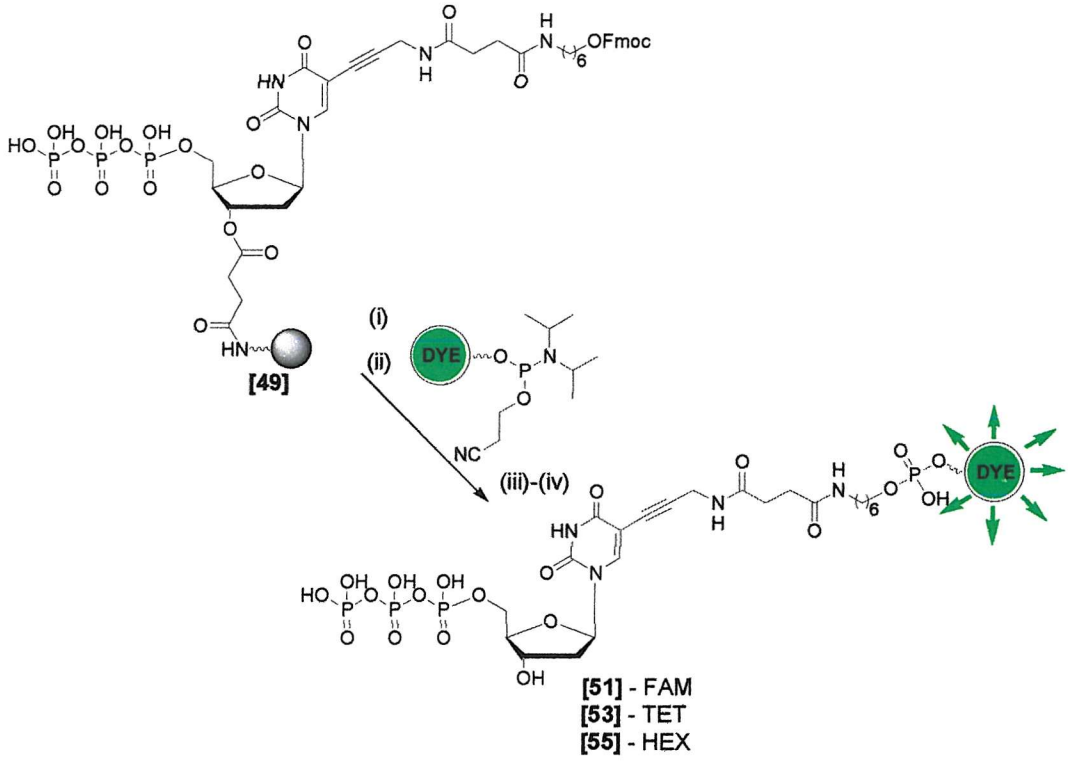



Figure 2.23 – i, Fluorescein phosphoramidite, ii, tetrachlorinated-fluorescein phosphoramidite, iii, hexachlorinated-fluorescein phosphoramidite

The idea was to introduce these dye phosphoramidites to the resin-bound modified triphosphate **[49]** on a DNA synthesiser (figure 2.24). Therefore, the appropriate amount of resin for 1.0 μmole scale oligonucleotide synthesis was utilised on an ABI 394 DNA synthesiser and the commercially available FAM, HEX and TET phosphoramidites were coupled to the resin using standard phosphoramidite chemistry[191].




*Reagents and conditions:* i, 20% piperidine in DMF, r.t., 10mins, ii, reactive dye phosphoramidite, tetrazole, iii, Iodine/pyridine/THF, iv, conc. aq. ammonia, r.t., 20 mins, 56% (FAM), 29% (TET), 35% (HEX)

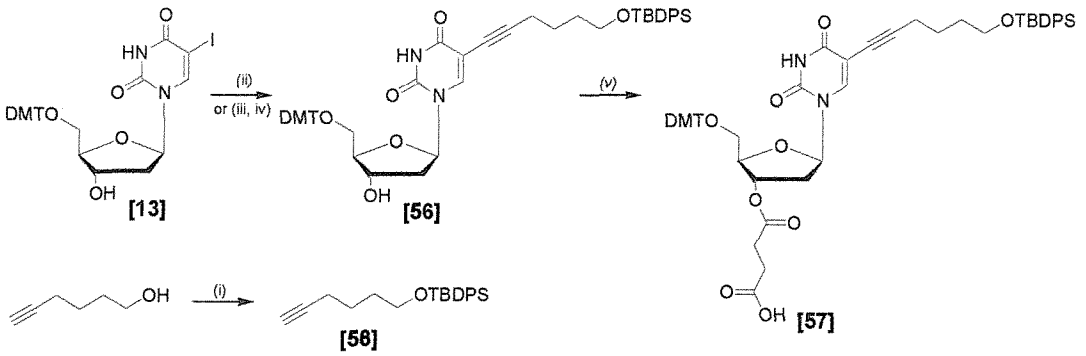
Figure 2.24 – Addition of dye phosphoramidites to resin **[49]** on DNA synthesiser

Cleavage of the fluorescently labelled triphosphates from the resin with ammonia was efficient and purification by anion exchange HPLC proceeded smoothly to give the labelled triphosphates **[51, 53, 55]** as coloured lyophilised solids in 29-56% yield.

### 2.3.5 Synthesis of an additional modified nucleoside

The introduction of fluorescent labels during PCR provides an alternative to traditional methods for the synthesis of labelled probes for use in hybridisation assays. This method relies on the recognition and incorporation of a modified nucleotide triphosphate by a DNA polymerase enzyme (see section 1.4.3.1). Previous studies within our laboratory have shown that the structure and length of the linker arm attaching the fluorescent dye to the triphosphate is crucial for successful PCR. Unpublished results have shown that replacement of the triple bond by more flexible spacers, for example saturated hydrocarbons, causes inhibition of PCR. To investigate which modifications may be tolerated, an alternative nucleoside with a protected terminal hydroxyl group (**[57]**, Figure 2.25) was synthesised by the two routes shown. However the synthesis was found to be more efficient by utilising hexyn-1-ol protected as a *t*-butyldiphenylsilyl ether in the Sonogashira reaction thereby following route (i, ii, v). Previous work in the Oswel laboratory had shown that extensive use of the phosphoramidite of the Fmoc nucleoside **[47]** for labelling oligonucleotides led to cleavage of the oligonucleotide from the resin, presumably due to extended treatment with piperidine. Therefore, the TBDPS protecting group was chosen for its increased stability towards acidic hydrolysis that the TBDMS group[252] thereby allowing the selective removal of the DMT group.

Attachment of compound **[57]** to a CPG solid support and conversion to the resin-bound triphosphate **[60]** proceeded in good yield. This allowed the appropriate amount of resin for 1.0 μmole scale oligonucleotide synthesis to be utilised on an ABI 394 DNA synthesiser and the commercially available FAM, HEX and TET phosphoramidites once again introduced using standard methods. Cleavage from the resin and purification was accomplished without complication, using the conditions described for triphosphates **[51, 53, 55]**, giving the FAM, TET and HEX labelled triphosphates **[62, 64, 66]** as colored lyophilised solids in 37-60% overall yield.




*Reagents and conditions*: i, *t*-butyldiphenylsilyl chloride, triethylamine, DMAP, DCM, 0°C to r.t., 12 hrs, 99%, ii, copper (I) iodide, triethylamine, compound **[58]**, $Pd(PPh_3)_4$, DMF, r.t., 12 hrs, 85%, iii, copper (I) iodide, triethylamine, hexyn-1-ol, $Pd(PPh_3)_4$, DMF, r.t., 18 hrs, 77%, iv, compound **[59]**, *t*-butyldiphenylsilyl chloride, triethylamine, DMAP, DCM, 0°C to r.t., 15 hrs, 97%, v, succinic anhydride, DMAP, pyridine, r.t., 12 hrs, 91%.

Figure 2.25 – Synthesis of modified nucleoside **[57]** with a protected hydroxyl terminus

## 2.3.6 CyDye Labelled Triphosphates

There is considerable commercial interest in having CyDye labelled triphosphates for applications such as DNA microarrays (see section 1.5.2). The triphosphate labelling methodology established using dye phosphoramidites was proposed to be a straight forward, efficient route to these compounds.

Using standard methods the commercially available Cy3 and Cy5 phosphoramidites were introduced to resin **[49]** and **[60]** producing a pink and blue coloured resin respectively. As before cleavage from the resin using either ammonia or ammonia : ethanol (3:1) produced highly coloured solutions (figure 2.26), however purification of these compounds was extremely problematic.

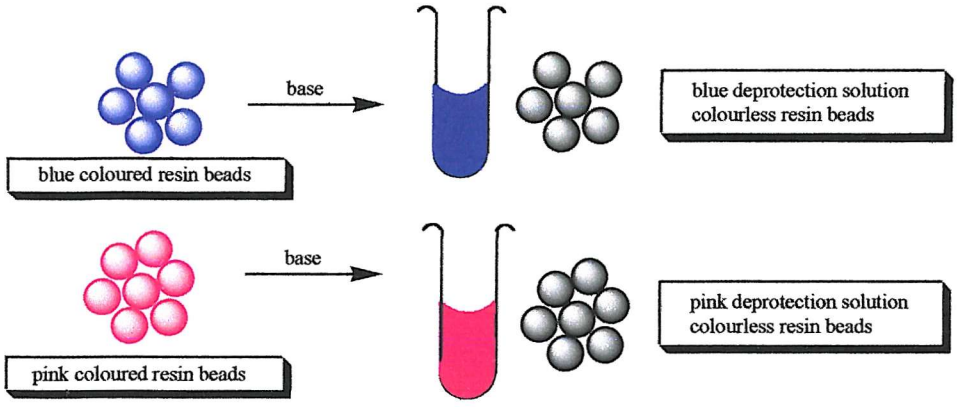



Figure 2.26 – Representation of CyDye introduction and deprotection

*2.3.6.1 Methods of Purification*

Purification by anion exchange HPLC using various buffer systems including gradients of ammonium acetate with acetonitrile and triethylammonium acetate with acetonitrile, was investigated. However disappointing results were obtained (figure 2.27 i). Despite the HPLC chromatogram showing no peak corresponding to unlabelled triphosphate, the only significantly coloured peak to elute was found to correspond to unreacted dye. As a result of these difficulties, it was thought that reverse-phase HPLC may be a more suitable system for purifying these compounds. Unfortunately this too gave unsatisfactory results (figure 2.27 ii).

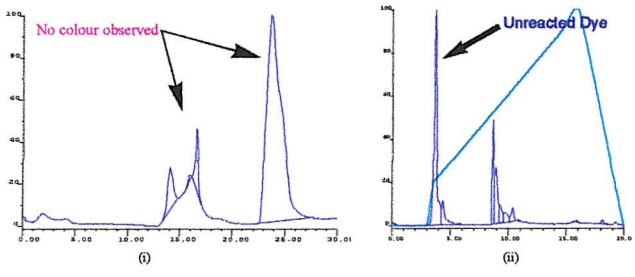



Figure 2.27 – i, Anion-exchange HPLC and ii, RP-HPLC of CyDye compounds

To simplify the purification process and make it more economical, preparative TLC was deemed a more suitable method of purification for these labelled compounds. A

solution obtained after cleavage from the resin was conc. *in vacuo* and dissolved in methanol. This was loaded onto the TLC plate and eluted with a mixture of *n*-butanol water and acetic acid (3:1:1). Unfortunately despite promising results from the TLC plate, analysis of the isolated band by HPLC, in various solvent systems showed a highly impure compound.

### *2.3.6.2 Solution-phase coupling*

The disappointing results from the initial methodology called for re-examination of an alternative strategy for introducing the Cyanine dyes. Since modified triphosphates have been derivatised in solution by reaction with *N*-hydroxysuccinimide esters of dyes in a simple labelling reaction it was considered beneficial to react a modified triphosphate in solution with an active ester of the cyanine dyes. This compound should be easier to purify and would provide a standard with which to aid in subsequent purification of CyDye-labelled triphosphates.

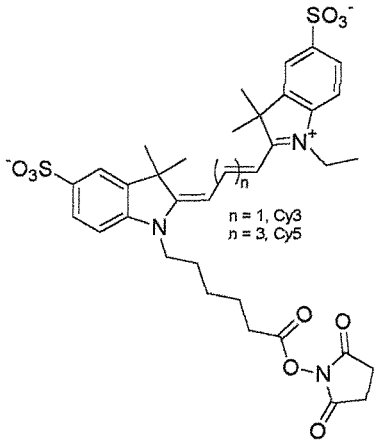



Figure 2.28 – Structure of commercially available, Cy3 and Cy5 active esters

Cy3 and Cy5 active esters were purchased from Amersham Pharmacia (figure 2.28). These were then reacted with the fully deprotected terminal amino group of triphosphate **[67]** obtained after cleavage from resin **[38]**. The coupling reaction was

performed by adding a solution of the dye in DMF to the amine in aqueous potassium hydrogen phosphate whilst protecting from light[179]. The product was readily purified by reverse phase HPLC to give triphosphate **[69]** and **[71]** in 40% and 41% yield respectively. Mass spectrometry confirmed isolation of the correct product.

### *2.3.6.3 Solid-phase coupling*

With a successful method for the synthesis and purification of CyDye-labelled triphosphates in hand, adaptation of this method to the solid-phase was investigated. Conveniently CPG resins which, proved to be the most suitable for triphosphate synthesis, do not require swelling and are compatible with aqueous buffer. Therefore transferring the conditions used in the solution-phase coupling of cyanine dyes to the solid-phase should be straightforward. Greater yields of labelled triphosphate were expected by this method as an excess of reagents can be employed to drive the reaction to completion. Hence, an excess of the dye in a solution of DMF was added to resin **[38]** (previously treated with piperidine to remove the protecting group) in aqueous potassium hydrogen phosphate buffer and protected from the light. Again, after extensive washing a coloured resin was observed. Two alternative cleavage conditions for removing the triphosphate from the resin were investigated, ammonia and water : methanol : tributylamine (2:1:1). Highly coloured deprotection solutions were produced with both conditions and satisfyingly, purification of these solutions by RP-HPLC proceeded smoothly (figure 2.29). Good yields of purified labelled triphosphate were obtained using both ammonia and water : methanol : tributylamine (2:1:1) however the latter proved more effective (86% c.f. 78% for Cy5 and 88% c.f. 80% for Cy3). Pleasingly this solid-phase method did indeed produce labelled triphosphates in greater yield than the corresponding solution-phase method.

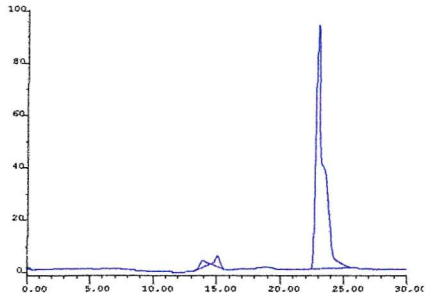



Figure 2.29 – RP-HPLC of triphosphate obtained from solid-phase coupling of Cy3

## 2.4 Conclusions and Future Work

A novel protocol for the solid-phase synthesis of labelled nucleoside-5'-triphosphates has been established. This method alleviates the constraints associated with solution phase methodologies and is a significant improvement both in time, efficiency and simplicity. The inherent advantages of solid-phase chemistry ensure higher yields are obtained as reactions are driven to completion. Reactions are simpler as only a final purification step is required. The advantages of this methodology are summarised in figure 2.30.

High yields of unlabelled triphosphate are routinely obtained on a CPG support and yields of labelled triphosphate are generally good. Use of a single regioisomer of the rhodamine dyes and slight optimisation of the reaction conditions for example a change of solvent or use of an alternative coupling agent such as TOTU may improve the yields of these compounds. CyDye-labelled triphosphates, which have significant applications in DNA microarrays can be obtained in excellent yield. However further endeavours are required to make this system compatible with CyDye phosphoramidites.

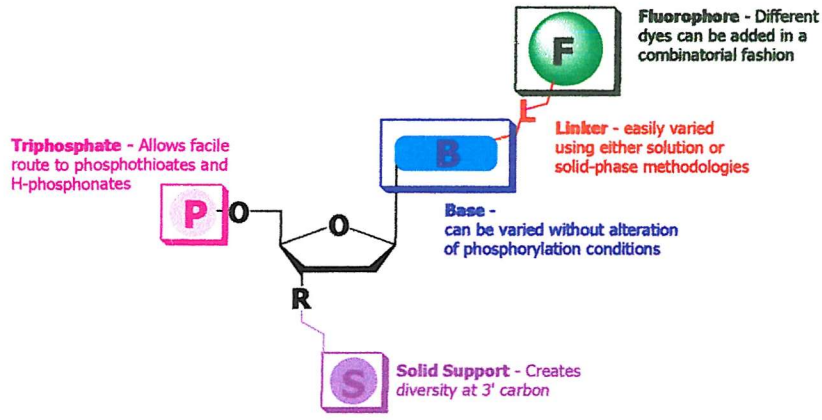



Figure 2.30 – Schematic representation of advantages of this methodology

Easy routes to a wide range of nucleoside derivatives are possible. In the future, phosphorothioates[192] and H-phosphonates[193] that have useful applications in antisense drug therapies may by easily synthesised by simply changing the iodine oxidation conditions to introduce sulphur. If required dideoxynucleotide triphosphates, or equivalent chain terminators used for Sanger[151] sequencing reactions may also be synthesised by simply changing the linker attaching the nucleoside to the solid support for a linker which releases for example an amino group. Alternatively by changing the position of attachment to the solid-support to, for example, the C5 position, and attaching the fluorescent dye to the 3' position of the sugar (figure 2.31 i), fluorescent chain terminators may be synthesised. In this system the fluorescent dye would be acting as the blocking group preventing chain elongation by the polymerase enzyme thus causing chain termination. Furthermore, after cleavage from the resin a second label may be introduced (figure 2.31 ii) such as an additional dye or an enzyme, thereby creating the potential for signal amplification by FRET or from the production of multiple signal-generating species by the enzyme.

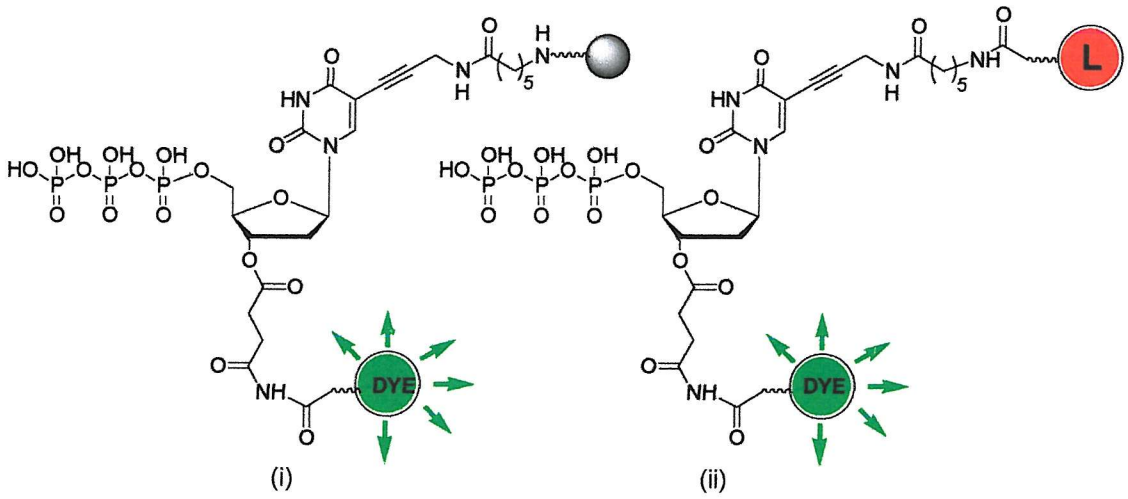



Figure 2.31 – Representation of i, resin-bound fluorescent chain terminator ii, a multiply labelled chain terminator.

# Chapter 3

# Biological Evaluation

# 3.0 Biological Evaluation

This chapter explains the microbiological assays performed in order to assess the suitability of the nucleoside triphosphate analogues **[40, 44-46, 51, 53, 55, 58, 62, 64, 66, 69, 71]** as substrates for DNA polymerase enzymes.

## 3.1 Introduction

Enzymatic labelling of DNA involves the incorporation of modified nucleoside triphosphates by a DNA polymerase enzyme (figure 3.1). Therefore it is important to synthesis nucleoside triphosphate analogues that do not interfere with or hinder the polymerase activity of the enzyme thereby preventing their incorporation into DNA.

In order to determine whether the fluorescently labelled triphosphate analogues **[40, 44-46, 51, 53, 55, 58, 62, 64, 66, 69, 71]** were suitable substrates for DNA polymerase enzymes, a series of PCR labelling experiments were performed.

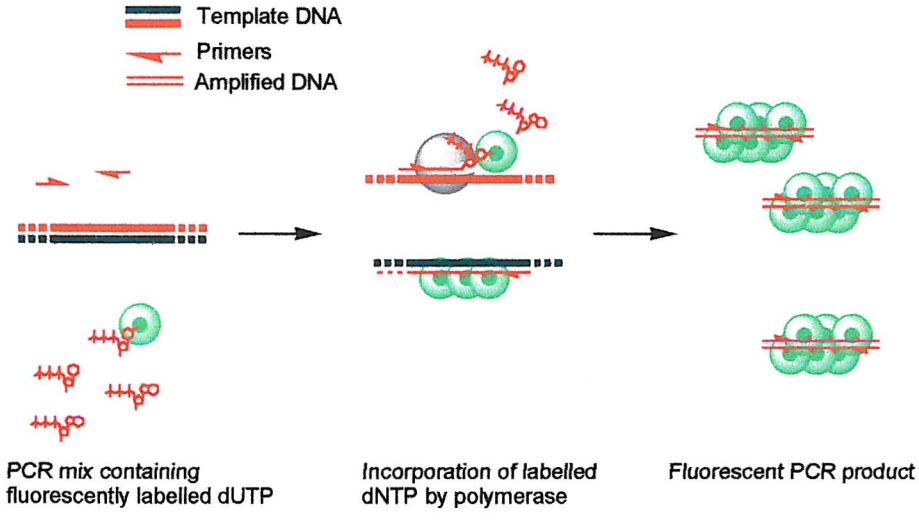



Figure 3.1 – Representation of incorporation of modified dUTPs during PCR producing labelled DNA

Previous work in the laboratory which, performed PCR labelling experiments in the presence of DNP labelled triphosphate analogues, demonstrated that increasing the molar ratio of dXTP (modified nucleoside triphosphate) to dTTP (thymidine triphosphate) present in the PCR experiment, resulted in a decrease in yield of a full length PCR product[58]. A similar effect was observed by Waggoner *et al*[108]. Here PCR experiments were performed with ratios of Cy3-dUTP to dTTP ranging from 0 to 10 and a steady decrease in the resulting yields was observed. However, in some cases the labelling reaction may be performed without inclusion of the natural dNTP in the reaction mixture. PCR products have successfully been labelled with 5-BrdUTP[128]; this analogue, which has a similar size to dTTP is incorporated by *Taq* DNA polymerase as efficiently as the natural substrate.

In order to investigate the effect of dXTP : dTTP molar ratios on PCR product yield, PCR experiments were performed in the presence of varying dXTP : dTTP ratios. Established protocols for PCR labelling reactions use a variety of concentrations of modified to unmodified dNTP's. For example, Flemming *et al*[126] suggest including *150μM of dTTP and 3μM bio-11-dUTP in PCR mixtures for the synthesis of 185-bp* biotinylated probes. Alternatively, Hiyoshi and Hosoi[89] used 33mM dTTP and 40mM fl-11-dUTP or rh-4-dUTP in PCR reactions. Having considered the ratios used in published labelling protocols, it was decided to perform a series of initial PCR experiments including 0-200μM dXTP and 200-0μM dTTP (figure 3.2). Different DNA polymerase enzymes were also investigated during PCR experiments.

| Experiment No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| dXTP : dTTP | 0:1 | 1:9 | 1:8 | 1:7 | 1:6 | 1.1 | 6:1 | 7:1 | 8:1 | 9:1 |
| % dTTP replaced with dUTP | 0 | 10 | 20 | 30 | 40 | 50 | 60 | 70 | 80 | 90 |

Figure 3.2 – Molar ratios of dXTP to dTTP used in PCR experiments incorporating dUTP analogues **[40, 44-46, 51, 53, 55, 58, 62, 64, 66, 69, 71]**. Also shown as % of dTTP replaced with dXTP

## 3.2 General PCR Method

PCR experiments using the modified triphosphates **[40, 44-46, 51, 53, 55, 58, 62, 64, 66, 69, 71]** were carried out using a 104mer synthetic oligonucleotide template **[122]** and 18mer synthetic primers **[120]** and **[121]**. The sequences of the template and primers used are shown in figure 3.3.

Unless otherwise stated, all PCR reaction mixtures were of 50μL volume, and contained the dNTP mixtures detailed in figure 3.2 and identical quantities of template, primers DNA polymerase enzyme, magnesium and PCR reaction buffer (concentrations are given in section 6.4.2). PCR reactions were carried out using the following temperature cycle: 50°C for 2 minutes, 95°C for 10 minutes followed by 22 cycles of 95°C for 0.5 minutes, 53°C for 0.5 minutes, 72°C for 0.5 minutes with a final extension period of 72°C for 7 minutes.

The PCR products obtained were analysed qualitatively by agarose gel electrophoresis of a 5μL aliquot of the crude reaction mixture. These products were then visualised by staining with ethidium bromide.

| Oligonucleotide | Sequence |
|---|---|
| Template **[122]** | GAATTCGAGCATCCTACCCACGATTCCGCTACGATCAACTAATACA CTTGGTATGAAACAATTTCCTCCCACTCACCCTGGCCCTATGCACG TGACACATAACC |
| Primer **[120]** | GGTTATGTGTCACGTGCA |
| Primer **[121]** | GAATCCGAGCATCCTACC |

Figure 3.3 – Sequences of template and primers used in PCR experiments incorporating dUTP analogues **[40, 44-46, 51, 53, 55, 58, 62, 64, 66, 69, 71]**

## 3.3 PCR labelling reactions with *Taq* DNA polymerase

The DNA polymerase enzyme *Taq* DNA polymerase, is a thermostable enzyme, with an optimum polymerisation temperature of 72°C[117] and is by far the most popular choice for the majority of PCR application, including the synthesis of labelled DNA.

*Taq* DNA polymerase was originally isolated from the extreme thermophile *Thermus aquaticus* by Trela *et al* in 1976[194] and was subsequently cloned and expressed *in E. coli* by Lawyer *et al* in 1989[195]. The enzyme has a molecular weight of approximately 96kDa and possesses 5'-3' polymerase and 5'-3' exonuclease activity[117, 195] but no 3'-5' exonuclease activity. The polymerase activity is highly processive and has a specific activity of approximately 150 nucleotides extended per second at its optimum temperature[196]. However, polymerisation occurs with relatively low fidelity compared to other enzymes used in PCR[197]. The error rate is approximately $1.1 \times 10^{-4}$ base substitutions per base pair[198], which is due, in part, to the lack of proof-reading activity of the enzyme[199].

Previous work has shown that *Taq* DNA polymerase is able to utilise a wide variety of nucleoside-5'-triphosphate substrate analogues (see section 1.4.3.1 and 1.5.3). It has been suggested that the ability of the enzyme to incorporate substrate analogues possessing bulky labels is due to the relatively open structure of the enzyme active site[196] however, detailed investigations have not been carried out.

Therefore it was envisaged that *Taq* DNA polymerase would be a suitable enzyme to perform initial PCR reactions with. Preliminary experiments were performed in the presence of the FAM-dUTP analogues **[40]**, **[44]**, **[51]** and **[62]** (figure 3.4) using the conditions described (see section 3.2 and 6.4.2).

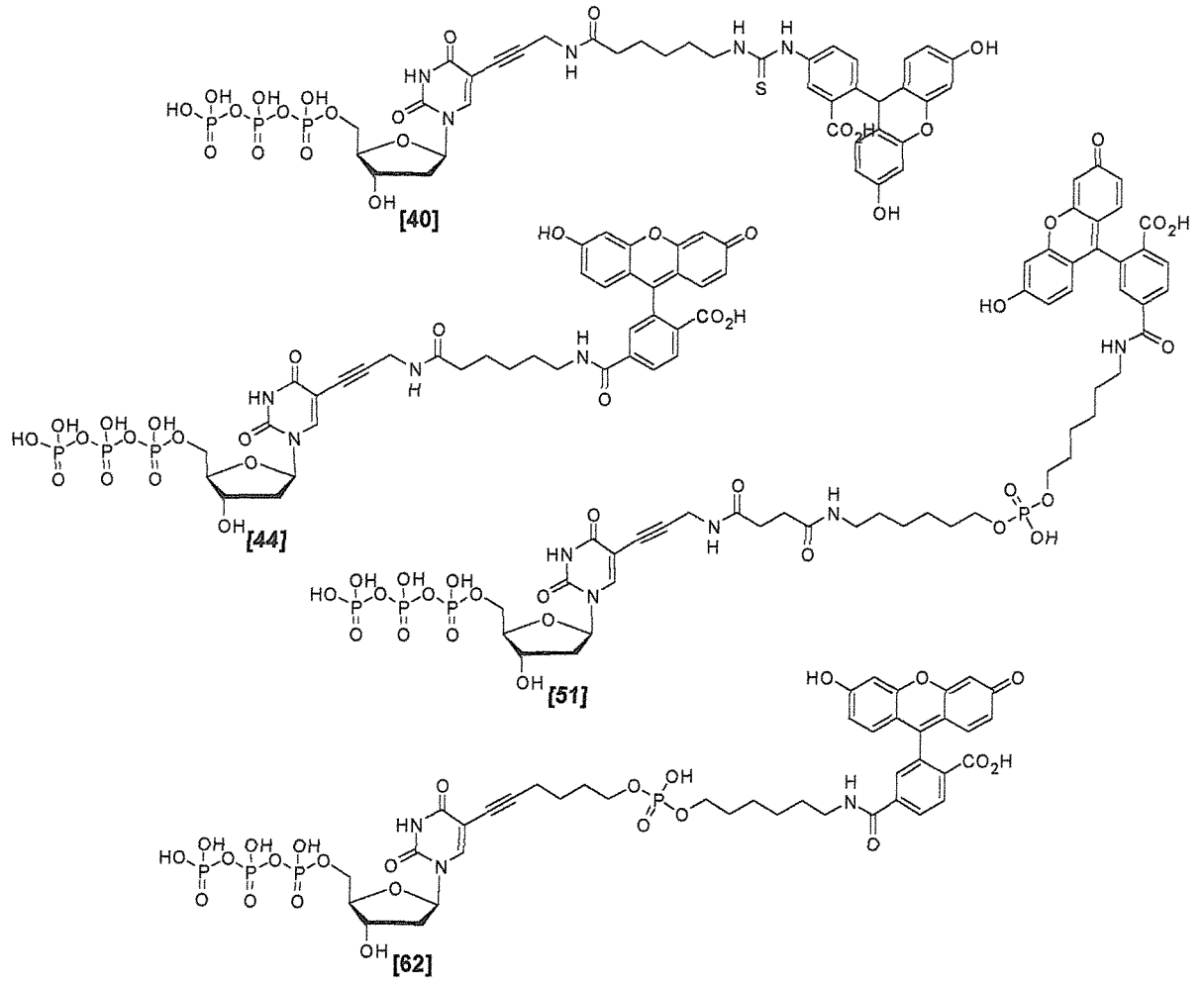



Figure 3.4 – Structures of the FAM-dUTP analogues **[40]**, **[44]**, **[51]** and **[62]** used in PCR experiments

It was anticipated that **[40]** and **[44]** would be incorporated by the DNA polymerase enzyme sufficiently well to produce a densely labelled PCR product. It was also assumed that the extended linker arm of **[51]**, which further removes the fluorescent dye from the nucleotide, would be beneficial, as it would minimise steric repulsion with the DNA duplex. However it was not clear what influence the extra phosphate group present in **[51]** would have on the PCR. Further more, no hypothesis could be made on what effect if any, the alternative structure of the linker arm of **[62]** which lacks an amide bond, would have on the success of PCR incorporation.

Agarose gel analysis of the PCR reactions carried out is shown in figure 3.5.

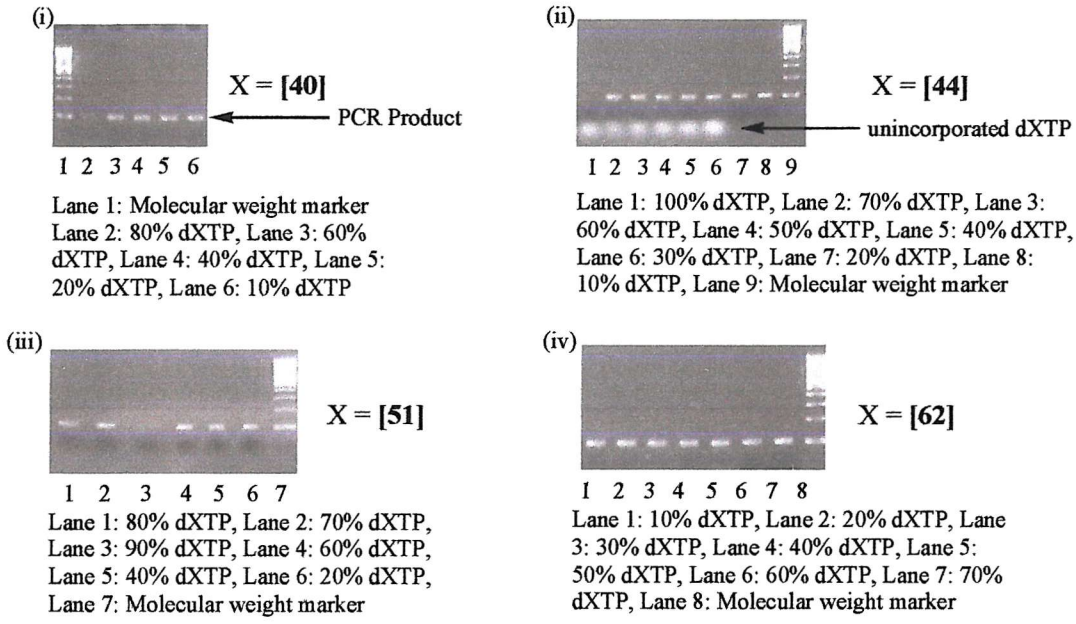



Figure 3.5 – Agarose gel analysis of PCR products synthesised with primer template sequences **[120-122]** in the presence of increasing dXTP to dTTP: i, triphosphate **[40]**, ii, triphosphate **[44]**, iii, triphosphate **[51]**, iv, triphosphate **[62]**

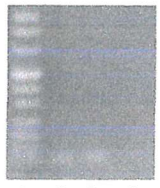
Qualitative analysis of the relative intensity of the bands corresponding to experiments 1-10 (figure 3.2), carried out for each dXTP, indicated that little decrease in yield was observed when up to 60% dTTP was replaced with dXTP for **[40]** and up to 70% dTTP was replaced with dXTP for **[44]**, **[51]** and **[62]**. Yields decreased sharply if 100% dTTP was replaced; a PCR product was formed in reasonable yield and purity if no dTTP was present only in the case of triphosphate **[44]**. However, in experiments in which no evidence of a product was observed by agarose gel, it could not be determined whether the reaction had failed completely, or the yield was too low to be detected by this method.

With successful PCR conditions in hand, the rhodamine labelled triphosphates **[45]** and **[46]** were evaluated in a similar PCR labelling reaction. Again, qualitative analysis of the relative intensity of the bands obtained from agarose gel

electrophoresis (data not shown), indicated that little decrease in yield was observed when up to 40% dTTP was replaced with dUTP analogues **[45]** and **[46]**.

Good results were also obtained from the PCR experiments performed in the presence of the TET and HEX labelled triphosphates **[53, 55]** and **[64, 66]**. After agarose gel analysis (data not shown), a good yield of PCR product was still observed for the TET labelled triphosphates when up to 60% dXTP replaced dTTP for triphosphate **[53]** and **[64]**. With the HEX labelled triphosphates a similar pattern was observed with good yields of PCR product when up to up 60% dXTP replaced dTTP for triphosphate **[55]** and up to 40% replacement for triphosphate **[66]**. These results suggest that there is no obvious benefit or detriment from either of the different structural linker arms employed.

The CyDye labelled triphosphates **[69]** and **[71]** were evaluated under the same PCR conditions. Rather disappointingly, the results obtained were similar to those obtained by Waggoner *et al*[108], with good yields of PCR product obtained when only 20% dTTP is replaced with dXTP for Cy3 triphosphate **[71]** (figure 3.6) and 30% dTTP replaced with dXTP for Cy5 triphosphate **[69]** (data not shown).



1 2 3 4

Lane 1: Molecular Weight Marker
Lane 2-4: 10-30% dTTP replaced with Cy3 dXTP **[71]**

Figure 3.6 – Agarose gel analysis of PCR products synthesised with primer template sequences **[120-122]** in the presence of increasing Cy3 dXTP **[71]** to dTTP with *Taq*

CyDye labelled triphosphates are generally employed in DNA microarray applications as these dyes are ideal for use as fluorescent probes because of their high extinction coefficients, high quantum yields and excellent photostability. However,

the low level of incorporation of these labelled triphosphates during PCR is a major drawback, drastically increasing costs. Therefore in a bid to improve the incorporation of these triphosphates a re-evaluation of the original PCR protocol was called for.

## 3.4 PCR labelling Experiments with $Vent_R^{®}$ (exo$^-$) DNA polymerase

A wide variety of DNA polymerases are commercially available for use in PCR[117] that provide variation in properties such as processivity, replication fidelity and associated activities. It was proposed that by changing the DNA polymerase enzyme used in the PCR experiments a higher proportion of CyDye-labelled triphosphates may be tolerated, thereby producing a probe with a greater labelling density. This would be of significant commercial interest with widespread applications.

$Vent_R^{®}$ (exo$^-$) DNA polymerase is a polymerase enzyme which is known to tolerate a wide range of nucleoside-5'-triphosphate substrate analogues and cosolvents[200]. The enzyme has been genetically engineered to eliminate the 3'-5' proof-reading exonuclease activity associated with the native enzyme and hence reducing the fidelity of polymerisation compared with the native enzyme. However the fidelity of this enzyme is still at a level two-fold higher than that of *Taq* DNA polymerase[201, 202]. $Vent_R^{®}$ (exo$^-$) is purified from a strain of *E. coli* that carries the Vent DNA polymerase gene from the archaea *Thermococcus litoralis*[203]. The enzyme has been successfully applied to primer extension reactions, thermal cycle sequencing and high temperature dideoxy-sequencing.

As a consequence of the high yields reported for primer extension reactions with $Vent_R^{®}$ (exo$^-$) and its ability to utilise a wide variety of nucleoside-5'-triphosphate substrate analogues, this enzyme was believed to be an extremely attractive

alternative to *Taq* DNA polymerase for incorporating CyDye labelled triphosphates **[69]** and **[71]**.

Initial PCR experiments using Vent$_R$® (exo$^-$) DNA polymerase were performed in the presence of the FAM labelled dUTP analogues **[44]** and **[62]**. This established the performance of Vent$_R$® (exo$^-$) under the PCR conditions previously exploited. Varying ratios of dTTP to dXTP was again investigated (figure 3.2) for a direct comparison of the incorporation ability of Vent$_R$® (exo$^-$) and *Taq* DNA polymerase. All other experimental parameters remained the same except for the reaction buffer. A Thermopol reaction buffer (see section 6.4.2) was used with Vent$_R$® (exo$^-$) DNA polymerase as this enzyme does not perform well in most commercially available *Taq* DNA polymerase buffers. Excellent results were obtained with this enzyme utilising the substrate analogues **[44]** and **[62]** (figure 3.7).

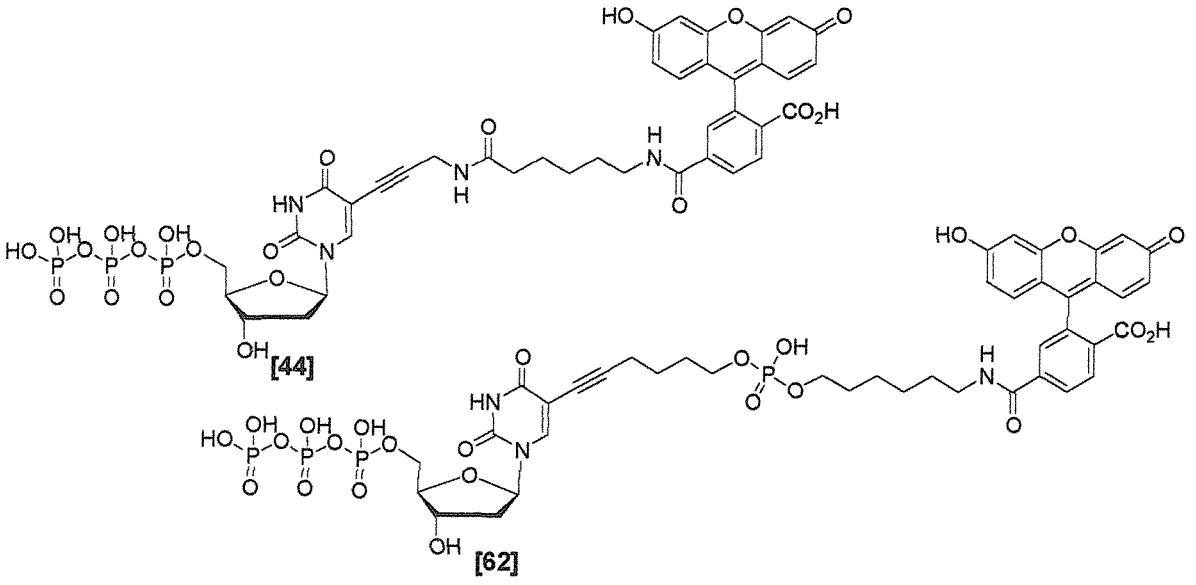
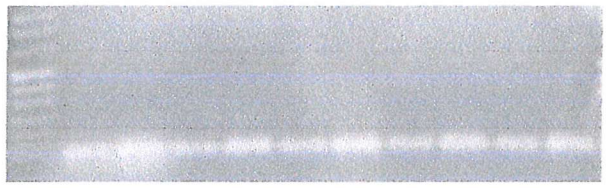



Figure 3.7 – Structures of FAM labelled triphosphates **[44]** and **[62]** used in PCR experiments with Vent$_R$® (exo$^-$)

Agarose gel analysis of the PCR experiments performed (figure 3.8 i) indicated that for both substrates **[44]** and **[62]** up to 90% replacement of the natural dTTP with the modified dXTP was tolerated whilst still producing a PCR product in good yield.

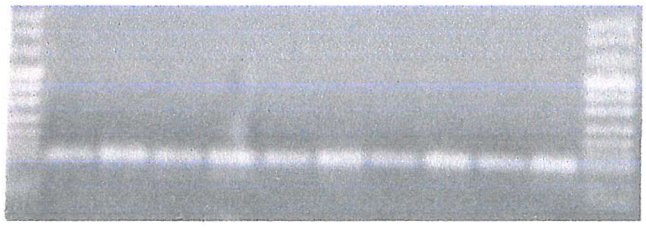
(i)



1 2 3 4 5 6 7 8 9 10 11

Lane 1: Molecular Weight Marker
Lane 2-6: 50-90% dTTP replaced with dXTP **[44]**
Lane 7-11: 50-90% dTTP replaced with dXTP **[62]**

(ii)



1 2 3 4 5 6 7 8 9 10 11 12

Lane 1 and 12: Molecular Weight Markers
Lane 2-6: 10-50% dTTP replaced with Cy3 dXTP **[71]**
Lane 7-11: 10-50% dTTP replaced with Cy5 dXTP **[69]**

Figure 3.8 – Agarose gel analysis of PCR products synthesised with primer template sequences **[120-122]** in the presence of increasing dXTP to dTTP: i, FAM dXTP's **[44]** and **[62]** with $Vent_R^{®}$ ($exo^-$), ii, Cy3 and Cy5 dXTP's **[71]** and **[69]** with $Vent_R^{®}$ ($exo^-$)

Following the successful incorporation of the FAM labelled substrate analogues with $Vent_R^{®}$ ($exo^-$) DNA polymerase, the next step was to examine the incorporation of the CyDye labelled triphosphate analogues **[69]** and **[71]** (figure 3.9) with this polymerase enzyme.

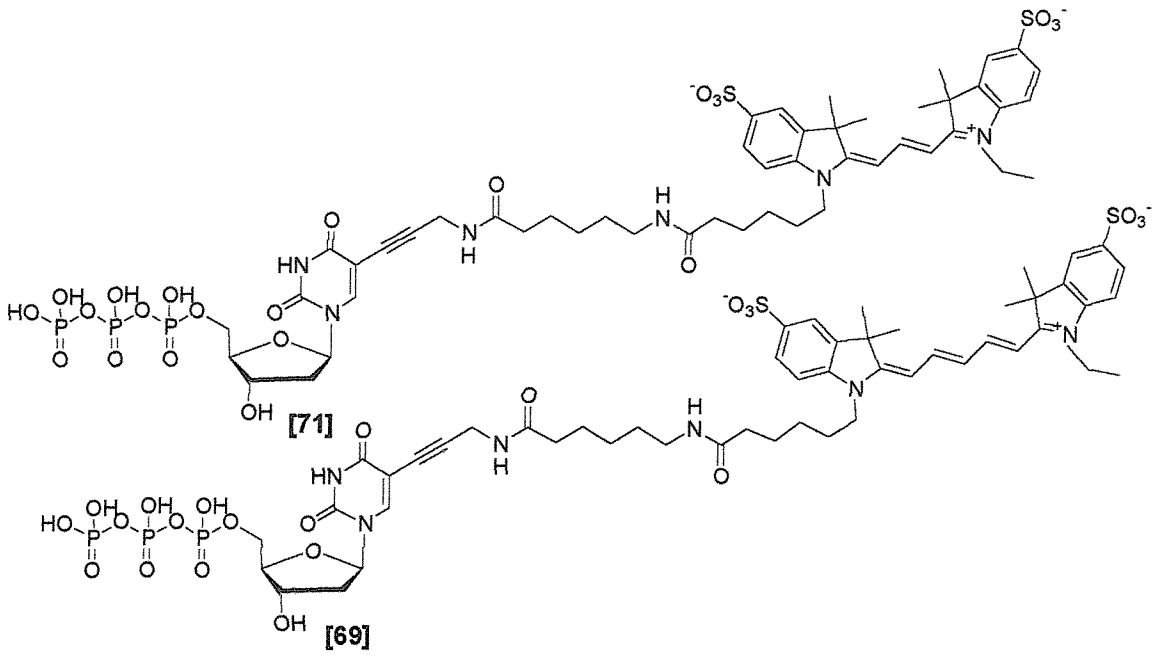



Figure 3.9 – Structures of CyDye labelled triphosphate **[69]** and **[71]** used in PCR experiments with $Vent_R^{®}$ (exo$^-$)

PCR experiments were performed in the presence of Cy3 triphosphate **[71]** and Cy5 triphosphate **[69]** with increasing ratios of dXTP to dTTP. Pleasing results were obtained from agarose gel analysis of these PCR experiments (figure 3.8 ii). Good yields of PCR product was still observed with $Vent_R^{®}$ (exo$^-$) when up to 50% dTTP was replaced with labelled triphosphate. This was a significant improvement over the results previously obtained with *Taq* DNA polymerase (figure 3.6). It is believed that the level of incorporation achieved with the TET and HEX labelled triphosphates together with the triphosphates labelled with TAMRA and ROX would also be improved with this enzyme.

## 3.5 Fluorescence Monitoring of PCR Products

Agarose gel analysis of PCR products cannot be used to verify that incorporation of the modified triphosphates is taking place. However, agarose gel analysis of a control

experiment (figure 3.10 i) performed in the presence of decreasing concentrations of dTTP only (figure 3.10 ii), indicated that a slight reduction in yield was observed in experiments containing only 10% of the initial dTTP concentration. No evidence of a PCR product was observed in experiments that contained no dTTP. These results suggest that dXTP incorporation occurred during PCR however further experimental evidence to support this hypothesis was required.

(i)

1 2 3 4 5 6 7 8

Lane 1 and 8: Molecular Weight Markers
Lane 2: 10% dTTP, Lane 3: 30% dTTP
Lane 4: 0% dTTP, Lane 5: 50% dTTP
Lane 6: 90% dTTP, Lane 7: 100% dTTP

(ii)

| dTTP conc. (μM) | 200 | 180 | 100 | 60 | 20 | 0 |
|---|---|---|---|---|---|---|
| % dTTP | 100 | 90 | 50 | 30 | 10 | 0 |

Figure 3.10 – Control experiment with dTTP only: i, agarose gel analysis of experiment, ii, table showing dTTP concentration used in experiment

It was proposed that fluorescence analysis of the purified PCR products would provide further confirmation of the incorporation of the fluorescently labelled triphosphates. The presence or absence of a fluorescent label in the PCR product may be noted by measuring the fluorescence emission at the emission maxima of the relevant dye.

### 3.5.1 Purification of PCR products

Fluorescence monitoring of the crude PCR mixture would provide ambiguous results as any fluorescence observed could possibly arise from unincorporated triphosphates. Therefore, three methods were assessed for the purification of the crude PCR reaction mixture:

*(i)* *Ethanol precipitation*

*(ii)* *DyeEx Spin Clean Up Kit*

(iii) *Agarose Gel Extraction*

Where possible ethanol precipitation is generally used for the purification of DNA as it is a relatively straightforward and cheap technique. Unfortunately this method proved to be unsuitable for purifying the PCR products. Agarose gel analysis of an aliquot of the purified DNA was found to still contain unincorporated fluorescent triphosphates.

Therefore a second method was investigated. DyeEx Spin Clean up Kits (Qiagen) are routinely used for successfully removing excess dNTP's from DNA sequencing reactions. This therefore seemed a feasible technique for the purification of the PCR products. However yet again, this method proved unsuitable. Fluorescence monitoring of a negative control PCR reaction, performed omitting template **[122]**, purified using the DyeEx Spin Clean up Kit, gave positive fluorescence properties. This could only result from the ineffectual removal of unincorporated fluorescent triphosphates from the crude PCR mixture.

Agarose gel extraction was the final purification technique investigated. Theoretically this technique should give unambiguous results. The technique involves separation of the crude PCR product by gel electrophoresis. Since the sample is separated according to size, isolation of a single band provides a unique sample of known molecular weight. Digestion of the agarose thus provides a pure sample of DNA. Although this technique was more complicated and tedious than the other two methods, pure samples of DNA were indeed obtained.

## 3.5.2 Fluorescence Monitoring

With the pure PCR products in hand, fluorescence emission monitoring experiments were undertaken. Samples of the pure DNA, obtained from the PCR experiments with

the modified triphosphates were placed in a fluorimeter cuvette and the fluorescence emission monitored at the emission wavelength of the relevant dyes (figure 3.11).

| Dye | Excitation Wavelength (nm) | Emission Wavelength (nm) |
|---|---|---|
| FAM | 500 | 525 |
| TET | 525 | 550 |
| HEX | 540 | 565 |
| TAMRA | 555 | 580 |
| ROX | 585 | 610 |
| Cy3 | 550 | 570 |
| Cy5 | 649 | 670 |

Figure 3.11 – Excitation and emission wavelengths of the dyes used in the production of labelled DNA

Consistently the results from these experiments indicated the incorporation of the nucleoside-5'-triphosphate analogues by displaying the correct emission properties of the individual dyes. As expected, an increase in fluorescence intensity was observed for the DNA obtained from the PCR experiments that utilised a higher ratio of dXTP to dTTP (figure 3.12).

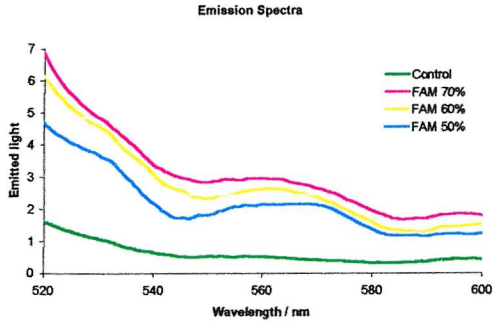



Figure 3.12 – Emission spectra of FAM-labelled DNA obtained from incorporation of triphosphate **[44]** as in figure 3.5ii

## 3.6 Conclusions and Future Work

The PCR experiments carried out in the presence of linker modified labelled dXTPs show that a product is formed in moderate to good yields when up to 90% dTTP is replaced with dXTP in the reaction mixture (figure 3.13). No additional advantage or disadvantage of the length of the linker attaching the dye to the nucleotide could be drawn from these experiments as no significant difference in results was observed for the dXTPs. However it was demonstrated that Vent$_R$® (exo$^-$) DNA polymerase was more tolerant to modified substrates than Taq DNA polymerase allowing for a higher proportion of dXTPs to be incorporated into the PCR product. This was of significant benefit for the CyDye labelled triphosphates. The observed decrease in yield when a particular percentage of dTTP is replaced with dXTPs is attributed to an increase in the formation of truncated products that cannot be amplified during PCR.

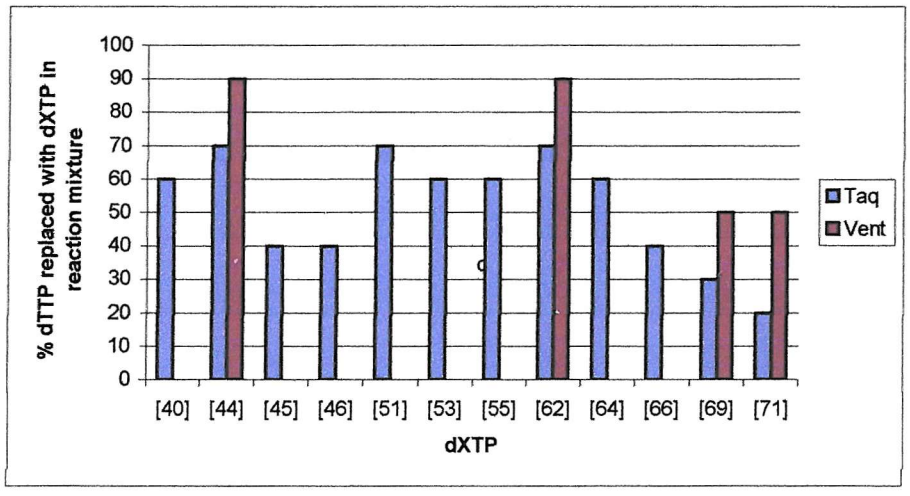



Figure 3.13 – Representation of the level of replacement of dTTP with dXTP achieved during PCR experiments using Taq and Vent$_R$® (exo$^-$) DNA polymerase enzymes.

Monitoring of the fluorescence emission spectra of DNA, obtained after PCR with the modified dXTPs, was considered a more meaningful method for detecting the

incorporation of modified triphosphates, than ethidium bromide staining of an agarose gel. The minimum detection level of an ethidium bromide stained agarose gel is ~5ng DNA[204], whereas previous work within the laboratory has shown that sensitivities in the picogram range have been achieved using fluorimetric detection techniques. Fluorescence-mediated detection of labelled PCR products verifies that dXTP incorporation has occurred. Signal intensity was seen to increase with the increased ratio of dXTP to dTTP included in the reaction mixture. Increased signal intensity is attributed to an increase in the amount of labelled PCR product. Therefore, the signal intensity may be further improved if enzyme concentrations and extension times are increased, as this is believed to increase the yield of PCR products.

A more precise investigation into the variation of labelling density with differing dXTP to dTTP ratios may be achieved by the enzymatic digestion of the PCR products produced.

Development of the incorporation of triphosphates containing multiple dyes (figure 3.14) is also of importance, for example in combinatorial labelling strategies. The use of such strategies also highlights the need for an increase in the number of dyes available with differing spectral properties, particularly long wavelength fluorescent dyes.

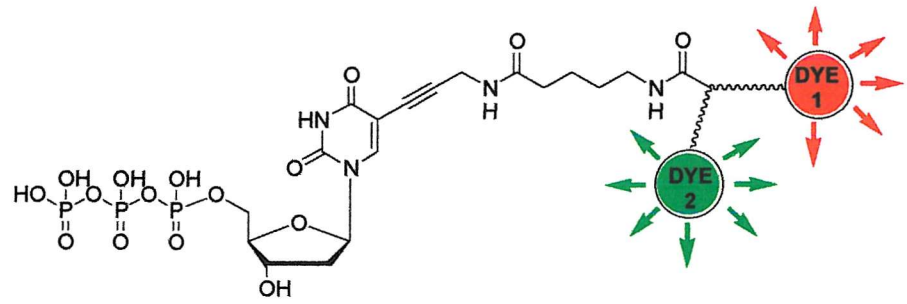



Figure 3.14 – Multiple dye labelled triphosphate for PCR incorporation studies

# Chapter 4

# Towards the Development of a Novel Real-Time PCR Assay System

# 4.0 Towards the Development of a Novel Real-Time PCR Assay System

This chapter explains the basis and development of a novel PCR based assay that requires only a probe and two primers with a single fluorescent dye to detect nucleic acid sequences.

## 4.1 Introduction

Both homogeneous (all components in solution) and heterogeneous (solid and solution-phase components) assay systems have been used to probe DNA sequences. However, homogeneous assays are considered to be more advantageous over their heterogeneous counterpart[205] as they do not rely on hybridisation of a probe to an insoluble support and therefore display faster hybridisation kinetics and the risk of contamination is minimised by the sealed-tube nature of the assay. In homogeneous assay formats there is no requirement for washing steps as the signal is generated only when the labelled probe is hybridised to the target.

Recently, the introduction of automated homogeneous PCR techniques has led to the development of novel assay systems that simplify the detection of PCR products. The absence of subsequent manipulations such as agarose gel electrophoresis significantly reduces the time needed for the analysis of results generated by these techniques. Indeed, in many cases, the results can be monitored in real-time.

Fluorescence [206, 207] is currently the favoured signalling technology for such homogeneous PCR assays. A number of techniques are currently available for specific sequence detection (see section 1.5.4, 1.5.5 and 1.5.6), with most of them relying on direct fluorescence or on energy transfer between a fluorophore and a proximal quencher molecule[208, 43].

These fluorescent probes have been used to discriminate between different alleles (variants of a single gene) and in particular, to Single Nucleotide Polymorphism substitutions (SNPs). SNPs represent the largest source of diversity found in mammalian genomes[209]. Some of these variations are directly linked to genetic diseases, though the vast majority are 'neutral'. Neutral variations are important because they are essential factors in linkage analysis that is used to identify genes responsible for complex disorders and in forensic analysis. Fast and automated genotyping methods are thus required for routine clinical diagnostics and forensic science. Some of the current alternatives to tedious sequence analysis[210] either miss some SNPs, or are too complex to enable high throughput assays.

Fluorescent probes provide a potential solution to the problem. Probes can be coupled with a wide variety of different fluorophores, allowing multiple targets to be identified in a single tube (a technique called 'multiplexing') (figure 4.1).

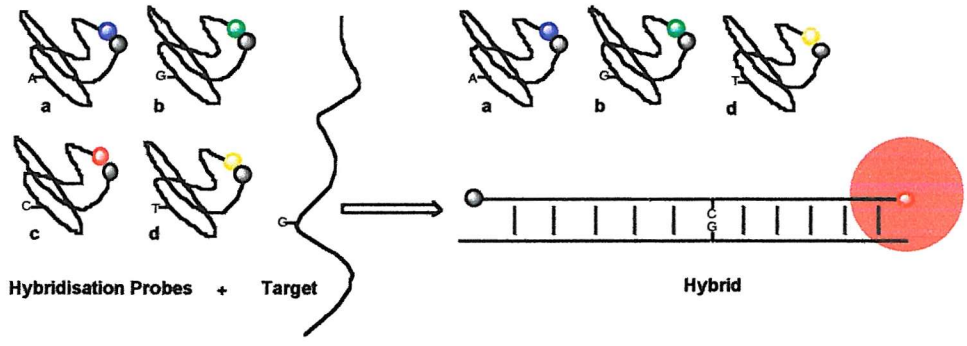



Figure 4.1 - Multiplexing. Only probe **c** is complementary to the target sequence. On binding to the target, **c** becomes fluorescent. Since probes **a**, **b** and **d** are not complementary to the target, they remain in a quenched form. The emission of the fluorophore can thus be used to identify the structure of the target.

Multiplex detection has previously been achieved using both Scorpion Primers[75] and Molecular Beacons[136] for the successful detection of SNPs. Both of these systems contain a fluorophore and a proximal quencher molecule attached to an

oligonucleotide (see sections 1.5.5 and 1.5.6) with detection arising from an increase in fluorescence when the fluorescent moiety is separated from the quencher.

A simpler assay that is amenable to high throughput genotyping programmes was proposed (figure 4.2). The assay required a probe to be labelled at the 5' end with a fluorescent dye, blocked against copying by a hexaethylene glycol PCR blocker at the 3' end and complementary to a known sequence on a target nucleic acid. Initially the probe will be fluorescent. During PCR, the target nucleic acid is in the presence of the probe, the unmodified dNTPs and a modified triphosphate labelled with a quencher molecule, (ideally non-fluorescent). A decrease in fluorescence will be observed when the probe hybridises and the fluorophore is quenched by the modified triphosphates incorporated into the nascent strand.

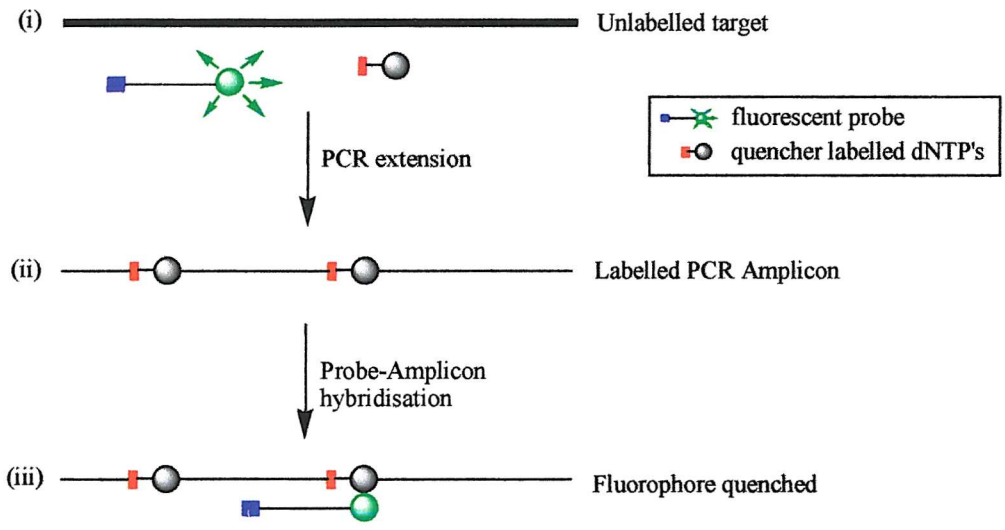



Figure 4.2 – Representation of novel assay (i) PCR reaction mixture containing target nucleic acid, labelled triphosphates and fluorescent probe, (ii) Incorporation of labelled triphosphates into PCR amplicon, (iii) Binding of probe to labelled amplicon resulting in loss of fluorescence

The assay format is homogeneous and potentially sensitive and specific. Theoretically the decrease in fluorescence observed can only arise from binding of the probe to the target amplicon (figure 4.3).

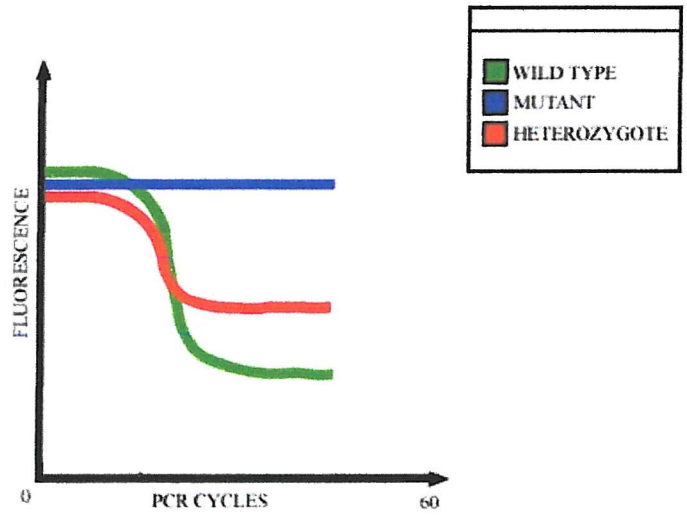



Figure 4.3 – Expected decrease in fluorescence with PCR cycles: i, normal DNA, ii, heterozgote, iii, homozgote mutant

In principle this assay can be adapted to mutation/allelic discrimination by monitoring the fluorescence at a temperature where the probe has dissociated from a target with a mismatch but remains bound to a fully complementary target. Discrimination between heterozygous and homozygous DNA should be discernible from melting analysis of the PCR product.

## 4.2 Synthesis of labelled triphosphates

The attachment of a suitable quencher to a modified triphosphate is the key starting point for assay development.

Routinely employed quenchers include 3-(4'-dimethylaminophenylazo) benzoic acid (Methyl-red) and 4,(4'-dimethylaminophenylazo) benzoic acid (DABCYL) (figure

4.4). These quenchers have been used successfully in systems such as Molecular Beacons and Scorpion Primers[211]. Due to its availability, Methyl-red was chosen as a suitable quencher for introduction into a modified triphosphate.

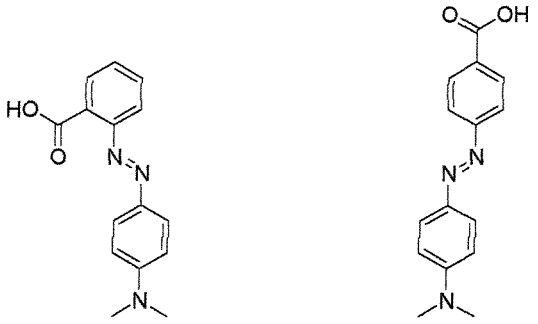


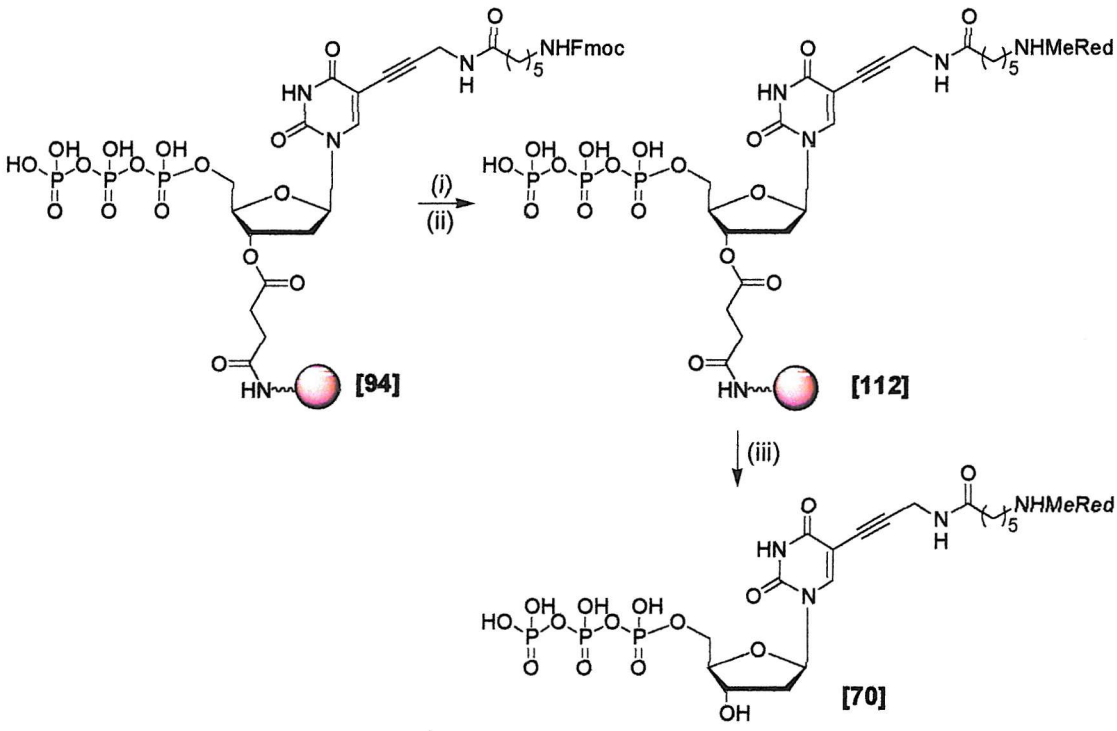

Figure 4.4 – Structure of commonly employed quenchers

## 4.2.1 Short-Chain Quencher Analogue

Coupling of the carboxylate group of methyl-red with an amino-modified triphosphate is a suitable method for introducing the quencher. This may be performed using either solution or solid-phase methodologies.

The methodology described in chapter 2 provided a straightforward route to an appropriately modified dU-5'-triphosphate **[67]**. As a consequence of the benefits found from attaching the cyanine dyes to this triphosphate on solid-phase (see section 2.3.6.3), it was decided to employ an identical approach for the introduction of methyl-red (figure 4.5). The resin **[38]** was treated with 20% piperidine in DMF to remove the Fmoc group and treated with a solution of methyl-red and EDC in pyridine. The resin was soaked in this solution for 12 hours before being washed and dried. The product was then cleaved from the resin using conc. aq. ammonia at room temperature for 20 minutes, and purified by anion-exchange HPLC giving the final product, as confirmed by mass spectrometry, as a red lyophilised solid in 39% yield. Yields were based on the resin-loading of the original nucleoside and was calculated

by measuring the concentration of a solution of the triphosphate using the extinction coefficient of the dye at the $\lambda_{max}$. For example, from 60mgs of resin with a loading of 21μmol $g^{-1}$, 451μg of pure labelled triphosphate was obtained, corresponding to a yield of 39%.

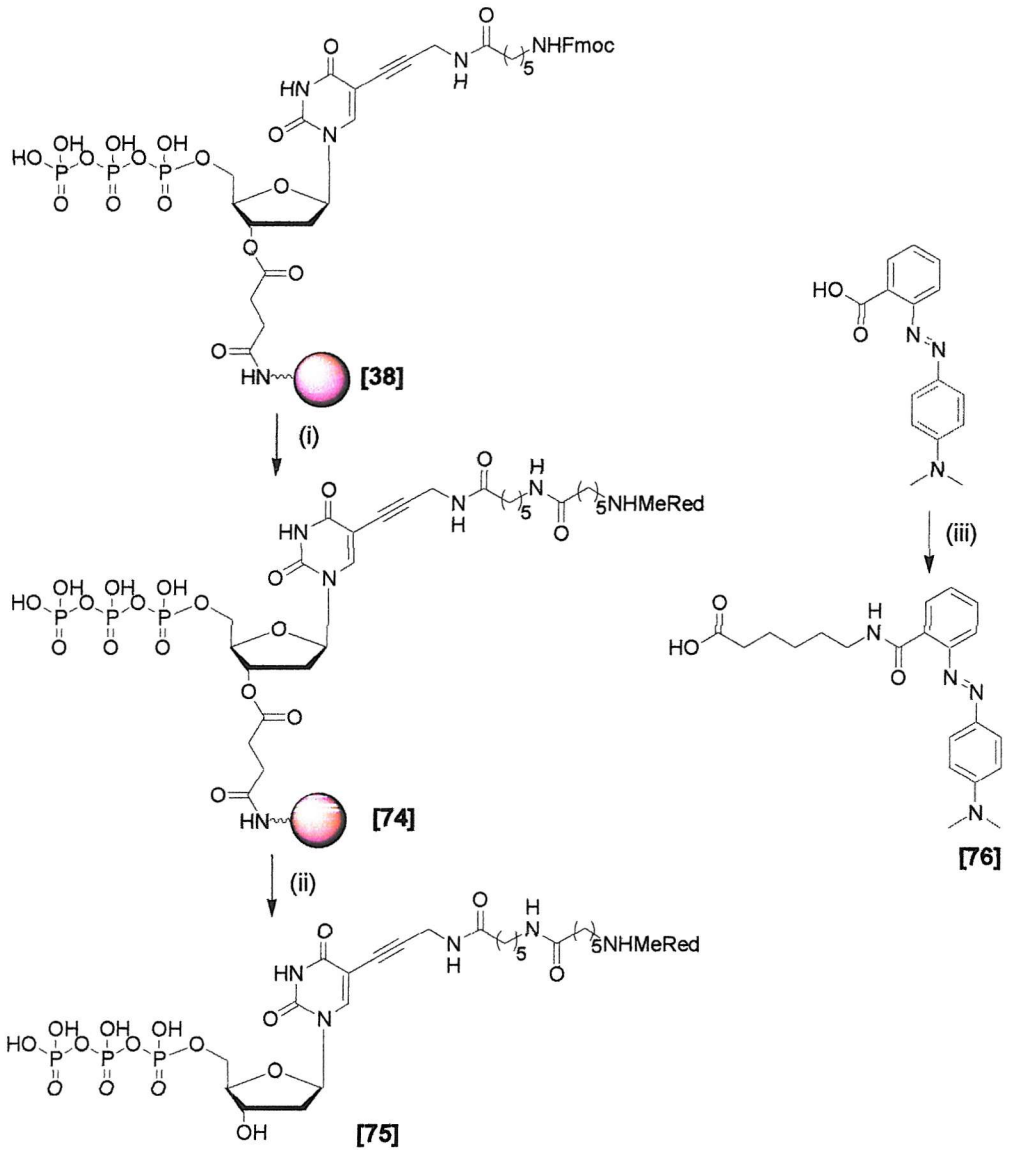



*Reagents and conditions*: i, 20% piperidine in DMF, r.t., ii, 10 mins, methyl-red, EDC, pyridine, r.t., 12 hrs, iii, conc.aq. ammonia, r.t., 20 mins, 39%

Figure 4.5 – Synthesis of triphosphate **[73]** labelled with methyl-red quencher

## 4.2.2 Long-chain Quencher Analogue

Good incorporation of the quencher-modified triphosphate is imperative for the success of the proposed assay. In an attempt to achieve this, a second labelled triphosphate **[75]**, which may prove to be more suitable was synthesised (figure 4.6). In this case the methyl red is attached to dU by a longer length linker.




*Reagents and conditions*: i, 20% piperidine in DMF, r.t., 10 mins, compound **[76]**, HOBt, DIC, pyridine, r.t., 2 hrs, ii, water : methanol : tributylamine (2:1:1), 60°C, 1.5 hrs, 58%, iii, 6-aminohexanoic acid, HOBt, DCC, $Et_3N$, DCM, r.t., 12 hrs, 61%

Figure 4.6 – Synthesis of the long-chain methyl-red quencher triphosphate **[75]**

We thought that this method would provide a better yield of labelled triphosphate as the coupling of the quencher moiety would be more efficient. We postulated that reaction of the carboxylate group of methyl-red with a relatively small alkyl-amino carboxylic acid such as 6-aminohexanoic acid, would proceed more readily than the

respective direct reaction with the larger triphosphate. This long-chain methyl red quencher molecule could then be coupled directly with the resin-bound triphosphate using carbodiimide coupling techniques.

Coupling of 6-aminohexanoic acid with methyl-red proceeded smoothly with DCC and HOBt to give the quencher derivative **[76]** in 61% yield. This was then reacted with the amino-terminus of resin bound triphosphate **[38]** (50mgs) (deprotected with piperidine as before) using carbodiimide chemistry thus providing the quencher-labelled triphosphate analogue on-resin as anticipated.

An aliquot of the resin was treated with ammonia, cleaving the product from the resin, and analysed by anion-exchange HPLC (figure 4.7 i). Despite the absence of the unlabelled triphosphate from the chromatogram, indicating that the reaction had gone to completion, a somewhat disappointing yield of labelled triphosphate was obtained (32%).

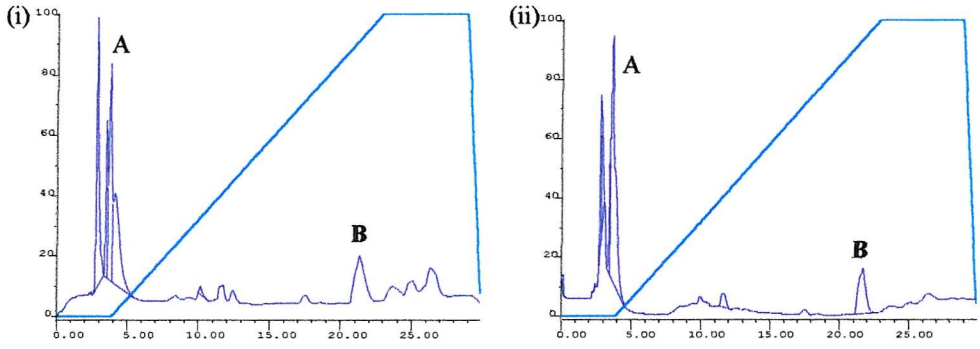



Figure 4.7 – Anion-exchange HPLC of labelled triphosphate **[75]** at 260nm, i, cleaved from the resin with ammonia, ii, further treatment of the resin with water : methanol : tributlyamine (2:1:1), A = excess dye, B = labelled triphosphate

This suggested that some product may still remain bound to the resin, which still remained red. Therefore an alternative method of cleavage was investigated. Experience gained from the rhodamine labelled triphosphates **[45]** and **[46]** indicated that treatment of the resin with water : methanol : tributylamine (2:1:1) at 60°C for

1.5 hours may be a more effective means of removing the product from the support. Therefore, the same aliquot of resin was treated in this manner and subsequently analysed by HPLC as before (figure 4.7 ii). A peak corresponding to labelled triphosphate was again observed. This concludes that effective cleavage of the labelled triphosphate is not possible with ammonia treatment of the resin.

Treatment of the remainder of the resin (40mgs) with water : methanol : tributylamine (2:1:1) at 60°C for 1.5 hours was performed. After subsequent purification by anion-exchange HPLC the methyl-red labelled triphosphate was obtained as a red-lyophilised solid in 58% yield (621μg), an improvement over the previous method in terms of yield and reaction times.

## 4.3 PCR Incorporation Studies

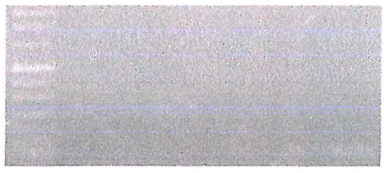
Before the quencher-labelled nucleoside-5'-triphosphates **[73]** and **[75]** could be employed in the proposed assay, evaluation of their incorporation by DNA polymerase enzymes was required.

### 4.3.1 *Taq* DNA Polymerase

Initial experiments were performed on triphosphates **[73]** and **[75]** using *Taq* DNA polymerase as this enzyme is routinely used with real-time PCR techniques such as Molecular Beacons, Scorpion Primers and Taqman assay[208].

Using the conditions described in chapter 3 and chapter 6 (section 3.2 and 6.4.2), a series of initial PCR experiments were performed including 0-200μM dXTP and 200-0μM dTTP (figure 3.2). Agarose gel analysis (figure 4.8) of the PCR experiments carried out indicated that that no PCR product was observed for either of the triphosphate substrates **[73]** or **[75]**. A PCR product was formed in the case of 100%

dTTP, performed as a control reaction, indicating that deterioration of reagents could not be the reason for the poor results. However, in the experiments in which no evidence of a product was observed by agarose gel, it could not be determined whether the reaction had failed completely, or the yield was too low to be detected by this method.



1 2 3 4 5 6 7

Lane 1: Molecular Weight Marker
Lane 2: 20% dXTP, Lane 3: 40% dXTP,
Lane 4: 60% dXTP, Lane 5: 80% dXTP,
*Lane 6: 90% dXTP, Lane 7: 100% dTTP*

Figure 4.8 – Agarose gel analysis of PCR experiments with methyl-red triphosphate **[75]** using *Taq* DNA polymerase.

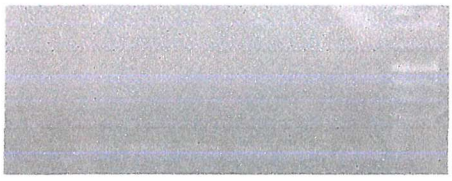
## 4.3.2 $Vent_R^{®}$ ($exo^-$) DNA polymerase

The improved levels of incorporation of modified triphosphates previously achieved when *Taq* DNA polymerase was replaced with $Vent_R^{®}$ ($exo^-$) DNA polymerase (see section 3.4) gave hope that this enzyme may be more suitable for incorporation of the methyl-red triphosphates **[73]** and **[75]**.

PCR experiments using $Vent_R^{®}$ ($exo^-$) DNA polymerase were performed in the presence of the methyl-red labelled dUTP analogues **[73]** and **[75]**. Varying ratios of dTTP to dXTP was again investigated (figure 3.2) for a direct comparison of the incorporation ability of $Vent_R^{®}$ ($exo^-$) and *Taq* DNA polymerase under the PCR conditions previously exploited. All other experimental parameters remained the same except for the reaction buffer. A Thermopol reaction buffer (see section 6.4.2) was used with $Vent_R^{®}$ ($exo^-$) DNA polymerase. Excellent results were obtained with

this enzyme utilising the substrate analogues **[73]** and **[75]**. Agarose gel analysis of the PCR experiments performed (figure 4.9) indicated that for substrates **[75]** up to 90% replacement of the natural dTTP with the modified dXTP was tolerated, whilst for substrate **[73]** up to 80% replacement of dTTP was tolerated whilst still producing a PCR product in good yield.

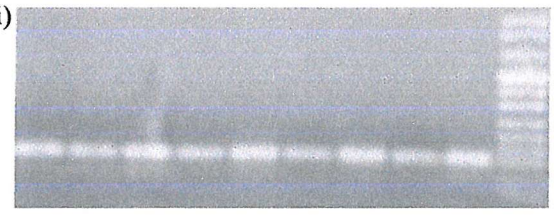
(i)



1 2 3 4 5 6 7 8

Lane 1: 100% dXTP, Lane 2:, 90% dXTP
Lane 3: 80% dXTP, Lane 4: 70% dXTP
Lane 5: 60% dXTP, Lane 6: 40% dXTP
Lane 7: 20% dXTP, Lane 8: Molecular Weight Marker

(ii)



1 2 3 4 5 6 7 8 9 10

Lane 1: 90% dXTP, Lane 2:, 80% dXTP, Lane 3: 70% dXTP, Lane 4: 60% dXTP, Lane 5: 50% dXTP, Lane 6: 40% dXTP, Lane 7: 30% dXTP, Lane 8: 20% dXTP
Lane 9: 10% dXTP, Lane 10: Molecular Weight Marker

Figure 4.9 – Agarose gel analysis of PCR products synthesised with primer, template sequences **[120-122]** in the presence of increasing dXTP to dTTP: i, Methyl-red dXTP **[73]** with $Vent_R^{®}$ ($exo^-$) ii, Methyl-red dXTP **[75]** with $Vent_R^{®}$ ($exo^-$)

## 4.4 Assay Evaluation

The quencher-labelled triphosphates had been synthesised and conditions established for their introduction into DNA enabling conditions for the assay (as described in section 4.1) to be designed and evaluated.

### 4.4.1 Real-Time PCR

The assay required a probe to be labelled at the 5' end with a fluorescent dye, blocked against extension by a hexaethylene glycol spacer at the 3' end and complementary to a known sequence on a target nucleic acid. The probe **[123]** was designed and synthesised to enable the probe to hybridise to the middle section of the PCR amplicon. The sequence of the probe is given in chapter 6 (see section 6.3.2)

To be amenable to high throughput genotyping programmes, the assay requires results to be generated in real-time preventing the bottleneck that occurs with gel-based methods. This can be achieved by using a Roche LightCycler, an instrument that possesses a 488 nm light source that is capable of continuous fluorescence monitoring and of very rapid temperature transitions. An added advantage of the LightCycler is its speed; PCR experiments can be performed and results obtained in less than 40 minutes.

### 4.4.2 PCR Experiments on the Roche LightCycler

#### *4.4.2.1 General Method*

PCR experiments using the modified triphosphates **[73]** and **[75]** were carried out using a 104mer synthetic oligonucleotide template **[122]** and 18mer synthetic primers **[120]** and **[122]**. The sequences of the template and primers used are shown in figure 3.3.

Unless otherwise stated, all PCR reaction mixtures were of 10μL volume, and contained the dNTP mixtures detailed in figure 3.2 and identical quantities of template, primers DNA polymerase enzyme, magnesium, PCR reaction buffer and BSA (concentrations are given in section 6.4.2). PCR reactions were carried out using the following cycling conditions: 95°C for 3 minutes followed by 100 cycles of

ramping to 95°C, then 52°C and monitoring for 3 seconds. A single fluorescence measurement was made for each cycle during the monitoring step.

On the LightCycler, PCR experiments are performed in glass capillaries and therefore require the addition of BSA (bovine serum albumin) to prevent reagents from sticking to the glass and hindering successful amplification.

*4.4.2.2 Experimental considerations*

The successful achievement of three separate experimental stages was required in order to confirm the efficacy of the overall assay system:

(i) PCR experiments in the presence of methyl-red dNTP's with SYBR Gold replacing the probe to establish that PCR is working sufficiently well on the LightCycler. SYBR Gold is a fluorescent intercalator that becomes fluorescent only on binding to DNA duplexes. Since the methyl-red dNTP's are non-fluorescent, SYBR Gold provids a means of signal generation.

(ii) PCR experiments using the conditions established in (i) with the addition of the probe to monitor the efficiency of PCR after addition of the probe.

(iii) PCR experiments omitting SYBR Gold thereby confirming that fluorescence quenching is solely from the probe hybridising to the PCR amplicon.

The logical starting point was to perform PCR experiments in the presence of differing ratios of dXTP to dTTP as before using $Vent_R^{®}$ ($exo^-$) DNA polymerase and including BSA and SYBR Gold in the reaction mixture. For the experiments, 1μL of SYBR Gold was added from a 1/1000 dilution of the stock, and 250ng $\mu L^{-1}$ BSA was added. The fluorescence output was monitored at each of the anneal/extend segments and plotted against cycle number. The fluorescence accumulation pattern allowed ready detection of PCR amplification by monitoring the increase in fluorescence. The results from these experiments were disappointing (figure 4.10). Poor results were obtained even with 100% dTTP indicating that the PCR was not working efficiently.

If the PCR was working as expected a much greater increase in fluorescence intensity should have been observed for 100% dTTP. Therefore a re-evaluation of the PCR conditions was called for.

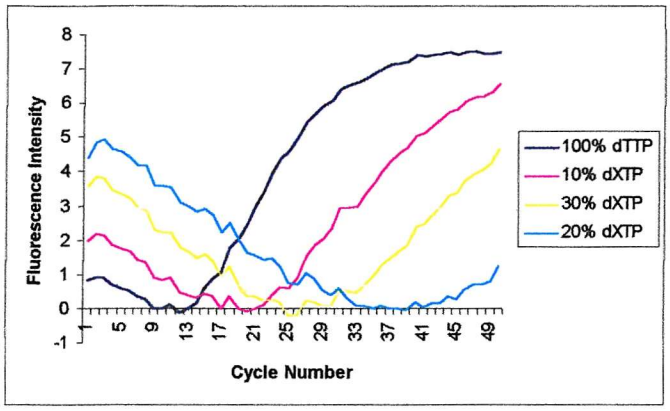



Figure 4.10 – Results obtained from PCR experiments in the presence of methyl-red triphosphate **[75]** using $Vent_R^{®}$ (exo$^-$) on Roche LightCycler, channel 1arithmetic mode

Conditions for successful PCR on the LightCycler using $Vent_R^{®}$ (exo$^-$) were required. No protocols for using $Vent_R^{®}$ (exo$^-$) DNA polymerase for real-time PCR could be found in the literature. Therefore, after reviewing basic PCR trouble shooting protocols, it was decided to perform a series of experiments using only the natural dNTP's at various different magnesium concentrations (figure 6.4). Higher concentrations of magnesium chloride are normally used in conjunction with *Taq* DNA polymerase on the LightCycler[211] than those used traditionally with $Vent_R^{®}$ (exo$^-$) DNA polymerase. $Mg^{2+}$ concentration was varied from 2mM to 16mM with little success (figure 4.11). The increase in fluorescence observed between cycles 8-13 arises too early to be due to a full length PCR product and most likely arises from primer dimers. Analysis of these PCR tubes by agarose gel electrophoresis with a molecular weight marker would provide confirmation of this hypothesis. Sadly this proof was not obtained.

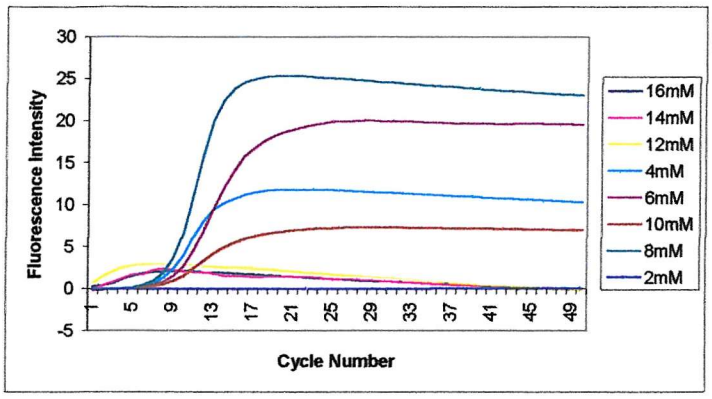



Figure 4.11 – Results obtained from PCR experiments at varying concentrations of magnesium using Vent$_R$® (exo$^-$) DNA polymerase on LightCycler, channel 1, arithmetic mode

*4.4.2.3 Conclusions*

These results together with the absence of literature on the use of Vent$_R$® (exo$^-$) DNA polymerase with the Roche LightCycler imply that perhaps this enzyme is not compatible with the rapid temperature transitions associated with this machine. Alternatively the addition of BSA, required for the LightCycler may in some way hinder the activity of the enzyme.

## 4.4.3 PCR Experiments on ABI7700 Genetic Analyser

The ABI 7700 Genetic Analyser is a real-time PCR instrument more similar to traditional PCR instruments than the Roche LightCycler. This instrument uses plastic tubes rather than glass capillaries and therefore does not require the addition of BSA. Hence this instrument was considered to be more suitable in this application. Previous research in the Oswel laboratory had established effective PCR conditions on the ABI7700 and so these were employed in conjunction with the methyl-red triphosphates.

*4.4.3.1 General Method*

PCR experiments using the modified triphosphates **[73]** and **[75]** were carried out using a pool of cDNA obtained from reverse transcription of Human heart total RNA **[126]** together with a 23mer synthetic primer **[124]** and 20mer synthetic primer **[125]**. The sequences of these primers are given in chapter 6 (see section 6.3.2).

Unless otherwise stated, all PCR reaction mixtures were of 50 or 25µL volume, and contained the dNTP mixtures detailed in figure 3.2 and identical quantities of template, primers DNA polymerase enzyme, magnesium and PCR reaction buffer (concentrations are given in section 6.4.2). PCR reactions were carried out using the following cycling conditions: 95°C for 3 minutes followed by 40 cycles of 95°C for 15 seconds, 60°C for 60 seconds and monitoring for 3 seconds. A single fluorescence measurement was made for each cycle during the monitoring step.

Satisfyingly, good results were obtained using the ABI7700 instrument (figure 4.12). Successful PCR was achieved when only the natural dNTP's were used and also when up 40% dXTP replaced dTTP for the methyl-red triphosphate **[73]** and **[75]**. These results support the hypothesis that this instrument is much more compatible with $Vent_R^{®}$ (exo$^-$) DNA polymerase.

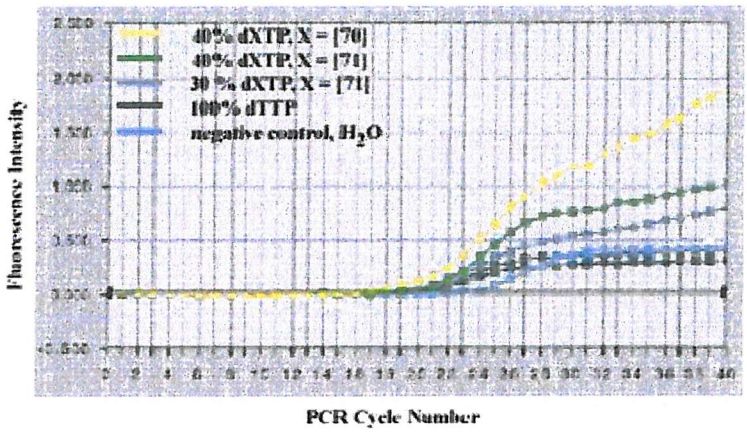



Figure 4.12 – Results obtained from PCR experiments in the presence of methyl-red triphosphate **[75]** using $Vent_R^{®}$ (exo$^-$) on an ABI7700 Genetic Analyser with SYBR Green detection

## 4.5 Conclusions and Further Work

The synthesis of two non-fluorescent triphosphate analogues **[73]** and **[75]** was accomplished. These were found to be suitable substrates for $Vent_R^{®}$ ($exo^-$) DNA polymerase enzyme when up to 90% of the natural dTTP was replaced with the modified dXTP using a standard Perkin Elmer GeneAmp 2400 Thermal Cycler.

On the basis of these results attention was turned to real-time PCR and the use of the Roche LightCycler. However despite several endeavours, successful PCR could not be obtained even when only natural dNTP's were used. The reason for the lack of success is attributed to an incompatibility of $Vent_R^{®}$ ($exo^-$) DNA polymerase with this instrument.

Greater success with real-time PCR was achieved by using an ABI 7700 Genetic Analyser. Good results were obtained when up to 40% of the quencher-labelled triphosphates **[73]** and **[75]** replaced dTTP. This is believed to be due to the similarities between this machine and regular thermal cyclers that have proved to successfully incorporate high levels of the triphosphates.

To study the assay further requires suitable conditions for the addition of the fluorescent probe to be established.

The principle of this assay might also be applied to Scorpion Primers (figure 4.13). PCR experiments could be performed using the quencher-labelled triphosphates and a modified Scorpion Primer. The Scorpion Primer comprises of an oligonucleotide probe, with a 5' fluorescent dye, that is attached to the 5'-end of one of the PCR primers. Read-through into the probe element is blocked by a hexaethylene glycol spacer. On binding to the target the primer is extended by polymerisation, the probe hybridises to the newly synthesised strand and fluorescence is subsequently quenched by the modified triphosphates. This system should combine the advantages of Scorpion Primers (see section 1.5.6) with a more simple system.

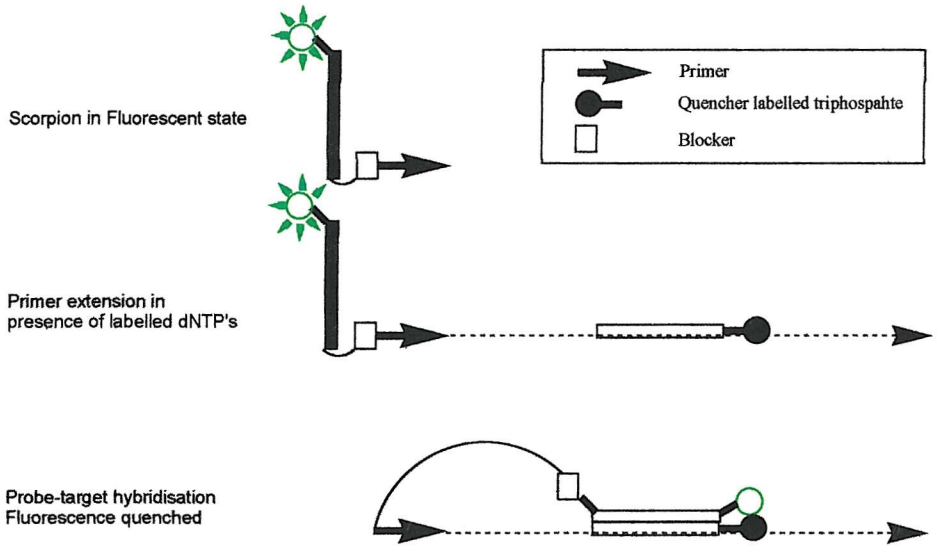



Figure 4.13 – Suggested application of assay to a modified Scorpion Primer system.

# Chapter 5

# Synthesis of Modified Nucleoside Derivatives

# 5.0 Synthesis of Modified Nucleoside Derivatives

This chapter is focussed on the synthesis and diagnostic and therapeutic applications of modified nucleic acid derivatives. The synthesis of a Peptide Nucleic Acid monomer and a C-nucleoside is described.

## 5.1 Introduction

Since the discovery of oligonucleotides as therapeutics, the search for nucleic acid mimetics with improved properties such as strengthened binding-affinity to complementary nucleic acids, increased biological stability and improved cellular uptake, has accelerated rapidly.

### 5.1.1 Peptide Nucleic Acids

The most interesting of the new nucleic acid mimetics to arise from previous research were discovered by Nielsen *et al*[212-215] and named Peptide Nucleic Acids (PNAs) since the entire sugar-phosphate backbone is replaced by an *N*-(2-aminoethyl)glycine polyamide structure (figure 5.1).

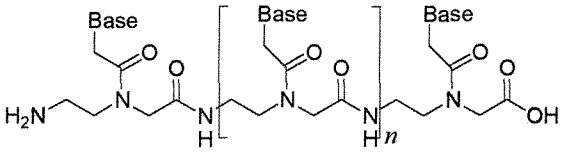


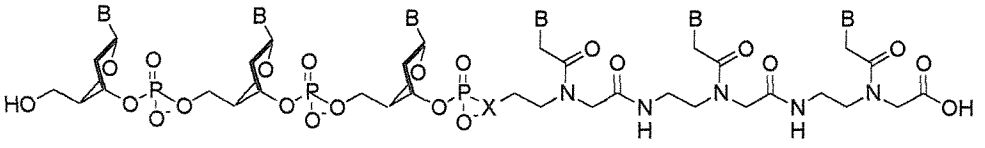

Figure 5.1 – Structure of Peptide Nucleic Acid oligomer

Despite the drastic structural change, PNAs result in nucleic acid mimetics with higher binding-affinity to complementary DNA and RNA than unmodified oligonucleotides[216] and obey the Watson-Crick base pairing rules. This astonishing

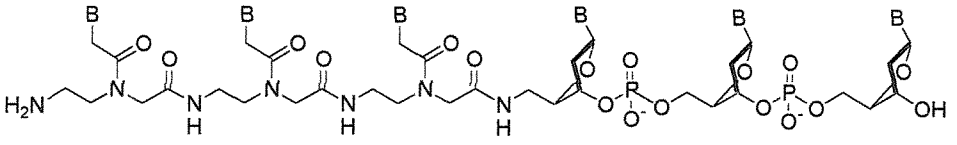
discovery paved the way forward for a new branch of research focussed on diagnostic and therapeutic applications of these highly interesting nucleic acid derivatives.

## 5.1.2 PNA / DNA Chimeras

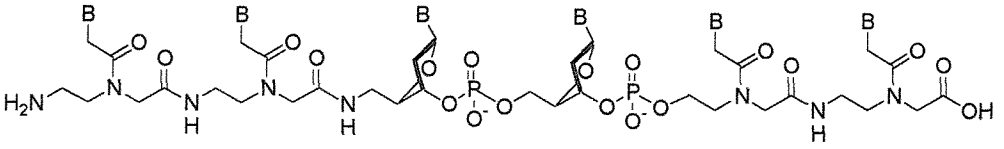
The combination of PNA and DNA to form PNA / DNA chimeras (figure 5.2) results in new structures which not only have excellent binding properties but can also assume biological functions such as a primer function for DNA polymerases. PNA / DNA chimeras with only one PNA unit at the 5'- and 3'-ends are 25 times more stable in human serum than the corresponding unmodified oligodeoxynucleotides[217].



5'-DNA-(linker)-PNA-(pseudo-3'), X = NH or O



(pseudo-5')-PNA-(linker)-DNA-3'



(pseudo-5')-PNA-(linker)-DNA-(linker)-PNA-(pseudo-3')

Figure 5.2 – PNA / DNA Chimeras

PNA belongs to a group of nucleic acid analogues that do not stimulate the enzyme RNaseH on duplex formation with RNA. This intracellular enzyme cleaves RNA and therefore, due to this lack of activity, PNA is not an effective antisense agent.

However PNA / DNA chimeras are able to stimulate RNA cleavage by RNaseH on formation of a chimera·RNA double strand[218]. RNA cleavage occurs at the ribonucleotides that base pair with the DNA part of the chimera.

### 5.1.3 Strand Invasion

In 1993 Nielsen *et al*[219] reported the ability of PNAs to displace one strand of a DNA double helix (figure 5.3) which is an inefficient process with natural oligonucleotides. Prerequisites for strand displacement by PNA are; a DNA duplex that is not too stable, such as in A-T rich regions and, most importantly, a low salt concentration (<50mM NaCl)[219].

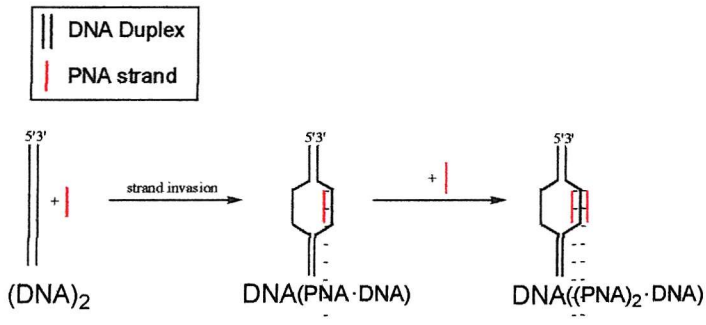



Figure 5.3 – Representation of the process of strand invasion by PNA

Transcription inhibition by PNA can occur by strand invasion when a homopyrimidine PNA oligomer binds to the homopurine sequence of the template strand (figure 5.4 i). When two PNA strands form a triple helix with the non-template strand, transcription is activated since initiation of transcription involves the formation of an 'open' complex, in which approximately 12 deoxynucleotides are exposed as a single strand such that they can base-pair with the synthesised RNA (figure 5.4 ii).

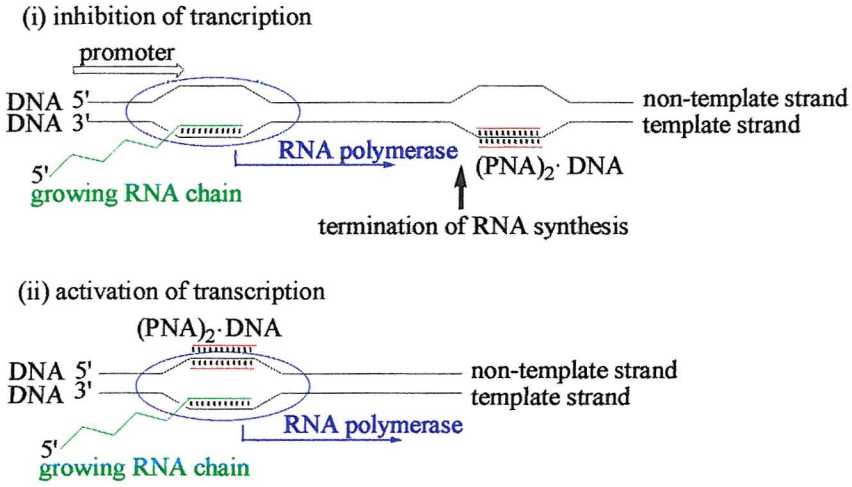



Figure 5.4 – Representation of, i, inhibition of transcription by strand invasion, ii, activation of transcription by strand invasion

The main obstacle to therapeutic use of the strand invasion principle is the stability of the natural DNA double strand, especially GC-rich sequences under physiological salt conditions[218].

## 5.1.4 Diagnostics

The screening of genetic mutations is aided by the exploitation of the following properties of PNA:-

i) PNAs form more stable hybrids and have enhanced sequence discrimination compared with oligonucleotides.

ii) In contrast to oligonucleotides, PNA can cause strand invasion in double-stranded DNA.

iii) On binding with PNA, the electrophoretic mobility of complementary nucleic acids is altered considerably due to the neutral character of PNA.

iv) The ability of PNA·DNA hybrids to form stable duplexes at low ionic strengths and increased temperatures.

The 'PCR clamping' method (figure 5.5) for the detection of point mutations[220] is based on the ability of PNAs to bind more strongly to complementary nucleic acids than oligonucleotides, combined with their inability to function as primers in the polymerase chain reaction (PCR). Targeting of the PNA oligomer, even partially, against the primer annealing site can block the formation of the PCR formation.

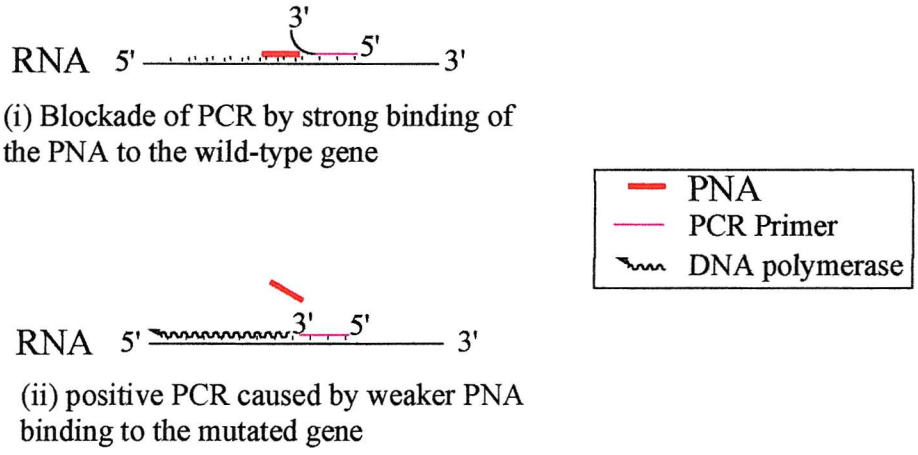


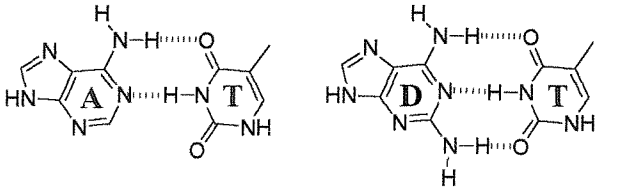

Figure 5.5 – Representation of the 'PCR Clamping' technique

PNA can also be used for the modulation of enzymatic cleavage. After strand invasion by PNA in a DNA duplex, the displaced DNA strand can be selectively cleaved by nuclease S1[220]. Also this enzyme can, in combination with two PNAs, be transformed into an 'artificial restriction enzyme' that cleaves both DNA strands and whose recognition sequence is determined by the PNA sequences employed. Conversely, it can be used to block DNA cleavage by restriction enzymes. The methylation of DNA may also be prevented by PNA sequence specifically[218].

## 5.2 Synthesis of PNA Monomers

The idea of this project was to design and synthesise a PNA monomer that will form a more stable base pair with thymidine than its natural complement adenosine, (figure 5.6) thus further enhancing DNA duplex invasion with PNA. Furthermore if a second

modified PNA monomer is designed and synthesised such that it can only form a stable base pair with adenosine, then pseudocomplentary PNA oligomers of this type will bind with high specificity and efficiency to their complementary target in double-stranded DNA. This process is termed "double duplex invasion".

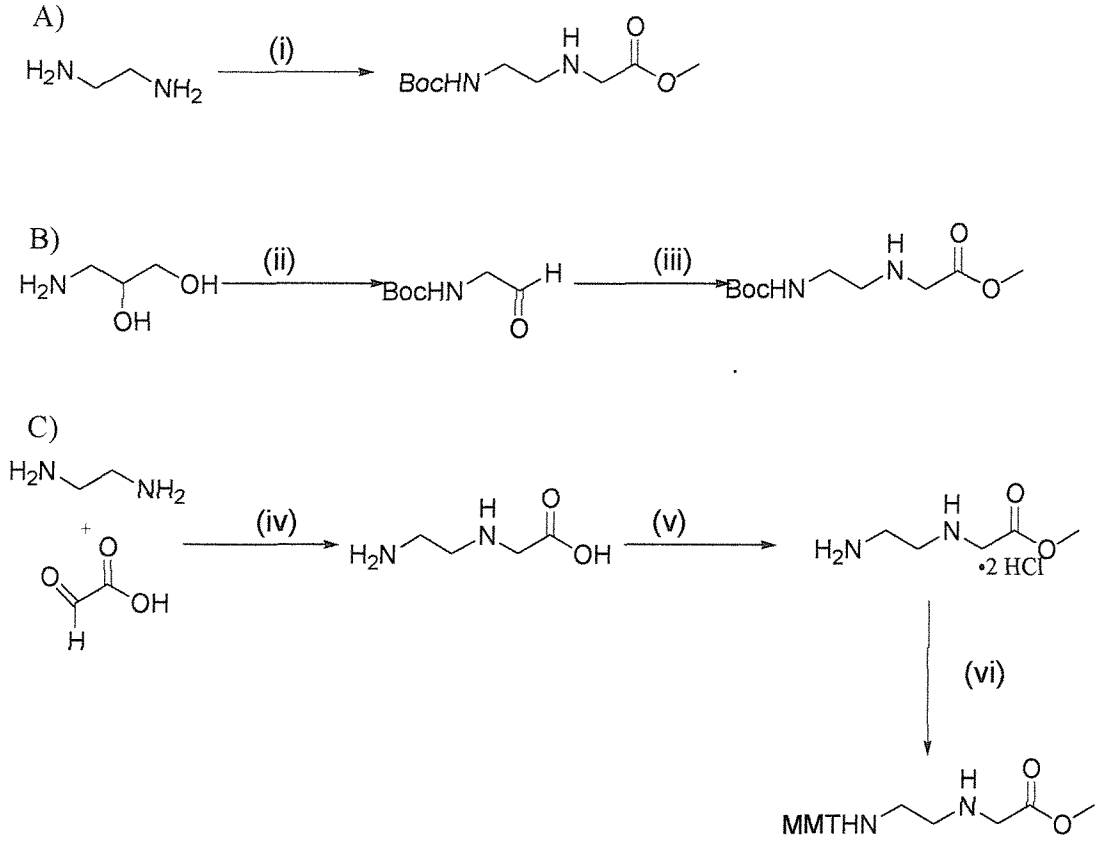



Adenine : Thymine 2,6-Diaminopurine : Thymine

Figure 5.6 – Representation of an adenine : thymine base pair and a 2,6-diaminopurine : thymine base pair.

## 5.2.1 Synthesis of PNA Backbone

The 'classical' PNA backbone is comprised of the amino acid *N*-(2-aminoethyl)glycine, where the secondary amino function is acylated with the nucleobase acetic acid derivative. The primary amino function is temporarily protected with either acid (Boc) or base (Fmoc) labile protecting groups. The most important synthetic routes to aminoethylglycine include alkylation of ethylenediamine with halogenoacetic acid derivatives, reductive amination with glycine esters and protected aminoacetaldehyde and reductive amination using ethylenediamine and glyoxylic acid (figure 5.7).



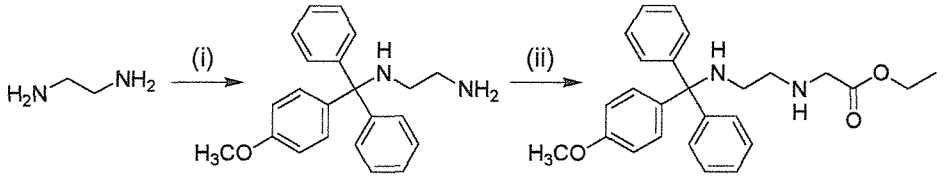

*Reagents and conditions*: i,$ClCH_2COOH$, MeOH/HCl, $(Boc)_2O$, dioxane / $H_2O$, ii, $(Boc)_2O$, NaH, $H_2O$, $KIO_4$, $H_2O$, iii, $H_2NCH_2COOMe$ HCl, NaOAc, $H_2$, Pd/C, iv, $H_2$/Pd/C, $H_2O$, v, MeOH/HCl, vi, MMT-Cl, $Et_3N$, DMF

Figure 5.7 - Literature routes to aminoethylglycine[221-223].

The use of Boc protected monomers is unsuitable for the synthesis of PNA / DNA chimeras, as the strongly acidic conditions (TFA / HF) necessary for deprotection cause depurination of DNA. However the MMT protected monomers can be deprotected under much milder conditions (TCA) and, when used in combination with on orthogonal protecting group on the nucleobases, allow deprotection conditions that are compatible with standard oligonucleotide synthesis. Therefore, the method of Heimer *et al*[221] (figure 5.7A), involving the alkylation of ethylenediamine with chloroacetic acid was adapted towards the synthesis a MMT protected *N*-(2-aminoethyl)glycine ester. However despite several endeavours, the desired product could not be isolated in a reasonable yield. An alternative route based on that

proposed by Uhlmann *et al*[222] (figure 5.7C) which, involved the reductive amination of ethylenediamine and glyoxylic acid monohydrate was investigated. Unfortunately, attempts to perform this reaction at atmospheric pressure were unsuccessful. Presumably, in this instance hydrogenation must be performed in a hydrogenation vessel at 0.3 bar. More success may be attained at atmospheric pressure by selecting a different reducing agent such as zinc and HCl, sodiumcyanoborohydride, sodium triacetoxyborohydride or iron pentacarbonyl and alcoholic potassium hydroxide.

Since limited success was achieved *via* published procedures, an alternative synthetic strategy was investigated (figure 5.8).




*Reagents and conditions*: ethylenediamine, monomethoxytritylchloride, diisopropylethylamine, DMF, r.t., 5 hrs, 76%, ii, ethylbromoacetate, diisopropylethylamine, DMF, r.t., 3 hrs, 42%

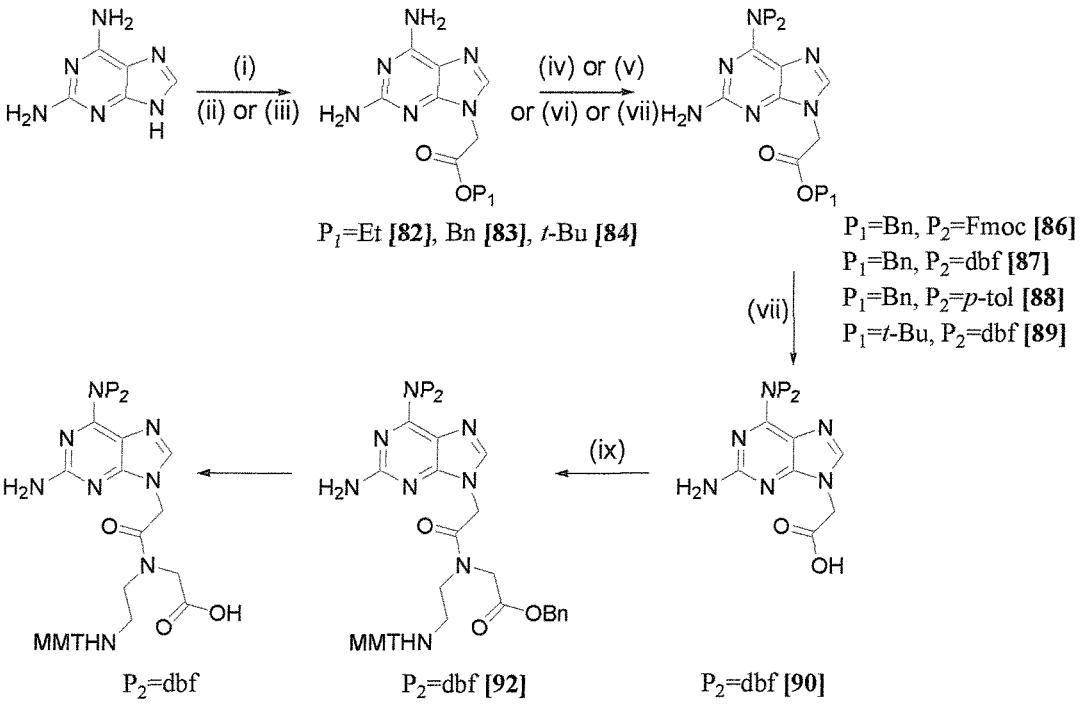
Figure 5.8 – Synthesis of MMT protected aminoethylglycine ethyl ester **[81]**

Ethylenediamine was mono-protected using 4-monomethoxytritylchloride producing compound **[80]** in 76% yield with only a small amount of the ditritylated compound being evident. This was then treated with a slight excess of ethylbromoacetate and diisopropylethylamine for 3 hours producing compound **[81]** in 42% yield. Evidence from mass spectrometry and NMR indicated that an equal amount of the diacetylated product had been formed (39%) however, this reaction has not been optimised. Formation of the diacetylated product may be minimised in future manipulations by adding a smaller quantity of both the base and the ethylbromoacetate.

## 5.2.2 Synthesis of Modified Adenine PNA Monomer

Literature states that the substitution of adenine by 2,6-diaminopurine[223, 224] increases the stability of complementary DNA, RNA and PNA duplexes. In some cases the sequence specificity was also improved upon incorporation of 2,6-diaminopurine in both DNA and PNA[224]. Additionally it has been demonstrated that a homopurine PNA decamer containing six 2,6-diaminopurines can form a strand displacement complex with a double-stranded DNA target and that this complex is sufficiently stable to be analysed by band shift assay[224]. The methodology used by Nielsen *et al*[224] was therefore adapted towards the synthesis of a similar 2,6-diaminopurine PNA monomer. Commercially available 2,6-diaminopurine was deprotonated with sodium hydride and then *N*-alkylated with ethylbromoacetate producing compound **[82]** in reasonable yield (51%). Low yields were stated in the literature[224] for this particular reaction presumably due to formation of the *N*7-alkylated isomer. Despite considerable endeavours to optimise this intractable reaction, the yield could not be significantly improved. Furthermore by employing base labile protecting groups on the exocyclic amine of the base, the need for the selective hydrolysis of an ethyl ester in the presence of the base labile protecting groups is required. Therefore, to increase the scope of viable protecting groups for the exocyclic amines, the 2,6-diaminopurin-9-yl acetate was re-synthesised incorporating both a benzyl ester and a *t*-butyl ester (figure 5.9).

*N*9-alkylation of 2,6-diaminopurine with the corresponding bromoacetate produced compounds **[83]** and **[84]** in 67% and 87% yield respectively. Compound **[83]** was then treated separately with Fmoc-succinimide, di-butyl formamidine **[85]** and *p*-toluoyl chloride producing compounds **[86]**, **[87]** and **[88]** respectively in reasonable yields (47%-72%). Compound **[89]** was produced in 47% yield by reacting compound **[84]** with di-butyl formamidine **[85]** in DMF. This was then treated with 50% TFA in DCM to yield compound **[90]** in 94% yield. The 2-amino group did not require protection as it was found to be sufficiently unreactive to cause no complications in future manipulations.




*Reagents and conditions*:: i, sodium hydride, DMF, r.t., 2 hrs, ethylbromoacetate, r.t., 1 hr, 51%, ii, sodium hydride, DMF, r.t., 2 hrs, benzylbromoacetate, r.t., 1 hr, 67%, iii, sodium hydride, DMF, r.t., 1 hr, *t*-butylbromoacetate, r.t., 1 hr, 87%, iv Fmoc-succinimide, dioxan, r.t., 12 hrs, 55%, v, compound **[85]**, DMF, r.t., 12 hrs, 47%, vi, *p*-toluoyl chloride, pyridine 0°C-r.t., 2 hrs, 72%, vii, compound **[85]**, DMF, r.t., 12 hrs, 47%, viii, TFA, DCM, r.t., 1 hr, 94%, ix, TOTU, diisopropylethylamine, , DMF, r.t., 5 hrs, 30%

Figure 5.9 – Synthesis of 2,6-diaminopurine PNA monomer

In order to accommodate the changes made to the PNA base, the backbone was re-synthesised using a benzyl ester to protect the carboxylic acid and compound **[91]** was produced in 81% yield by treating *N*1-(monomethoxytrityl) ethylenediamine with benzyl-2-bromoacetate.

The product of coupling the backbone **[91]** with the base **[90]**, as suggested by $^{1}H$ and $^{13}C$ NMR data, was found to proceed in 40% yield by employing the coupling additive TOTU. It is believed that this product can be debenylated in the presence of the isolated double bond using lithium 4,4-di-*t*-butylbiphenyl ($Li^{+}DBB^{-}$) in a procedure similar to that used by Ireland and Smith[225].

## 5.2.3 Synthesis of Modified Thymine PNA monomer

As mentioned previously, an analogue of the base thymine that will have a higher binding affinity to its natural complement than its unnatural 2,6-diaminopurine complement was required. Replacement of a hydrogen bond acceptor with a group unable to participate in hydrogen bonding would provide the required analogue. However this can lead to undesirable tautomerisation, therefore a 2-pyridone derivative was necessary as this ring system generally prefers the carbonyl tautomer[226] (figure 5.10).

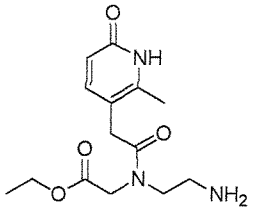
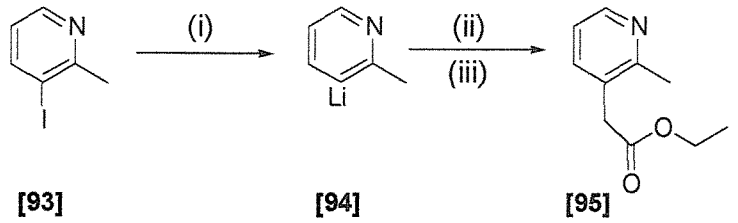



Figure 5.10 – Proposed modified thymine PNA monomer

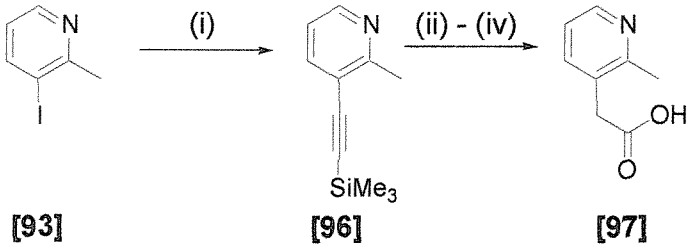
However with regards to PNA, this in itself generated problems. In order to be coupled to compound **[91]**, a suitably protected acetic acid derivative needed to be added to the heterocycle. A paper by Gilman and Spatz[227] describing conditions for the synthesis of various pyridyllithiums from the appropriate iodopyridine suggested that it may be possible to convert compound **[93]** to **[94]**. It was then believed that **[94]** could then undergo lithium-copper exchange and subsequent reaction with ethyl-2-bromoacetate utilising the conditions described by Velkov *et al*[228] would yield the model compound **[95]** (figure 5.11). Unfortunately, despite several endeavours this was unsuccessful.




*Reagents and conditions*: i, *n*-butyllithium, THF, ii, copper iodide, iii, ethylbromoacetate

Figure 5.11 – Model reaction conditions for the synthesis of a modified thymidine PNA monomer

However an alternative model synthetic route (figure 5.12) which may prove more successful, involves an initial palladium-catalysed coupling reaction of **[93]** with (trimethylsilyl) acetylene[229] to give compound **[96]** which upon hydroboration with dicyclohexylborane (DCHBH)[230, 231] and oxidation to **[97]** should provide a feasible route to the target compound.




*Reagents and conditions*: i, trimethylsilylacetylene, *bis*triphenylphosphine palladium dichloride, triethylamine, THF, ii, dicyclohexylborane, iii, sodium hydroxide, iv, hydrogen peroxide

Figure 5.12 – Alternative route to modified thymidine PNA monomer

## 5.2.4 Conclusions

The synthesis of a 2,6-diaminopurine PNA monomer **[92]** that has the potential to form a more favourable base pair with its natural complement thymidine, has been

accomplished (as confirmed by $^{1}H$ and $^{13}C$ NMR only). This was achieved through the coupling of a suitably protected aminoethylglycine backbone **[91]** with the base moiety **[90]**. Incorporation of this monomer into a PNA oligomer should significantly enhance the process of strand invasion by a PNA oligomer.

Limited success was achieved with the synthesis of a modified thymidine PNA monomer for the reasons discussed. However a more feasible route has been proposed (figure 5.11).

## 5.3 C-Nucleosides

Due to the difficulties in synthesising a suitable PNA analogue of thymidine, attention was focussed on synthesising the corresponding DNA analogue.

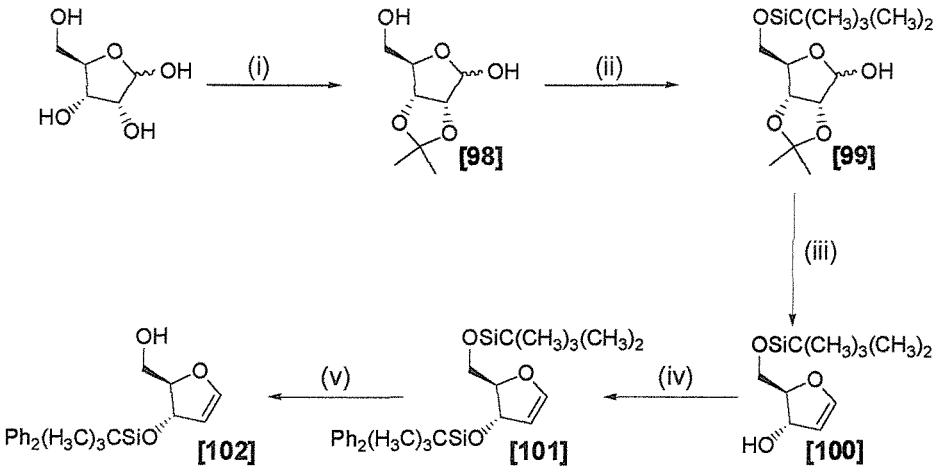
### 5.3.1 Introduction

Nucleoside analogues which are only minimally altered with respect to the natural counterpart prove valuable tools for probing enzymatic binding or catalysis and when present in DNA or RNA, can be used to probe related recognition or catalytic events involving sequence specific ligands[232], proteins[233], or enzymes[234]. The deletion of a functional group which is potentially involved in a critical interaction, without otherwise altering the functional group character of the nucleoside have proven to be the simplest modifications. McLaughlin *et al*[235] have synthesised modified purine and pyrimidine nucleosides whereby a single functional group has been excised from the otherwise common nucleoside. However with pyrimidines this can lead to problems such as an undesirable tautomeric change which can change hydrogen-bonding character. By preparing the corresponding C-nucleoside of these types of derivatives, the aforementioned problems can be eliminated.

An efficient route to C-glycosides developed by Daves *et al*[236-238] involving, as a key step, the palladium-mediated coupling of a suitably protected glycal[239, 240] with an iodo-substituted heterocyclic aglycon in a regio- and stereospecific manner, was considered to be the most attractive method for the synthesis of the DNA monomers. This method generates solely the β-isomer of the C-nucleoside.

## 5.3.2 Synthesis of the Ribose Sugar

The glycal[239, 240] **[102]** was prepared from commercially available D-ribose (figure 5.13). The first step involved converting the 1, 2-diol functionality of D-ribose to the corresponding acetonide **[98]** utilising the method of Kiso and Hasegawa[241]. The 5'-hydroxyl group was then protected as its *t*-butyldimethylsilylether producing **[99]** in reasonable yield. This was then converted to the ribofuranosyl chloride (carbon tetrachloride, tris(dimethylamino)phosphine) and a subsequent reductive fragmentation (lithium in ammonia)[239] produced compound **[100]** in reasonable yield. Protection of the free hydroxyl group of **[100]** with *t*-butyldiphenylsilyl chloride proceeded smoothly to give **[101]** in high yield. However deprotection of the TBDMS group of **[101]** using the conditions described by Daves[240] was problematic. Careful monitoring of the reaction, which was performed numerous times with varying conditions revealed that the reaction was extremely sensitive to the batch of TBAF used and was therefore difficult to reproduce. Different reagents to TBAF, including pyridinium *p*-toluenesulfonate and triethylamine trihydrofluoride were investigated for deprotection of this TBDMS group. Unfortunately unsatisfactory results were obtained and so despite the difficulty in producing consistent results, the method of Daves[240] was used throughout.

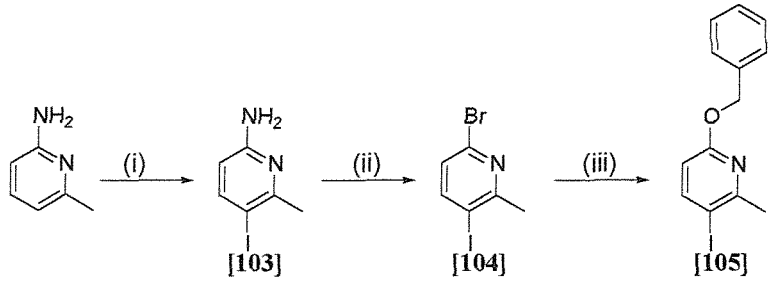



*Reagents and conditions*: i, 2,2-dimethoxypropane, *p*-toluene sulfonic acid, DMF, r.t., 3 hrs, 63%, ii, *t*-butyldimethyl silyl chloride, imidazole, DMF, r.t., 24 hrs, 94%, iii, carbontetrachloride, tris(dimethylamino)phosphine, lithium, ammonia, -78°C-r.t., 3 hrs, 66%, iv, *t*-butyldiphenylsilyl chloride, imidazole, DMF, r.t., 2 hrs, 96%, v, tetra-*n*-butylammonium fluoride, *THF*, -22°C-r.t., 2 hrs, 38%

Figure 5.13 – Synthesis of glycal **[102]**[239, 240]

## 5.3.3 Synthesis of the Heterocyclic Base

The synthesis of a novel hetrocyclic thymine base analogue was accomplished by utilizing the conditions described by McLaughlin *et al*[235] (figure 5.14).




*Reagents and conditions*: i, periodic acid dihydrate, iodine, acetic acid, sulfuric acid, 80°C, 4 hrs, 65%, ii, hydrobromic acid, bromine, sodium nitrite, -20°C-0°C, 1 hr, 72%, iii, sodium hydride, benzyl alcohol, DMF, 0°C-r.t., 3 hrs, 75°C, 16 hrs, 50%

Figure 5.14 – Synthesis of novel thymine heterocyclic base analogue

The commercially available 2-amino-6-picoline was selectively iodinated at the para-position by treatment with periodic acid, iodine and acetic acid to produce compound **[103]** in 65% yield. Although 2D NMR experiments suggested formation of the *p*-isomer, a crystal structure of **[103]** was also obtained to confirm the position of iodination (figure 5.15). Bromination of compound **[103]** was achieved in 72% yield by treatment with hydrobromic acid, bromine and sodium nitrite. This was then converted to the benzyl ether by treatment with sodium hydride and benzyl alcohol to produce **[105]** in 50% yield.

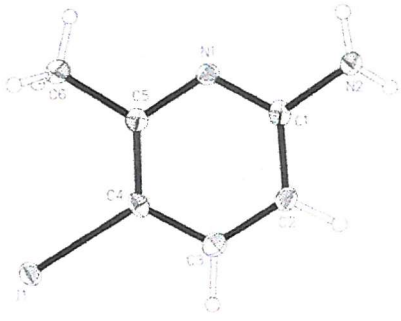
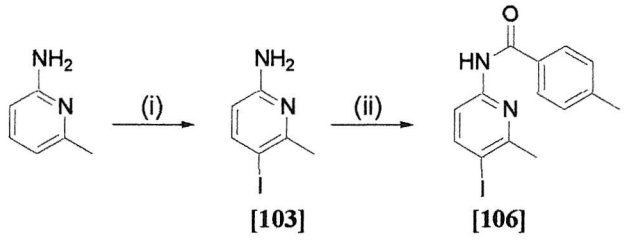


Figure 5.15 – Crystal structure obtained of compound **[103]**

A novel cytosine base analogue, **[106]** was also successfully synthesised from **[103]** by treatment with *p*-tolouyl chloride (figure 5.16).



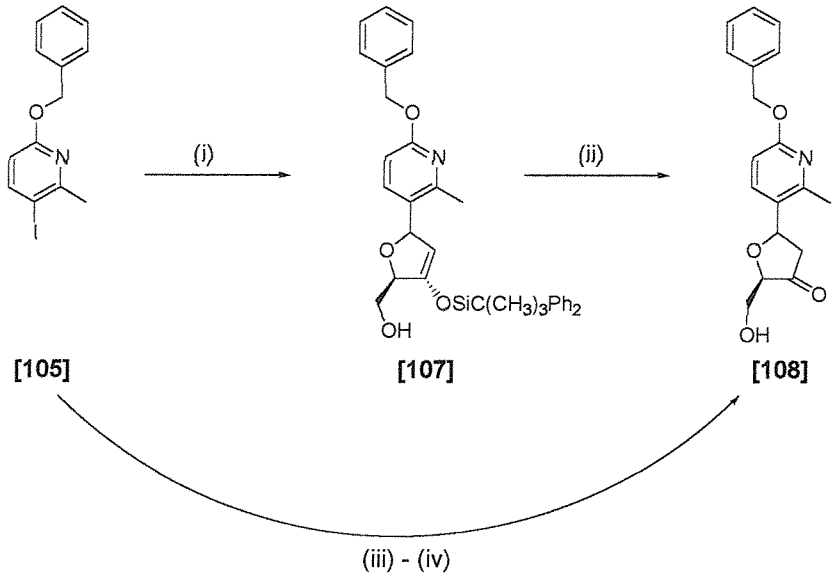

**[103]** **[106]**

*Reagents and conditions*: i, periodic acid dihydrate, iodine, acetic acid, sulfuric acid, 80°C, 4 hrs, 65%, ii, *p*-toluoylchloride, pyridine, 0°C-r.t., 18 hrs, 82%

Figure 5.16 – Synthesis of novel modified cytosine base analogue

## 5.3.4 Generation of C-Nucleosides

Glycal **[102]** was used in the palladium-mediated coupling reaction with **[105]** (figure 5.16 route i and ii) using the conditions employed by Hsieh and McLaughlin[235] to produce compound **[107]** in low, unoptimised yield. This was then treated with TBAF and acetic acid to deprotect the TBDPS group but again problems were encountered and results were disappointing. Unfortunately, successful conditions to **[108]** have not been attained. The palladium mediated coupling reaction with **[105]** was performed using glycal **[101]** (figure 5.17 route iii and iv) as literature evidence[242] stated that the reaction with protecting groups on both the 3' and 5' positions proceeds to give almost exclusively β-products. Indeed NMR evidence suggested that the reaction was successful, however a higher ratio of α-isomer than that reported in the literature[242] was evident. Again deprotection of this coupled product was again unsuccessful.




*Reagents and conditions*: i, *bis*(dibenzyllideneacetone) palladium (0), 1,3-*bis*(diphenylphosphino)propane, acetonitrile, r.t., 25 mins, compound **[102]**, tributylamine, aetonitrile, 80°C, 12 hrs, 24%, ii, tetrabutylammonium fluoride, acetic acid, THF, iii, palladium (0), 1,3-*bis*(diphenylphosphino)propane, acetonitrile, r.t., 25 mins, compound **[101]**, tributylamine, aetonitrile, 80°C, 12 hrs, iv, tetrabutylammonium fluoride, acetic acid, THF

Figure 5.17 – Coupling of glycal **[101]** and **[102]** with heterocyclic base **[105]** to give C-nucleoside

### 5.3.5 Conclusions

Novel thymine and cytosine base analogues **[105]** and **[106]** have been successfully synthesised. Palladium mediated coupling of analogue **[105]** with both a fully protected glycal **[101]** and mono-protected glycal **[102]** was accomplished. Coupling with glycal **[102]** gave almost exclusively the desired β-isomer, however with glycal **[101]** a significant proportion of α-isomer was observed in the NMR. This is believed to be due to the absence of the stereodirecting influence of the 5'-hydroxyl group.

Reasons for the disappointing results observed for the deprotection of **[107]** are not fully understood. However it is believed to be due to a poor source of fluoride ion. Alternatively it has been suggested that the coupling reaction can lead to unstable products that lose further protecting groups to form the more stable ketone[242] (figure 5.18). *However, no evidence to support this hypothesis was obtained from* NMR.


Figure 5.18 – Representation of the proposed loss of protecting groups by Townsend *et al*[242]

## 5.4 Association of Nucleobases

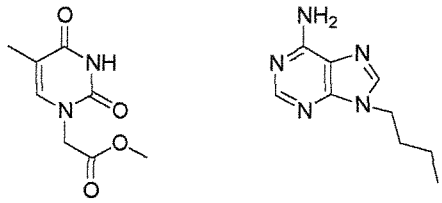
### 5.4.1 Introduction

Association of nucleobases by specific hydrogen bonds is a major determinant of nucleic acid structure. In addition to the standard Watson-Crick base pairs, other configurations frequently occur in DNA and RNA due to the multiple proton donor

and acceptor sites available i.e. reverse Watson-crick base-pairs are observed in parallel-stranded DNA[243] and Hoogsteen hydrogen bonding in triplexes[244]. Numerous theoretical[245-247] studies and some experimental studies[248-250] on the association of nucleobases have been performed. Whilst a considerable amount of thermodynamic data for base-base interactions in aprotic solvents has been collected, with the exception of Weisz *et al*[251], there is no significant evidence in the literature of the detailed structure of preferred association modes of homo- and heterodimers of nucleobases in solution.

## 5.4.2 NMR Studies

NMR studies probing hydrogen bonding interactions of heterocyclic base analogues provides information on the preferred association modes of nucleobases in solution. By monitoring hydrogen bonding interactions at the level of the nucleobase alone, problems such as undesirable tautomeric change, may be highlighted and rectified at an early stage. To this end, compounds **[109]** and **[110]** (figure 5.19) were synthesised in order to have a suitable thymine-adenine base pair with which to perform initial experiments.

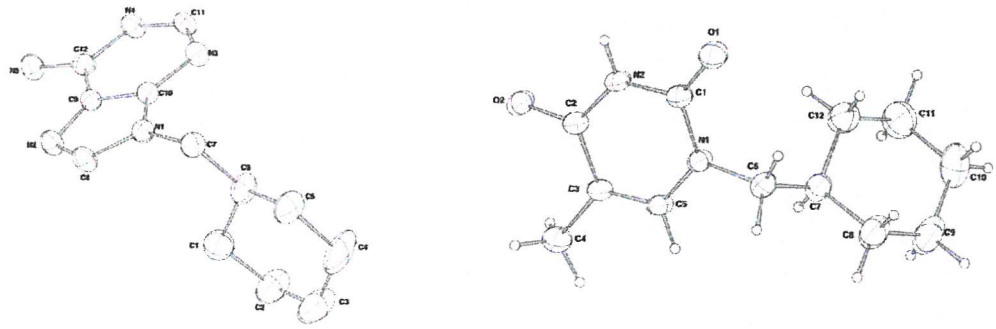



**[109]** **[110]**

Figure 5.19 – Thymin-1-ylacetic acid methyl ester **[109]** and *N*9 butyl adenine **[110]** synthesised for initial NMR experiments

Unfortunately, compounds **[109]** and **[110]** were not sufficiently soluble in $CDCl_3$ at the concentration range required to perform suitable NMR titrations. It was proposed

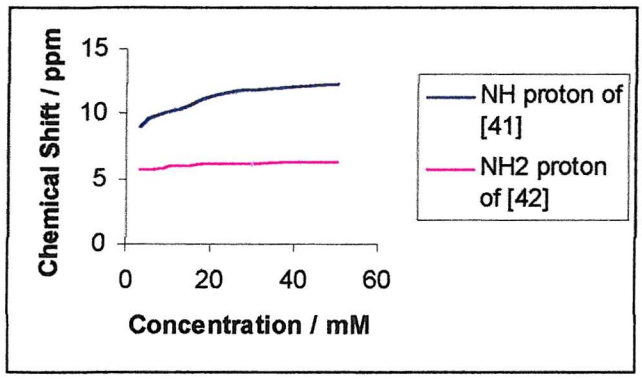
that by incorporating a cyclohexyl group to the nucleobase, solubility would be increased, therefore the derivatives *N*1-cyclohexylmethylthymine **[111]** and *N*9-cyclohexylmethyladenine **[112]** were synthesised and were found to be highly soluble in $CDCl_3$ (up to 100mM). Reasonable yields (32-41%) of the products were obtained by treating the respective base with sodium hydride and bromomethylcyclohexane. It was assumed that *N*7 alkylation of adenine and and *N*3 alkylation of thymine was responsible for the yields of product observed. Crystal structures of the isolated products were obtained to confirm that the correct regio-isomer was obtained (figure 5.20).

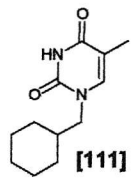


(i) (ii)

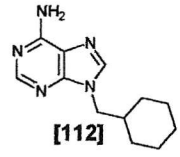
Figure 5.20 – Crystal Structures of i, *N*9-cyclohexylmethyladenine **[112]** and ii, *N*1-cyclohexylmethylthymine **[111]**

These compounds **[111]** and **[112]** were then used in preliminary dilution studies by diluting a 1:1 mixture of the two components in the concentration range 50-3mM and recording the $^1H$ NMR spectrum of each at room temperature on a Brucker AM400 spectrometer. Results from these experiments are summarised in figure 5.21.




[111]




[112]

Figure 5.21 - NMR titration results from thymine and adenine derivatives **[111]** and **[112]**

To compare these results with a molecule capable of forming an additional hydrogen bond with thymine, a 2,6-diaminopurine derivative **[113]** was synthesised. Again a crystal structure was obtained to confirm alkylation at *N*9 had occurred (figure 5.22).

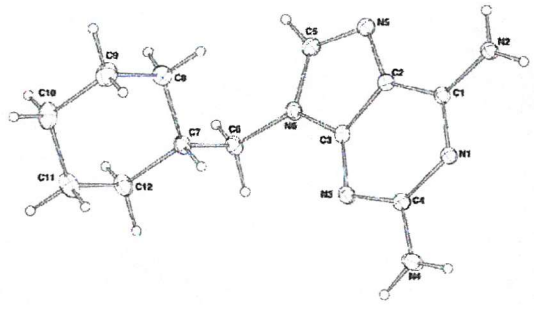


Figure 5.22 – Crystal structure of *N*9-cyclohexylmethyl-2,6-diaminopurine **[113]**

A series of dilution studies were then performed on compounds **[111]** and **[113]** by diluting a 1:1 mixture of the two components in the concentration range 50-3mM and recording the $^{1}H$ NMR spectrum of each at room temperature. Results from these experiments are summarised in figure 5.23.

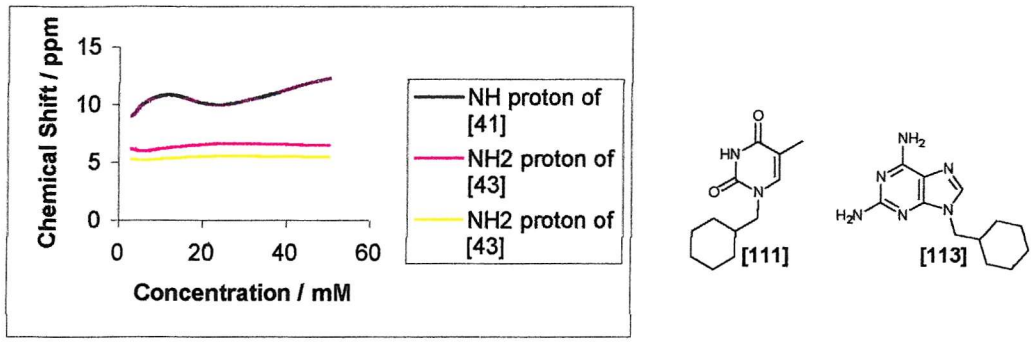



Figure 5.23 - NMR titration results from thymine and 2,6-diaminopurine derivatives **[111]** and **[113]**

A further series of dilution experiments were performed on a modified thymine and the 2,6-diaminopurine derivative. The purpose of these experiments was to investigate the influence of the methyl group on the chemical shift of the 2-amino protons of **[113]**. Results are shown in figure 5.24.

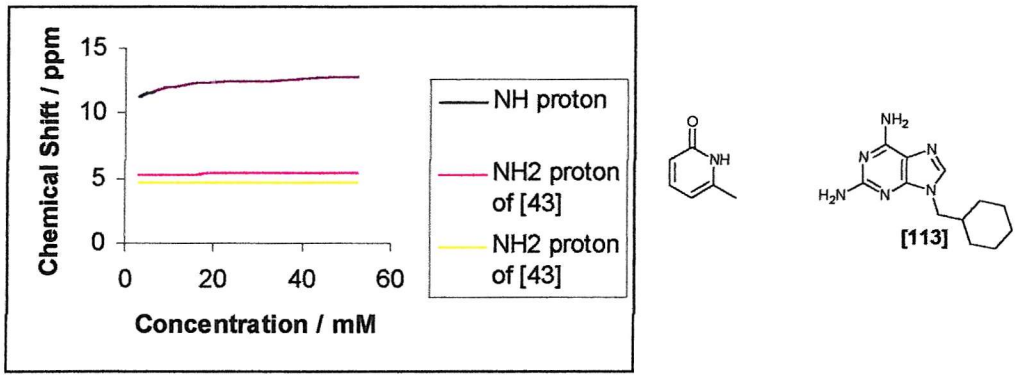



Figure 5.24 - NMR titration results from modified thymine and 2,6-diaminopurine derivatives

Although differences in chemical shift were observed, information on the detailed structure of the associates is limited due to the fast exchange on the NMR time scale at ambient temperature. Therefore, experiments with the modified thymine and 2,6-diaminopurine derivatives were conducted at –10°C. At this temperature one of the

2,6-diaminopurine $NH_2$ peaks was broadened beyond detection and significant precipitation was observed as solubility decreased with decreasing temperature. However, it was observed that both 2,6-diaminopurine $NH_2$ peaks do not respond equally to the modified thymine analogue as may be expected. One peak shifts significantly further downfield and also gives a much stronger nOe to the imino-proton of the modified thymine analogue.

### 5.4.2 Conclusions and Further Work

Base analogues have been synthesised **[111]-[113]** which are sufficiently soluble in $CDCl_3$ to perform a series of dilution experiments allowing information on base-base interactions to be gathered. A change in chemical shift has been observed for each of the hydrogen bond donors with change in concentration. In the simplest terms, with decreasing concentration an upfield shift in NH or $NH_2$ protons is observed. This is consistent with a decrease in the extent of H-bonding since the upfield shift of the H-donor corresponds to higher electron density at this proton.

IR and calorimetric studies can provide additional physical data on base-base interactions. A few tentative IR studies have been performed.

This data together with the correct crystallographic data would provide definite proof of the preferred association of nucleobases in solution.

# Chapter 6

# Experimental

# 6.0 Experimental

## 6.1 Preparation of Compounds

### 6.1.1 General Methods

All reactions requiring anhydrous conditions were performed in dry glassware under an atmosphere of argon. Acetonitrile, dichloromethane, dimethylsulfoxide, pyridine and triethylamine were distilled from calcium hydride. Ether and tetrahydrofuran were freshly distilled from sodium wire and benzophenone. Methanol was distilled from magnesium and iodine. Anhydrous carbon tetrachloride, diisopropylethylamine and *N*, *N*-dimethylformamide were purchased from Aldrich. 1,3-Dichloro-1,1,3,3-tetraisopropyldisiloxane was purchased from Cruachem. Other reagents were purchased from Aldrich/Sigma or Lancaster.

Column chromatography was carried out under pressure on Merck 60-mesh silica. TLC was carried out using Merck Kieselgel $60F_{24}$ (0.22mm thickness, aluminium backed). Compounds were visualised by irradiation at 254nm or staining with *p*-anisaldehyde (4-methoxybenzaldehyde) : glacial acetic acid : concentrated sulphuric acid : ethanol (5:1:1:100) or ninhydrin : ethanol (1:100) or concentrated $H_2SO_4$ : ethanol (1:10) or phosphomolybdic acid : ethanol (1:10) or 4,4'-bismethylaminobenzehydrol : acetone (1:100). Solvent systems A-Q are described:

A: 2-propanol : $H_2O$ : $NH_4OH$ 7:1:2

B: *n*BuOH : AcOH : $H_2O$ : EtOAc 1:1:1:1

C: $CH_2Cl_2$ : MeOH : AcOH 9:1:1

D: EtOAc : MeOH : $NH_4OH$ 5:1:1

E: EtOAc : MeOH : $Et_3N$ 5:1:1

F: $CH_2Cl_2$ : MeOH 8:2

G: $CH_2Cl_2$ : MeOH 9:1

H: EtOAc : $Et_3N$ 5:1

I: EtOAc

J: $CH_2Cl_2$

K: EtOAc : Hexane 4:1

L: EtOAc : Hexane 3:3

M: EtOAc : Hexane 1:1

N: $CH_2Cl_2$ : $Et_2O$ 1:1

O: EtOAc : Hexane 3:7

P: $Et_2O$ : Petrol 7.5:2.5

Q: $Et_2O$ : Petrol 1:2

Anion-exchange HPLC and Reverse-phase HPLC were performed on a Gilson HPLC system, controlled by Gilson 7.12 software using a Dionex NucleoPac100 HPLC column (4 x 250mL) and an ABI aquapore octyl (C8) column 300 Å pore size respectively. Elution of triphosphates was monitored by UV detection (260nm). Buffer systems consisted of

(1) Buffer A: Water with 10% acetonitrile; Buffer B: 1M $NH_4OAc$ with 10% acetonitrile; flow rate 2.5 mL/min.

(2) Buffer A: Water with 20% acetonitrile; Buffer B: 1M $NH_4OAc$ with 20% acetonitrile; flow rate 2.5 mL/min.

(3) Buffer A: 0.1M triethylammonium acetate; Buffer B: 0.1M triethylammonium acetate with 70% acetonitrile; flow rate 1.0 mL/min.

with gradient programmes as described in figure 6.1. Analytical injections were monitored at 260nm, 0.1 AUFS, preparative injections were monitored at 260nm, 0.5-1.0 AUFS. The triphosphates were collected manually.

| Time / minutes | System 1 % B | Flow rate mL/min |
|---|---|---|
| 0 | 0 | 0 |
| 2 | 0 | 2.5 |
| 15.5 | 100 | 2.5 |
| 16 | 100 | 2.5 |
| 16.5 | 0 | 2.5 |
| 19.9 | 0 | 2.5 |
| 20 | 0 | 0 |

| Time / minutes | System 2 % B | Flow rate mL/min |
|---|---|---|
| 0 | 0 | 0 |
| 4 | 0 | 2.5 |
| 23 | 100 | 2.5 |
| 26 | 100 | 2.5 |
| 26.5 | 0 | 2.5 |
| 29.9 | 0 | 2.5 |
| 30 | 0 | 0 |

| Time / minutes | System 3 % B | Flow rate mL/min |
|---|---|---|
| 0 | 0 | 0 |
| 3 | 0 | 1 |
| 3.5 | 20 | 1 |
| 15 | 100 | 1 |
| 20 | 100 | 1 |
| 25 | 0 | 1 |
| 29.9 | 0 | 1 |
| 30 | 0 | 0 |

Figure 6.1 – HPLC gradient programmes.

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

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

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

Proton NMR was recorded at 300 MHz or 400 MHz and carbon at 75.5 MHz or 100.6 MHz on a Brucker AM300 or AM400 spectrometer in either deuterated chloroform or D6-DMSO or D6-benzene or $D_2O$. The multiplicities of carbon-13 signals were determined using distortionless enhancement by phase transfer (DEPT) spectral editing technique.

Mass spectra were recorded on a Fisons VG platform instrument or MALDI TOF.

Microanalytical data was obtained from University College, London or Medac Ltd, Analytical and chemical consultancy services, Brunel Science Centre, Surrey.

## 6.1.2 Preparation of Resins

Polystyrene resins were supplied by Glen Research, Tentagel resins were supplied by Novabiochem, Hypogel resins were obtained from Rapp Polymere and CPG resins were obtained from Link Technologies. All other reagents were obtained from Aldrich/ Sigma or Lancaster. Solvents were distilled as described previously. All glassware was pre-soaked in trimethylsilyl chloride : dichloromethane (1:20) for 0.5 hour, rinsed thoroughly with dichloromethane and oven dried.

Synthesiser columns for packaging of the resins were obtained from Glen Research.

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

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

### *6.1.2.1 General Method for attachment of nucleoside to resin*

To a solution of the nucleoside (5 eq) in the minimum volume of anhydrous pyridine was added HOBt (5 eq) and the solution stirred under an argon atmosphere for 10 minutes. The reaction mixture was then added to the commercial amino-resin with DIC (5 eq) and gently agitated under a stream of argon gas for 16 hours before being filtered, washed extensively with pyridine (x 3) and DCM (x 5) and dried *in vacuo*. A

small sample of resin was removed to ascertain the resin loading (section 6.1.3.1). The resin was then treated with a solution of acetic anhydride : 2, 6-lutidine in THF : 1-methylimidazole in THF (1:1) for 15 minutes, the resin filtered, washed with pyridine (x 3), THF (x 3) and DCM (x 5) and dried over $P_2O_5$ *in vacuo*. A small sample of resin was removed to verify the resin loading (section 6.1.3.1).

### *6.1.2.2 General Method for phosphorylation of resins*

The tritylated resin was treated with a solution of 5% TCA in DCM for 5 minutes, before being filtered and washed extensively with DCM (x 5) and acetonitrile (x 5) and dried *in vacuo*. The resin was then treated with a 1M solution of salicylphosphochoridite[21f] in anhydrous dioxan suspended in a mixture of pyridine : DMF (1 : 3) and gently agitated under a stream of argon gas for 15 minutes . The resin was then filtered, washed extensively with dioxan (x 3) and acetonitrile (x 5) and dried *in vacuo*. The resin was then treated with a 0.5M solution of *bis*-(tributylammonium) pyrophosphate in anhydrous DMF and tri-*n*-butylamine and gently agitated under a stream of argon gas for 20 minutes. The resin was then treated with a solution of iodine : water : pyridine in THF (3:1:1) and gently agitated under a stream of argon gas for 30 minutes. The resin was filtered and washed extensively with DMF (x 3), THF (x 3) and acetonitrile (x 5) and dried over $P_2O_5$ *in vacuo* for 12 hours.

### *6.1.2.3 General method for labelling with dye active esters or equivalent*

The resin was treated with 20% piperidine in DMF for 10 minutes, the resin washed with DMF (x 3) and DCM (x 5) and dried *in vacuo*. A solution of the dye (5eq) and HOBt (5eq) in anhydrous pyridine (1mL) was stirred for 10 minutes before being added to the resin with DIC (5.5eq). The resin suspension was agitated gently with a stream of argon gas for 90 minutes before being washed extensively with pyridine (x 3), DMF (x 3), DCM (x 5) and dried over $P_2O_5$ *in vacuo*.

*6.1.2.4 General method for labelling with dye phosphoramidites*

The resin was treated with 20% piperidine in DMF for 10 minutes, the resin washed with DMF (x 3) and DCM (x 5) and dried *in vacuo*. The resin was packaged into a Glen research column suitable for an ABI 394 solid-phase DNA/RNA synthesiser. The amount of resin used was determined by the resin loading and the scale of the synthesis (0.2μmol). Using the standard assembly cycle of, coupling, capping and iodine oxidation procedures, the dye phosphoramidite was attached.

*6.1.2.5 General method for ammonia cleavage of triphosphate from resin*

The resin was treated with conc. aqueous ammonia at room temperature for 30 minutes, the resin filtered and washed with ammonia followed by methanol. The filtrate was concentrated *in vacuo*, the residue dissolved in water (0.5mL) and passed through a column of Dowex $Li^+$ eluting with water (1mL).

*6.1.2.6 General method for automated cleavage on DNA synthesiser*

The resin was treated with conc. aqueous ammonia at room temperature for 5 minutes, on an ABI 394 solid-phase DNA/RNA synthesiser, the resin filtered and washed with ammonia followed by methanol. The filtrate was concentrated *in vacuo*, the residue dissolved in water (0.5mL) and passed through a column of Dowex $Li^+$ eluting with water (1mL).

*6.1.2.7 General method for alternative basic cleavage of triphosphate*

The resin was treated with a solution of water : methanol : tributylamine (2:1:1) for 1.5 hours at 60°C, the resin filtered and washed with methanol. The filtrate was

concentrated *in vacuo*, the residue dissolved in water (0.5mL) and passed through a column of Dowex $Li^+$ eluting with water (1mL).

## 6.1.3 Determination of resin loading and yield of triphosphate

Loading of resins were calculated by either trityl assay or ninhydrin assay where appropriate.

*Yields of unlabelled triphosphate were based on the loading of the original nucleoside* on the resin and were calculated by measuring the concentration of a solution of the triphosphate using the extinction coefficient of the base at the $\lambda_{max}$. This was then converted to a weight of triphosphate (no hydrate or salt form). For example, from 352mg of resin with a loading of 21μmol $g^{-1}$, 6mg of pure triphosphate was obtained, corresponding to a yield of 81%.

Yields of labelled triphosphate were again based on the loading of the original nucleoside on the resin and were calculated by measuring the concentration of a solution of the triphosphate using the extinction coefficient of the dye at the $\lambda_{max}$. This was then converted to a weight of triphosphate. For example, from 48mgs of resin with a loading of 21μmol $g^{-1}$, 70μg of pure labelled-triphosphate was obtained, corresponding to a yield of 56%.

### *6.1.3.1 Trityl Analysis*

A sample of resin was weighed and suspended in either trichloroacetic acid : dichloromethane (1:20 v:v, 25mL) or hydrochloric acid : ethanol (3:2 v:v, 25mL) and shaken for 5 minutes. The absorbance of 1mL of the orange solution was measured at 495nm on a Perkin-Elmer UV/Vis spectrometer. The amount of 4,4'-dimethyloxytrityl cation present was calculated as follows:

| | | |
|---|---|---|
| Absorbance for 1mL (at 495nm) | | = **a** |
| Absorbance for 25mL | a x 25 | = **b** |

[The absorbance of 1μmol of $DMT^+$ at 495nm = 71.7 absorbance units]

| | | |
|---|---|---|
| For **c**g of resin | b /71.7 | = **d**μmol |
| Resin loading (amount of $DMT^+$ present) | d / c | = **e**μmol$g^{-1}$ |

*Example:*

| | | |
|---|---|---|
| Absorbance for 1mL (at 495nm) | | = 1.698 |
| Absorbance for 25mL | 1.698 x 25 | = 42.45 |
| For 0.012g of resin | 42.45 / 71.7 | = 0.59μmol |
| Resin loading | 0.59 / 0.012 | = 49μmol$g^{-}$ |

*6.1.3.2 Ninhydrin Assay*[176]

A sample of resin was weighed and treated with a solution of ninhydrin reagent A : reagent B (3:1) and heated at 100°C for 5 minutes. The contents were then filtered into a 25mL volumetric flask, washing with 3 x 0.5$cm^3$ aliquots of a 0.5M solution of tetraethylammonium chloride in dichloromethane and made up to volume with 60% ethanol in water. The absorbance of 1mL of the blue solution was measured at 570nm on a Perkin-Elmer UV/Vis spectrometer. The amount of free primary amino groups present was calculated as follows:

| | | |
|---|---|---|
| Absorbance for 1mL (at 570nm) | | = **a** |
| Absorbance for 25mL | a x 25 | = **b** |

[The absorbance of 1μmol of Ninhydrin at 570nm = 15000 absorbance units]

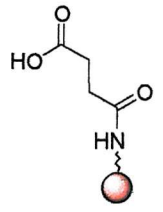
| | | |
|---|---|---|
| For **c**mg of resin | b (1000)/15000 | = **d**μmol |
| Resin loading (amount of $NH_2$ present) | d (1000)/ **c** | = **e**μmol$g^{-1}$ |

*Example:*

| | | |
|---|---|---|
| Absorbance for 1mL (at 570nm) | | = 0.669 |
| Absorbance for 25mL | 0.669 x 25 | = 16.725 |
| For 10mg of resin | 16.725 (1000) / 15000= 1.115μmol | |
| Resin loading | 1.115 (1000) / 10.2 | = 109.31μmol$g^{-1}$ |

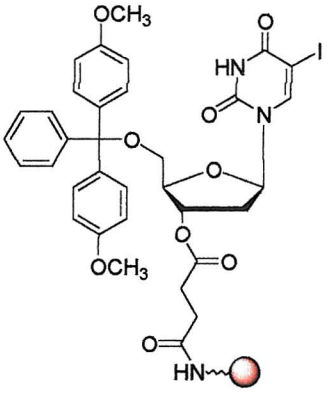
## 6.1.4 List of Compounds

## 6.1.5 Experimental




**Succinamide resins [1-4]**

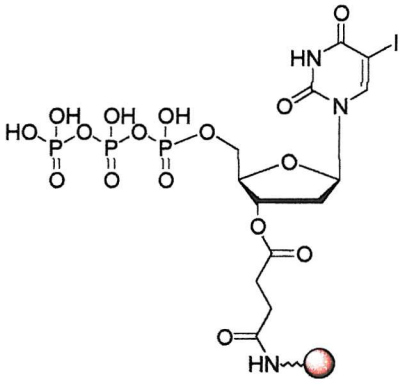
The amino functionalised PS (1.15g), TG (1.00g), HG (1.10g) and CPG (1.80g) resins were washed with anhydrous pyridine and to each resin was added a solution of succinic anhydride (50 eq) and DMAP (10 eq) in the minimum volume of anhydrous pyridine. The resins were gently agitated under a stream of argon gas for 1 hour before being filtered and washed extensively with pyridine (x 3) and DMF (x 5). The resins were dried over $P_2O_5$ *in vacuo* for 12 hours. A small sample of each resin was removed to ascertain the resin loading (section 6.1.3.2) which was found to be 1.28mmol $g^{-1}$, 98%, 0.98mmol $g^{-1}$, 92%, 0.75mmol $g^{-1}$, 99% and 68μmol $g^{-1}$, 85% respectively.




**5-Iodo-5'-O-(4,4'-dimethoxytrityl)-2'-deoxyuridine-3'-O-succinamide resins [5-8]**

The succinyl PS (0.75g, 0.96mmol), TG (0.78g, 0.76mmol), HG (0.91g, 0.68mmol) and CPG (1.37g, 93 μmol) resins **[1-4]** were soaked with a solution of DIC (5 eq),

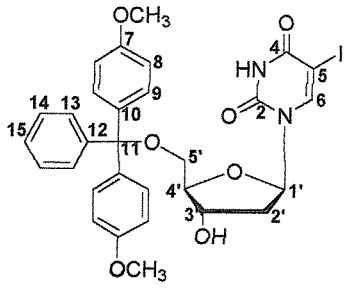
compound **[13]** (5 eq) and DMAP (0.5 eq) in the minimum volume of anhydrous pyridine. The resins were gently agitated under a stream of argon gas for 16 hours before being filtered and washed extensively with pyridine (x 5) and DMF (x 5). The resins were dried over $P_2O_5$ *in vacuo* for 12 hours. A small sample of each resin was removed to ascertain the resin loading (section 6.1.3.1). The remainder of the resin was capped by treatment with a solution of pentafluorophenol (5 eq), DIC (5 eq) and DMAP (0.5 molar equiv) in a minimum volume of anhydrous pyridine for 16 hours. The resin was washed with pyridine (x 3) and DCM (x 5) before being treated with piperidine for 15 minutes. Any remaining amino groups were capped with a solution of acetic anhydride : 2, 6-lutidine in THF : 1-methylimidazole in THF (1:1) for 15 minutes, the resin washed with THF (x 3) and DCM (x 5) and dried over $P_2O_5$ *in vacuo*. A small sample of each resin was used to verify the loading (section 6.1.3.1) and was found to be 0.41mmol $g^{-1}$, 32%, 0.37mmol $g^{-1}$, 38%, 0.15mmol $g^{-1}$, 40% and *20μmol* $g^{-1}$, *60% respectively*.



**5-Iodo-2'-deoxyuridine-5'-triphosphate-3'-O-succinamide resins [9-12]**

The nucleoside PS (0.61g, 0.25mmol), TG (0.63g, 0.23mmol), HG (0.77g, 0.12mmol) and CPG (0.95g, 19μmol ) resins **[5-8]** were treated using general method 6.1.2.2. A small sample of each resin was removed to ascertain the resin loading and treated with conc. aq. ammonia at r.t. for 20 minutes and concentrated *in vacuo*. The residues were dissolved in water and purified by anion exchange HPLC (Buffer system1)

giving the following yields and loading of pure triphosphate for each resin: 7%, 82μmol $g^{-1}$, 17%, 74μmol $g^{-1}$, 30%, μmol $g^{-1}$, 73%, 58μmol $g^{-1}$.




$C_{30}H_{28}IN_2O_7$
Mol. Wt.: 655.46
C, 54.97; H, 4.31; I, 19.36; N, 4.27; O, 17.09

**5-Iodo-5'-O-(4,4'-dimethoxytrityl)-2'-deoxyuridine [13]**[179]

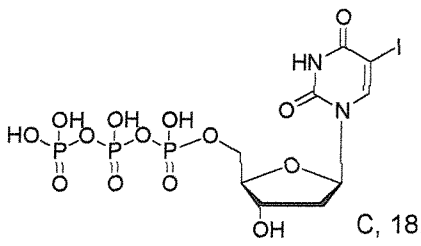
To a solution of 5-iodo-2'-deoxyuridine (10.548g, 29.8mmol) in anhydrous pyridine (50mL) was added drop-wise a solution of 4,4'dimethoxytrityl chloride (11.107g, 32.8mmol) in anhydrous pyridine (50mL). The reaction mixture was stirred under an argon atmosphere for 12 hours before being concentrated *in vacuo*. The residue was dissolved in DCM (400mL) and washed with sat. KCl (3 x 200mL) followed by brine (200mL). The organic phase was separated, dried ($Na_2SO_4$) and the solvent removed *in vacuo*. The crude product was purified by wet-flash column chromatography eluting with a gradient of methanol in DCM (0-6%) to give the title compound as a white foam which was dried in a vacuum desiccator over $P_2O_5$ for 24 hours (18.767g, 28.6mmol, 96%).

$R_f$(G) 0.35; LRMS ($ES^+$ mode): *m/z* 303.2 $[DMT]^+$, 678.9 $[MNa]^+$; $\lambda_{max}$ (MeOH): 281, 230nm; *mp*: 133-134°C (*Lit. mp*: 133-135°C).

IR ($CH_2Cl_2$): ν = 3599.2 (m), 3368.9 (m), 2935.8 (m, br), 1714.9 (s), 1693.1 (s) 1264.0 (s) $cm^{-1}$.

$\delta_H$($CDCl_3$) 2.31 (1H, *dt*, *J* = 15.4, 5.9 Hz, **H2'$^a$**) 2.52 (1H, *dt*, *J* = 10.3, 2.9 Hz, **H2'$^b$**) 3.35-3.41 (2H, m, **H5'**) 3.80 (6H, s, **$OCH_3$**) 4.12 (1H, d, *J* = 2.9Hz, **H4'**) 4.57 (1H, t, *J* =2.9 Hz, **H3'**) 6.34 (1H, dd, *J* = 7.4, 5.9 Hz, **H1'**) 6.86 (4H, d, *J* = 8.8 Hz, **H9**) 7.21-7.36 (7H, m, **H8+H14+H15**) 7.43 (2H, d, *J* = 7.4 Hz, **H13**) 8.16 (1H, s, **H6**).

$\delta_C(CDCl_3)$ 41.6 (**C2'**) 55.5 (**0$CH_3$**) 63.7 (**C5'**) 68.9 (**C4'**) 72.7 (**C11**) 85.8 (**C3'**) 86.7 (**C1'**) 87.2 (**C5**) 113.5 (**C8**) 127.3 (**C15**) 128.2 (**C13**) 128.3 (**C14**) 130.2 (**C9**) 135.5 (**C10**) 144.5 (**C6**) 150.3 (**C12**) 158.8 (**C7**) 160.4 (**C2**) 173.9 (**C4**).

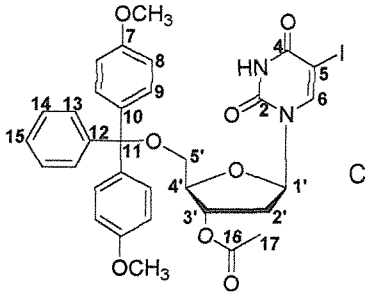


$C_9H_{14}IN_2O_{14}P_3$
Exact Mass: 593.87
C, 18.20; H, 2.38; I, 21.36; N, 4.72; O, 37.71; P, 15.64

**5-Iodo-2'-deoxyuridine-5'-triphosphate [14]**

The triphosphate PS **[9]** (0.61g, 0.25mmol), TG **[10]** (0.63g, 0.23mmol), HG **[11]** (0.77g, 0.28mmol) and CPG resin **[12]** (0.95g, 19μmol) were cleaved using general method 6.1.2.5 and purified by anion exchange HPLC (Buffer system 1). The appropriate fraction was lyophilised repeatedly to give the title compound as a white lyophilised solid (1.8μmol, 7% for PS, 39μmol, 17% for TG, 84μmol, 30% for HG and 14μmol, 73% for CPG).

HPLC retention time (Buffer system 1): 11.11 minutes; LRMS ($ES^-$ mode): *m/z* 593.0 $[MH]^-$; $E_{max}$ ($H_2O$): 297nm (6,230).



$C_{32}H_{31}IN_2O_8$
Mol. Wt.: 698.50
C, 55.02; H, 4.47; I, 18.17; N, 4.01; O, 18.32

**5-Iodo-5'-O-(4,4'-dimethoxytrityl)-3'-O-acetyl-2'-deoxyuridine [15]**

To a solution of compound **[13]** (1.032g, 1.57mmol) in anhydrous pyridine (5mL) was added acetic anhydride (5mL, 5.410g, 53.0mmol) and the reaction mixture stirred under an argon atmosphere for 3 hours before being concentrated *in vacuo*. The

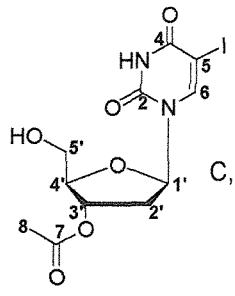
residue was purified by wet-flash column chromatography eluting with 50% diethyl ether in DCM to give the title compound as a cream coloured foam which was dried in a vacuum desiccator over $P_2O_5$ for 24 hours (698mg, 1.00mmol, 64%).

$R_f$(N) 0.53; LRMS ($ES^+$ mode): *m/z* 699.6 $[MH]^+$; $\lambda_{max}$(MeOH): 281, 230nm.

IR ($CH_2Cl_2$): ν = 3599.2 (m), 3368.9 (m), 2935.8 (m, br), 1714.9 (s), 1693.1 (s) 1264.0 (s) $cm^{-1}$.

$\delta_H$($CDCl_3$) *2.00* (3H,s, **H17**) 2.32 (1H, m, **H2'$^a$**) 2.48 (2H, dd, *J* = 14.6, 5.0 Hz, **H2'$^b$**) 3.37 (2H, ddd, *J* = 21.3, 10.8, 2.8 Hz, **H5'**) 3.72 (6H, s, **$OCH_3$**) 4.08 (1H, m, **H4'**) 5.36 (1H, d, *J* = 6.0 Hz, **H3'**) 6.25 (1H, dd, *J* = 8.8, 5.3 Hz, **H1'**) 6.78 (4H, d, *J* = 8.0 Hz, **H9**) 7.15-7.23 (7H, m, **H8+H14+H15**) 7.35 (2H, d, *J* = 7.1 Hz, **H13**) 8.10 (1H, s, **H6**).

$\delta_C$($CDCl_3$) 17.4 (**C17**) 41.6 (**C2'**) 63.7 (**C5'**) 68.9 (**C4'**) 85.8 (**C3'**) 86.7 (**C1'**) 87.2 (**C5**) 144.5 (**C6**) 160.4 (**C2**) 171.1 (**C16**) 173.9 (**C4**).




$C_{11}H_{13}IN_2O_6$
Mol. Wt.: 396.14
C, 33.35; H, 3.31; I, 32.04; N, 7.07; O, 24.23

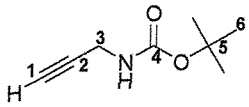
**5-Iodo-3'-O-acetyl-2'-deoxyuridine [16]**

Compound **[15]** (630mg, 0.90mmol) was dissolved in a solution of 5% TCA in DCM and stirred for 1hr. The reaction mixture was then diluted with DCM (200mL) and washed with $H_2O$ (3 x 100mL) followed by brine (100mL). The organic layer was separated, dried ($Na_2SO_4$) and the solvent removed *in vacuo*. The crude product was purified by wet-flash column chromatography eluting with 50% diethyl ether in DCM to give the title compound as a white micro-crystalline solid which was dried in a vacuum desiccator over $P_2O_5$ for 24hrs (331mg, 0.85mmol, 94%).

$R_f$(N) 0.24; LRMS ($ES^-$ mode): *m/z* 395.2 $[MH]^-$; $\lambda_{max}$ (MeOH): 281nm.

$\delta_H(CDCl_3)$ 2.10 (3H,s, **H17**) 2.41 (2H, m, **H2'**) 3.98 (2H, ddd, $J$ = 21.3, 10.8, 2.8 Hz, **H5'**) 4.17 (1H, m, **H4'**) 5.34 (1H, d, $J$ = 6.0 Hz, **H3'**) 6.24 (1H, dd, $J$ = 8.8, 5.3 Hz, **H1'**) 8.23 (1H, s, **H6**).

$\delta_C(CDCl_3)$ 17.4 (**C17**) 41.6 (**C2'**) 55.5 (**0CH$_3$**) 63.7 (**C5'**) 68.9 (**C4'**) 72.7 (**C11**) 85.8 (**C3'**) 86.7 (**C1'**) 87.2 (**C5**) 113.5 (**C8**) 127.3 (**C15**) 128.2 (**C13**) 128.3 (**C14**) 130.2 (**C9**) 135.5 (**C10**) 144.5 (**C6**) 150.3 (**C12**) 158.8 (**C7**) 160.4 (**C2**) 171.1 (**C16**) 173.9 (**C4**).




$C_8H_{13}NO_2$
Mol. Wt.: 155.19
C, 61.91; H, 8.44; N, 9.03; O, 20.62

**_N_-(_t_-butoxycarbonyl)propargylamine [17][100]**

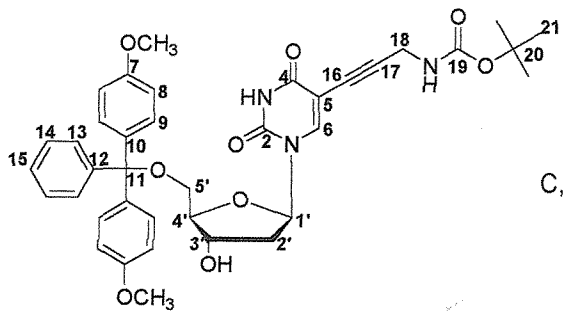
To a solution of 3-aminopropyne (2.750g, 50mmol) in anhydrous dichloromethane (200mL) was added di-*t*-butyldicarbonate (10.920g, 50mmol) and the solution stirred under an argon atmosphere for 3 hours. The solvent was removed *in vacuo* producing a yellow syrup, which was placed at –20°C to crystallise. The product was filtered and washed with hexane to yield the title compound as colourless crystals which were dried in a vacuum desiccator over $P_2O_5$ for 24 hours (4.418g, 28.5mmol, 57%).

Mp: 43-44°C (Lit. mp: 43-45°C).

IR: $\nu$ = 3053.8 (w), 2353.9 (w), 1736.3 (m), 1693.0 (s), 1465.0 (m), 1232.4 (m) $cm^{-1}$.

$\delta_H(CDCl_3)$ 1.45 (9H, s, **H6**) 2.22 (1H, s, **H1**) 3.93 (2H, s, **H3**) 4.80 (1H, s, br, **NH**).

$\delta_C(CDCl_3)$ 28.5 (**C6**) 30.5 (**C3**) 71.4 (**C1+C2**) 80.2 (**C5**) 155.4 (**C4**).



$C_{38}H_{41}N_3O_9$
Mol. Wt.: 683.75
C, 66.75; H, 6.04; N, 6.15; O, 21.06

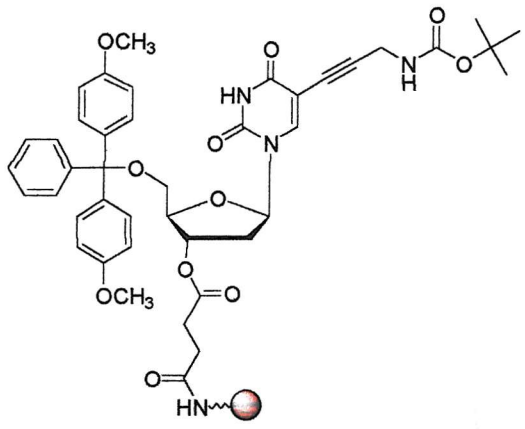
**5-[*N*-(*t*-butoxycarbonyl)propargylamino-5'-O-(4, 4'-dimethoxytrityl)]-2'-deoxyuridine [18]**

To a degassed solution of compound **[13]** (1.024g, 1.56mmol), copper (I) iodide (59mg, 0.31mmol, 0.2eq), tetrakistriphenylphosphine palladium (0) (180mg, 0.16mmol, 0.1eq) and triethylamine (3ml, XS) in anhydrous DMF (15mL), was added a degassed solution of compound **[17]** in anhydrous DMF (5mL). The solution was stirred under an argon atmosphere for 24 hours before the solvent was removed *in vacuo*. The residue was dissolved in ethylacetate (350mL) and washed with sat. KCl (3 x 50mL), brine (50mL) and 5% EDTA (3 x 50mL). The organic phase was separated, dried ($Na_2SO_4$)and concentrated *in vacuo*. The crude product was puified by wet flash column chromatography (silica pre-equilibrated with 1% $Et_3N$) eluting with methanol in dichloromethane (0-5%) to yield the title compound as a white foam which was dried in a vacuum desiccator over $P_2O_5$ for 24 hours (543mg, 0.79mmol, 51%).

$R_f$ (G) 0.54; LRMS ($ES^+$ mode): *m/z* 706.5 $[MNa]^+$ 722.5 $[MK]^+$; $\lambda_{max}$ (MeOH): 284, 232nm; *mp: 130-132°C; Found C: 65.96, H: 6.18, N: 5.94, $C_{38}H_{41}N_3O_9$ requires C:* 66.75, H: 6.04, N: 6.14%

IR: $\nu$ = 2360.2 (m), 1692.6 (s), 1508.0 (s), 1456.6 (m), 1250.3 (s) $cm^{-1}$.
$\delta_H(CDCl_3)$ 1.39 (9H, s, **H21**) 2.30 (1H, m, **H2'$^b$**) 2.56 (1H, m, **H2'$^a$**) 3.31-3.41 (2H, m, **H5'**) 3.78 (6H, s, **$OCH_3$**) 4.16 (1H, d, *J* = 2.9 Hz **H18**) 4.59 (1H, m, **H4'**) 4.84 (1H, m, **H3'**) 6.34 (1H, t, *J* = 6.6 Hz, **H1'**) 6.85 (4H, d, *J* = 8.8 Hz, **H9**) 7.19-7.38 (7H, m, **H8+H14+H15**) 7.43 (2H, d, *J* = 6.6 Hz, **H13**) 8.23 (1H, s, **H6**).

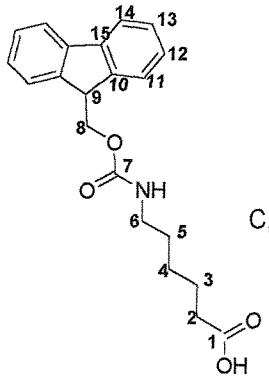
$\delta_C(CDCl_3)$ 28.3 (**C21**) 31.1 (**C18**) 41.6 (**C2'**) 55.3 (**$OCH_3$**) 63.7 (**C5'**) 72.2 (**C4'**) 74.0 (**C11**) 79.6 (**C16**) 85.6 (**C3'**) 86.7 (**C1'**) 87.0 (**C20**) 89.8 (**C17**) 99.7 (**C5**) 113.4 (**C8**) 127.0 (**C15**) 127.9 (**C13**) 128.1 (**C14**) 130.0 (**C9**) 135.5 (**C10**) 142.9 (**C6**) 144.6 (**C12**) 149.7 (**C7**) 155.2 (**C19**) 158.5 (**C2**) 162.6 (**C4**).




**5-*N*-(*t*-butoxycarbonyl) aminopropargyl-5'-O-(4, 4'-dimethoxytrityl)-2'-deoxyuridine-3'-O-succinamide resins [19-21]**

The succinyl PS (0.40g, 0.51mmol), TG (0.32g, 0.31mmol) and CPG (0.27g, 18μmol) resins **[1, 2, 4]** were treated twice with a solution of compound **[18]** (5 eq), DIC (5 eq) and DMAP (0.5 eq) in a minimum volume of anhydrous pyridine in two separate 16 hour couplings with only a washing step in between. After the second coupling, the resin was washed with pyridine (x 5) and dried over $P_2O_5$ *in vacuo* for 12 hours. A small sample of each resin was removed to ascertain the resin loading (section 6.1.3.1). The remainder of the resins were capped by treatment with a solution of pentafluorophenol (5 eq), DIC (5 eq) and DMAP (0.5 molar equiv) in a minimum volume of anhydrous pyridine for 16 hours. The resins were washed with pyridine (x 3) and DCM (x 5) before being treated with piperidine for 15 minutes. Any remaining amino groups were capped with a solution of acetic anhydride : 2, 6-lutidine in THF : 1-methylimidazole in THF (1:1) for 15 minutes, the resins washed with THF (x 3) and DCM (x 5) and dried over $P_2O_5$ *in vacuo*. A small sample of each

resin was used to verify the loading (section 6.1.3.1) which were found to be 0.46mmol $g^{-1}$, 36%, 0.43mmol $g^{-1}$, 44% and 42 μmol $g^{-1}$, 62% respectively.




$C_{22}H_{27}NO_4$
Mol. Wt.: 369.45
C, 71.52; H, 7.37; N, 3.79; O, 17.32

**6-(9-Fluorenylmethoxycarbonylamino)hexanoic acid [22]**

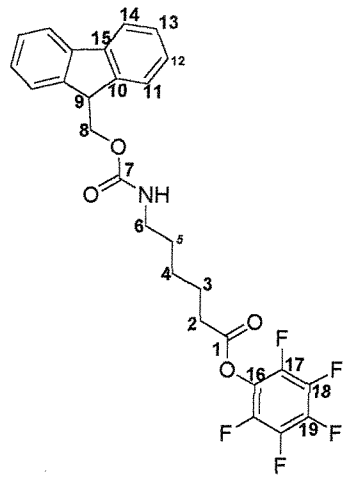
To a solution of 6-amino-1-hexanoic acid (565mg, 4.31mmol 1.2eq) in aq. sodium carbonate (10mL, 9%) was added a solution of *N*-(9-fluorenylmethyloxycarbonyloxy) succinimide (1.210g, 3.59mmol, 1.0eq) in DMF(10mL). The solution was stirred for 20 minutes, diluted with ethylacetate (300mL) and washed with $H_2O$ (3 x 100mL). The organic phase was separated, dried ($Na_2SO_4$) and the solvent removed *in vacuo*. The crude product was purified by wet flash column chromatography eluting with a gradient of methanol in dichloromethane (0-6%) to yield the title compound as a white crystalline solid which was dried in a vacuum desiccator over $P_2O_5$ for 24 hours (612mg, 1.73mmol, 93%).

$R_f$ (G) 0.36; LRMS ($ES^+$ mode): *m/z* 370.4 $[MH]^+$; $\lambda_{max}$ (MeOH): 300, 289, 265nm; mp: 118-120°C.

IR: ν = 3337.6 (m), 2940.3 (w), 1687.6 (s), 1530.8 (s), 1269.7 (m) $cm^{-1}$.

$\delta_H(CDCl_3)$ 1.26-1.71 (6H, m, **H4** + **H3** + **H5**) 2.36 (2H, t, *J* = 7.4 Hz, **H2**) 3.20 (2H, dd, *J* = 12.9, 7.0 Hz, **H6**) 4.22 (1H, t, *J* = 6.6 Hz, **H9**) 4.42 (2H, d, *J* = 7.4 Hz, **H8**) 4.85 (1H, s, br **NH**) 7.28-7.35 (2H, m, **H12**) 7.41 (2H, t, *J* = 7.0 Hz, **H13**) 7.60 (2H, d, *J* = 7.4 Hz, **H11**) 7.78 (2H, d, *J* = 7.4 Hz, **H14**).

$\delta_C$($CDCl_3$) 24.4 (**C4**) 26.3 (**C3**) 29.7 (**C5**)34.0 (**C2**) 40.9 (**C6**) 47.4 (**C9**) 66.7 (**C8**) 120.1 (**C13**) 125.2 (**C14**) 127.2 (**C12**) 127.8 (**C11**) 141.5 (**C15**) 144.1 (**C10**) 156.6 (**C7**) 173.9 (**C1**).



$C_{27}H_{22}F_5NO_4$
Mol. Wt.: 519.46
C, 62.43; H, 4.27; F, 18.29; N, 2.70; O, 12.32

**6-(9-Fluorenylmethoxycarbonylamino)hexanoic acid pentafluorophenyl ester [23]**[256]

To a solution of compound **[22]** (265mg, 0.82mmol) in anhydrous DMF (3mL) was added a solution of pentafluorophenol (166mg, 0.90mmol, 1.1eq) and dicyclohexylcarbodiimide (169mg, 0.82mmol, 1.0eq) in anhydrous DMF (2mL) and the solution stirred under an argon atmosphere for 24 hours. The solution was filtered, the filtrate evaporated *in vacuo* to dryness giving the crude ester as a white waxy solid. This was re-crystallised from 95% ethanol/ 1% acetic acid to give the title compound as white needles which were dried in a vacuum desiccator over $P_2O_5$ for 24 hours (360mg, 0.69mmol, 85%).

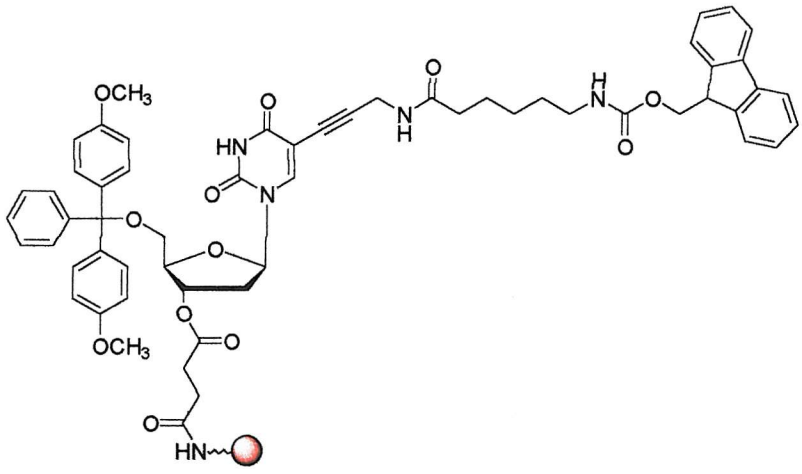
$R_f$ (G) 0.94; $\lambda_{max}$ (MeOH): 300, 282, 264nm; mp: 126-127°C (Lit. mp: 126-127°C).

IR: $\nu$ = 3328.1 (m), 2907.2 (w), 2361.3 (m), 1784.6 (m), 1694.5 (s), 1517.9 (s) 1267.4 (m) $cm^{-1}$.

$\delta_H$($CDCl_3$) *1.04-1.96 (6H, m,* ***H4+H3+H5*** *) 2.69 (2H, t, J = 7.4 Hz,* **H2***) 3.24 (2H, dd, J =* 12.5, 5.9 Hz, **H6**) 4.23 (1H, t, *J* = 6.6 Hz, **H9**) 4.43 (2H, d, *J* = 6.6 Hz, **H8**) 4.82

(1H, s, br, **NH**) 7.28-7.35 (2H, m, **H12**) 7.42 (2H, t, $J$ = 7.4 Hz, **H13**) 7.61 (2H, d, $J$ = 7.4 Hz, **H11**) 7.78 (2H, d, $J$ = 7.4 Hz, **H14**).

$\delta_C(CDCl_3)$ 24.4 (**C4**) 25.7 (**C3**) 29.7 (**C5**) 33.3 (**C2**) 34.1 (**C6**) 47.4 (**C9**) 66.7 (**C8**) 120.1 (**C13**) 123.8 (**C16**) 125.2 (**C14**) 127.2 (**C12**) 127.8 (**C11**) 132.7 (**C18**) 136.4 (**C19**) 140.6 (**C17**) 141.5 (**C15**) 144.2 (**C10**).




**5-[6-(9-Fluorenylmethoxycarbonylamino)hexanoyl-3-propargylamino]-5'-O-(4,4'-dimethoxytrityl)-2'-deoxyuridine-3'-O-succinamide resins [24-27]**

*Method 1:*

The nucleoside PS (0.25g, 0.12mmol), TG (0.31g, 0.13mmol),and CPG (60mg, 2.5μmol) resins **[19-21]** were treated with 90% TFA in ethanedithiol (2mL) for 10 minutes, washed with DMF (x 3) and DCM (x 5), neutralised with 20% triethylamine in DCM and washed again with DCM (x 5) and dried *in vacuo*. The resins were then treated twice with a solution of compound **[23]** (2.5 eq) and HOBt (2.5 eq) in a minimum volume of anhydrous DMF in two separate 45 minute couplings with only a washing step in between. After the second coupling the resins were washed with DMF (x 3) and DCM (x 5) and dried *in vacuo*. The resins were then treated with a solution of 4, 4'-dimethoxytritylchloride (50 eq) in the minimum volume of anhydrous pyridine in two separate 16 hour couplings. After the second coupling the resins were washed with pyridine (x 3) and DCM (x 5) and dried over $P_2O_5$ *in vacuo*

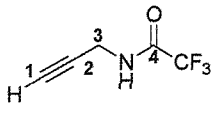
for 12 hours. A small sample of each resin was removed to ascertain the resin loading (section 6.1.3.1) which was found to be 0.31mmol $g^{-1}$, 86%, 0.39mmol $g^{-1}$, 91% and 33μmol $g^{-1}$, 78% respectively.

*Method 2:*

The amino PS (0.32g, 0.41mmol), TG (0.36g, 0.35mmol), HG (0.18g, 0.14mmol) and CPG (50mg, 3.4μmol $g^{-1}$) resins **[1-4]** were treated with a solution of DIC (5 eq), compound **[34]** (5 eq) and DMAP (0.5 eq) in the minimum volume of anhydrous pyridine. The resins were gently agitated under a stream of argon gas for 16 hours before being filtered and washed extensively with pyridine (x 3), DMF (x 3) and DCM (x 5). The resins were dried over $P_2O_5$ *in vacuo* for 12 hours. A small sample of each resin was removed to ascertain the resin loading (section 6.1.3.1) which was found to be 0.17mmol $g^{-1}$, 13%, 0.26mmol $g^{-1}$, 21%, 0.15mmol $g^{-1}$, 29% and 49μmol $g^{-1}$, 72% respectively.

*Method 3:*

Compound **[39]** was attached to the HG (0.16g, 0.12mmol) and CPG (80mg, 6.4μmol) solid supports using general method 6.1.2.1 giving a loading of 0.35mmol $g^{-1}$, 23% and 52μmol $g^{-1}$, 65% respectively.




$C_5H_4F_3NO$
Mol. Wt.: 151.09
C, 39.75; H, 2.67; F, 37.72; N, 9.27; O, 10.59

**_N_-trifluoroacetylpropargylamine [28]**[252]

Propargylamine (6.23ml, 5.00g, 90.8mmol) was added dropwise to a solution of ethyltrifluoroacetate (14.0ml, 118.0mmol, 1.3eq) in anhydrous methanol at 0°C. When addition was complete the reaction was allowed to warm to room temperature and stirred under an argon atmosphere overnight. The solvent was removed *in vacuo*, the residue dissolved in dichloromethane (200ml) and washed with sat. $NaHCO_3$ (2 x

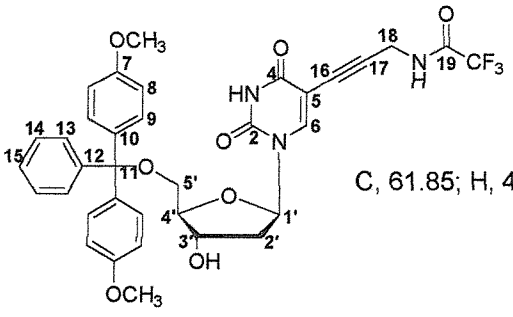
200ml) followed by sat. KCl (2 x 200ml). The organic phase was separated, dried ($Na_2SO_4$) and the solvent removed *in vacuo*. The crude product was purified by distillation at reduced pressure to give the title compound as a colourless liquid (7mm Hg, 10.850g, 77%).

$R_f$(F) 0.25; LRMS (ES$^-$ mode): *m/z* 264.1 [MTFA]$^-$; bp: 65°C (7mm Hg)

IR ($CH_2Cl_2$): $\nu$ = 3428.6 (m), 3301.8 (s), 2259.7 (*w*), 1734.0 (s) 1541.5 (m) 1269.7 (s) cm$^{-1}$.

$\delta_H$($CDCl_3$) 2.31 (1H, t, $J$ = 2.2 Hz, **H1**) 4.14 (2H, dd, $J$ = 5.1, 2.9 Hz **H3**) 7.25 (1H, br s, **NH**).

$\delta_C$($CDCl_3$) 29.6, 29.7 (**C3**) 73.0 (**C1**) 77.3 (**C2**) 117.6 (**$CF_3$**) 157.4 (**C4**).




$C_{35}H_{32}F_3N_3O_8$
Mol. Wt.: 679.64
C, 61.85; H, 4.75; F, 8.39; N, 6.18; O, 18.83

**5-Trifluoroacetylaminopropargyl-5'-O-(4, 4'-dimethoxytrityl)-2'-deoxyuridine [29]**[253]

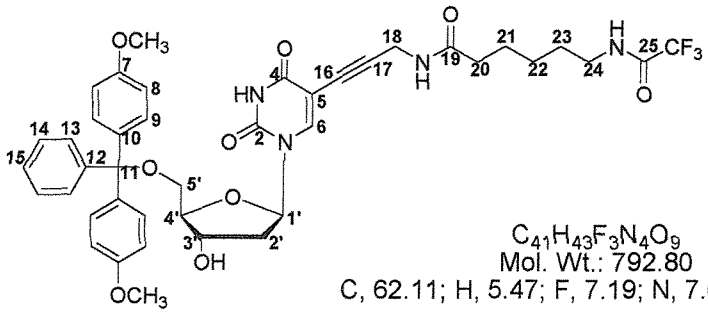
Compound **[13]** (1.531g, 2.33mmol) was dissolved in anhydrous, degassed DMF (10ml). To this solution was added successively copper (I) iodide (88.8mg, 0.47mmol, 0.2eq), anhydrous triethylamine (5.0ml, XS), compound **[28]** (1.056g, 6.99mmol, 3.0eq) and tetrakis(triphenylphosphine)palladium (0) (0.269g, 0.23mmol, 0.1eq). The reaction mixture was stirred under an argon atmosphere for 24 hours before being concentrated *in vacuo*. The residue was dissolved in dichloromethane (250ml) and washed with sat. KCl (3 x 100ml), brine (100ml) and 5% EDTA (3 x 100ml). The organic phase was separated, dried ($Na_2SO_4$) and the solvent removed *in vacuo*. The crude product was purified by wet flash column chromatography (silica

pre-equilibrated with 1% $Et_3N$) eluting with methanol in dichloromethane (0-4%) to yield the title compound as a cream foam which was dried in a vacuum desiccator over $P_2O_5$ for 24 hours (1.225g, 1.8mmol, 77%).

$R_f$ (G) 0.39; LRMS ($ES^+$ mode): *m/z* 702.4 $[MNa]^+$ 718.4 $[MK]^+$; $\lambda_{max}$ (MeOH): 283, 233*nm*.

$\delta_H(CDCl_3)$ 2.33 (1H, m, **H2'$^b$**) 2.57 (1H, m, **H2'$^a$**) 3.37 (2H, m, **H5'**) 3.78 (6H, s, **$OCH_3$**) 3.93 (2H, d, *J* = 6.6 Hz, **H18**) 4.09 (1H, m, **H4'**) 4.60-4.61 (2H, m, **H3'+3'OH**) 6.37 (1H, t, *J* = 6.6 Hz, **H1'**) 6.85 (4H, d, *J* = 9.6Hz, **H9**) 7.20-7.35 (7H, m, **H8+H14+H15**) 7.43 (2H, d, *J* = 7.4 Hz, **H13**) 8.23 (1H, s, **H6**).

$\delta_C(CDCl_3)$ 30.7 (**C18**) 41.9 (**C2'**) 55.4 (**0$CH_3$**) 63.8 (**C5'**) 72.1 (**C4'**) 72.7 (**C11**) 85.8 (**C3'**) 86.7 (**C1'**) 87.1 (**C5**) 93.5 (**C16**) 99.0 (**C17**) 113.5 (**C8**) 127.1 (**C15**) 128.1 (**C13**) 130.1 (**C14**) 130.2 (**C9**) 135.7 (**C10**) 143.3 (**C6**) 144.7 (**C12**) 151.3 (**$CF_3$**) 158.7 (**C7**) 165.3 (**C19**).




$C_{41}H_{43}F_3N_4O_9$
Mol. Wt.: 792.80
C, 62.11; H, 5.47; F, 7.19; N, 7.07; O, 18.16

**5-Trifluoroacetylaminopentylcarbonylaminopropargyl-5'-O-(4, 4'-dimethoxytrityl)-2'-deoxyuridine [30]**[254]

Compound **[29]** (1.271g, 1.95mmol) was treated with a two molar solution of methanolic methylamine (10ml) for 1hour. The reaction mixture was then concentrated *in vacuo*, the residue dissolved in dichloromethane (250mL) and washed with sat. KCl (3 x 100mL). The organic phase was separated, dried ($Na_2SO_4$) and the solvent removed *in vacuo*. The residue was dissolved in anhydrous DMF (10mL) and to the solution was added compound **[32]** (0.758g, 2.34mmol, 1.2eq) and anhydrous triethylamine (0.68ml, 4.91mmol, 2.5eq). The solution was allowed to stir under an

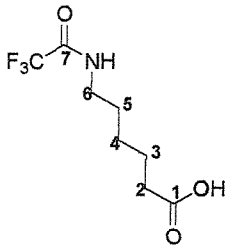
argon atmosphere for 2 hours whereupon the solvent was removed *in vacuo*, the residue dissolved in dichloromethane (400mL) and washed with sat. KCl (3 x 100mL) followed by brine (150mL). The organic phase was separated, dried ($Na_2SO_4$) and concentrated *in vacuo*. The crude product was purified by wet flash column chromatography (silica pre-equilibrated with 1% $Et_3N$) eluting with methanol in dichloromethane (0-5.5%) to yield the title compound as a white foam which was dried in a vacuum desiccator over $P_2O_5$ for 24 hours (0.823g, 1.04mmol, 53%).

$R_f$ (G) 0.40; LRMS ($ES^+$ mode): *m/z* 810.4 $[MNH_4]^+$

IR ($CH_2Cl_2$): $\nu$ = 3435.1 (w), 3058.5 (w), 3937.4 (w) 1721.0 (m), 1698.0 (m) 1508.5 (m) 1266.3 (s) $cm^{-1}$.

$\delta_H$($CDCl_3$) 1.29 (2H, dd, *J* = 14.7, 8.1 Hz, **H22**) 1.54 (4H, dt, *J* = 14.7, 7.0 Hz, **H21**+**H23**) 1.94 (2H, t, *J* = 7.0 Hz **H20**) 2.28 (1H, m, **H2'**$^b$) 2.51 (1H, m, **H2'**$^a$) 3.28-3.38 (4H, m, **H24**+**H5'**) 3.77 (6H, s, **OCH$_3$**) 3.85 (2H, t, *J* = 5.5 Hz, **H18**) 4.10 (1H, m, **H4'**) 4.53 (1H, m, **H3'**) 5.79-5.93 (4H, br, 3x**NH**+**OH**) 6.33 (1H, t, *J* = 6.6 Hz, **H1'**) 6.84 (4H, d, *J* = 8.8 Hz, **H9**) 7.21-7.38 (7H, m, **H8**+**H14**+**H15**) 7.43 (2H, d, *J* = 7.4 Hz, **H13**) 8.15 (1H, s, **H6**).

$\delta_C$($CDCl_3$) 24.6 (**C22**) 26.1 (**C21**) 28.4 (**C23**) 30.1 (**C20**) 35.6 (**C18**) 39.7 (**C24**) 41.8 (**C2'**) 55.4 (**0CH$_3$**) 63.8 (**C5'**) 72.1 (**C4'**) 72.7 (**C11**) 85.9 (**C3'**) 86.8 (**C1'**) 87.1 (**C5**) 89.2 (**C16**) 99.5 (**C17**) 113.5 (**C8**) 127.1 (**C15**) 128.0 (**C13**) 128.2 (**C14**) 130.2 (**C9**) 135.7 (**C10**) 143.2 (**C6**) 144.7 (**C12**) 150.3 (**CCF$_3$**) 158.7 (**C7**) 163.7 (**C19**) 172.6 (**C25**).

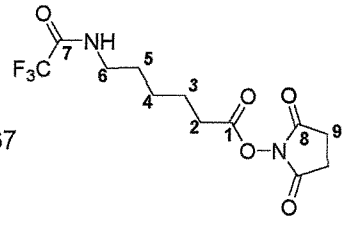


$C_8H_{12}F_3NO_3$
Mol. Wt.: 227.18
C, 42.29; H, 5.32; F, 25.09; N, 6.17; O, 21.13

**6-(*N*-trifluoroacetylamino)-hexanoic acid [31]**

To a suspension of 6-aminohexanoic acid (7.412g, 56.50mmol) in anhydrous methanol (100ml) was added ethyltrifluoroacetate (10.1ml, 12.041g, 184.75mmol, 1.5eq) and anhydrous triethylamine (11.8ml, 8.576g, 84.75mmol, 1.5eq). After 45 minutes, dissolution was complete. Dowex $H^+$ 50WX8-100 (50g) was added and the mixture stirred for a further 30 minutes whereupon the mixture was filtered, the resin washed with methanol (200ml) and the solvent removed *in vacuo*. The waxy white residue was then co-evaporated with dichloromethane followed by chloroform to yield a white solid that required no further purification. The product was dried in a vacuum desiccator over $P_2O_5$ for 24 hours (12.092g, 53.23mmol, 94%).

$R_f$ (C) 0.26; LRMS ($ES^-$ mode): *m/z* 340.1 $[MTFA]^-$.
IR ($CH_2Cl_2$): $\nu$ = 3433.5 (m), 2944.4 (m), 1725.8 (s), 1546.4 (m) 1269.3 (s) $cm^{-1}$.
$\delta_H(D_2O)$ 1.18-1.28 (2H, m, **H3**) 1.49 (4H, ddd, $J$ = 32.5, 15.3, 7.4 Hz **H4**+**H5**) 2.05 (2H, t, $J$ = 7.4 Hz, **H2**) 2.85 (2H, t, $J$ = 7.4 Hz, **H6**).
$\delta_C(D_2O)$ 24.1 (**C4**) 25.7 (**C3**) 28.0 (**C5**) 33.5 (**C2**) 45.7 (**C6**) 118.0 (**$CF_3$**) 156.5 (**C7**) 174.5 (**C1**).



$C_{12}H_{15}F_3N_2O_5$
Mol. Wt.: 324.25
C, 44.45; H, 4.66; F, 17.58; N, 8.64; O, 24.67

**6-(*N*-trifluoro-acetylamino)-hexanoic acid *N*-hydroxysuccinimyl ester [32][254]**

To a solution of compound **[31]** (1.622g, 9.53mmol) in anhydrous *N*, *N*-dimethylformamide (5mL) was added *N*-hydroxysuccinamide (1.316g, 11.4mmol,

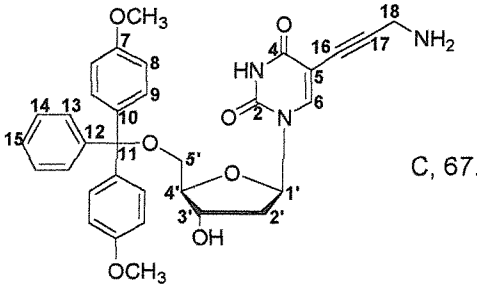
1.2eq) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (2.741g, 14.3mmol,1.5eq). The solution was allowed to stir under an argon atmosphere for 12 hours where after the solvent was removed *in vacuo*. The residue was dissolved in dichloromethane (250mL) and washed with sat. KCl (3 x 200mL). The organic phase was separated, dried ($Na_2SO_4$) and the solvent removed *in vacuo*. The crude product was purified by wet flash column chromatography eluting with methanol in dichloromethane (0-3%) to yield the purified product as a white solid which was dried in a vacuum desiccator over $P_2O_5$ for 24 hours (2.601g, 8.02mmol, 84%).

$R_f$ (G) 0.75; LRMS ($ES^-$ mode): *m/z* 437.2 $[MTFA]^-$; mp: 82-83°C.

IR ($CH_2Cl_2$): ν = 3433.9 (w), 2945.4 (w), 1815.2 (m), 1786.4 (m) 1741.9 (s) 1260.7 (s) $cm^{-1}$.

$\delta_H(CDCl_3)$ 1.42-1.53 (2H, m, **H3**) 1.63 (2H, dt, *J* = 15.7, 7.0 Hz **H4**) 1.79 (2H, dt, *J* = 14.7, 7.4 Hz, **H5**) 2.62 (2H, t, *J* = 7.0 Hz, **H2**) 2.84 (4H, s, **H9**) 3.36 (2H, q, *J* = 6.4 Hz, **H6**) 6.89 (1H, br, s, **NH**).

$\delta_C(CDCl_3)$ 24.2 (**C4**) 25.6 (**C9**) 25.7 (**C3**) 28.2 (**C5**) 30.9 (**C2**) 39.6 (**C6**) 114.1 (**$CF_3$**) 157.2, 157.7 (**C9**) 168.6 (**C7**) 169.5 (**C1**).




$C_{33}H_{33}N_3O_7$
Mol. Wt.: 583.63
C, 67.91; H, 5.70; N, 7.20; O, 19.19

**5-Propargylamino-5'-O-(4,4'-dimethoxytrityl)-2'-deoxyuridine [33]**[186]

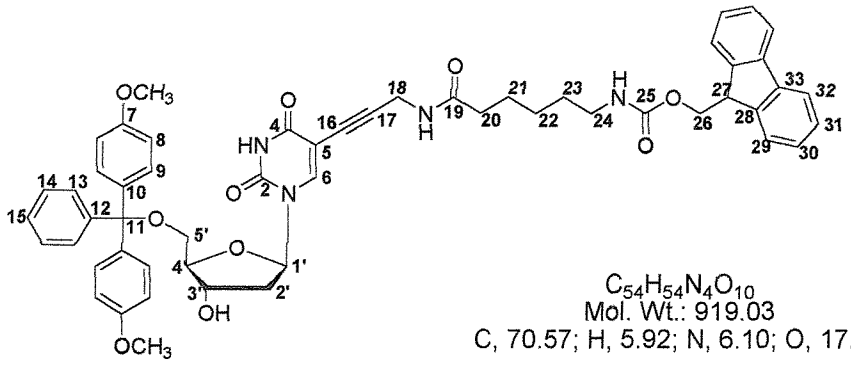
To a solution of compound **[13]** (6.825g, 10.4mmol) in anhydrous DMF (15mL) was added a solution of propargylamine (2.14mL, 1.718g, 31.2mmol), copper (I) iodide 495mg, 2.60mmol) and triethylamine (7.25mL, 5.264g, 52mmol) in anhydrous DMF

(20ml). To this was then added tetrakistriphenylphosphine palladium (0) (1.202g, 1.04mmol) and the solution stirred under an argon atmosphere for 2 hours. The reaction mixture was then reduced in volume, pre-absorbed onto silica and purified by wet-flash column chromatography eluting with a gradient of methanol in DCM (0-10%) to give the title compound as a pale yellow foam which was dried in a vacuum dessicator over $P_2O_5$ for 24hrs (5.390g, 9.25mmol, 89%).

$R_f$(D) 0.15; LRMS ($ES^+$ mode): *m/z* 584.5 $[MH]^+$

$\delta_H(CDCl_3)$ 2.22 (1H, m, **H2'$^a$**) 2.42 (1H, m, **H2'$^b$**) 3.21 (1H, dd, *J* = 10.8, 3.0 Hz, **H5'$^a$**) 3.36 (1H, dd, *J* = 11.0, 2.5 Hz, **H5'$^b$**) 3.40 (2H, s, **H18**) 3.71 (6H, s, 2x**OCH$_3$**) 4.02 (1H, m, **H4'**) 4.47 (1H, m, **H3'**) 6.26 (1H, t, *J* = 7.0 Hz, **H1'**) 6.77 (4H, d, *J* = 7.5 Hz, **H9**) 7.12-7.29 (7H, m, **H8+H14+H15**) 7.35 (2H, d, *J* = 7.0 Hz, **H13**) 8.10 (1H, s, **H6**).

$\delta_C(CDCl_3)$ 32.2 (**C18**) 42.0 (**C2'**) 55.7 (**0CH$_3$**) 64.0 (**C5'**) 72.4 (**C4'**) 73.5 (**C11**) 86.1 (**C3'**) 87.1 (**C1'**) 87.2 (**C5**) 95.0 (**C16**) 100.6 (**C17**) 113.5 (**C8**) 127.3 (**C15**) 128.3 (**C13**) 128.5 (**C14**) 130.4 (**C9**) 135.9 (**C10**) 143.0 (**C6**) 149.7 (**C12**) 159.0 (**C7**) 162.3 (**C2**) 163.1 (**C4**).



$C_{54}H_{54}N_4O_{10}$
Mol. Wt.: 919.03
C, 70.57; H, 5.92; N, 6.10; O, 17.41

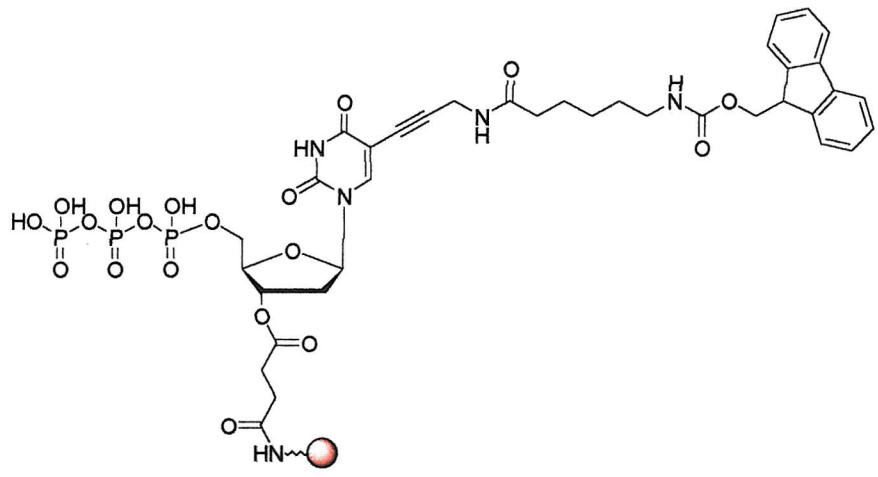
**5-[6-(9-Fluorenylmethoxycarbonylamino)hexanoyl-3-propargylamino]-5'-O-(4,4'-dimethoxytrityl)-2'-deoxyuridine [34]**[190]

To a solution of compound **[22]** (2.976g, 8.83mmol) in anhydrous pyridine (20mL) was added EDC (2.031g, 10.6mmol) and to the resulting suspension was added drop-wise a solution of compound **[33]** (5.146g, 8.83mmol) in anhydrous pyridine (25mL). The reaction mixture was stirred under an argon atmosphere for 12 hours before being concentrated *in vacuo*. The residue was dissolved in DCM (300mL) and washed with sat. KCl (3 x 150mL) followed by brine (150mL). The organic layer was separated, dried ($Na_2SO_4$) and the solvent removed *in vacuo*. The crude product was purified by wet-flash column chromatography eluting with ethylacetate to give the title compound as a pale yellow foam which was dried in a vacuum desiccator over $P_2O_5$ for 24 hours (4.377g, 4.77mmol, 54%).

$R_f$(I) 0.32; LRMS ($ES^+$ mode): *m/z* 936.5 $[MNH_4]^+$

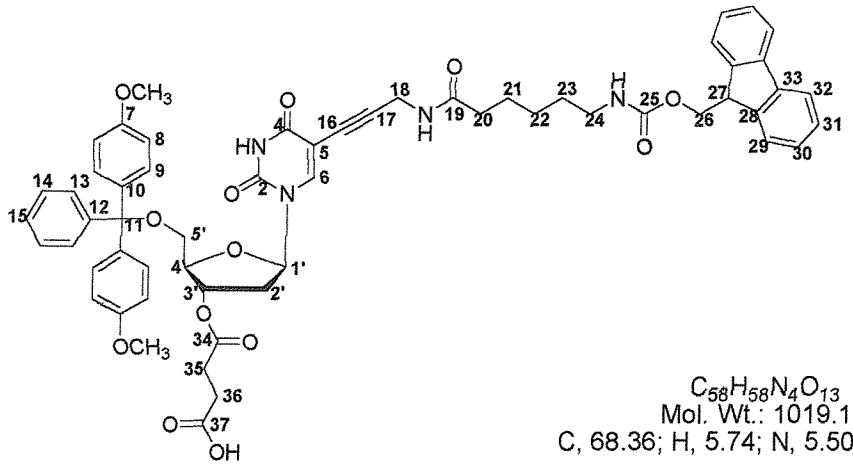
$\delta_H(CDCl_3)$ 1.62-1.77 (7H, m, **H21**+**H22**+**H23**) 2.24-2.33 (2H, m, **H2'**) 2.47 (2H, t, *J* = 5.9 Hz, **H24**) 3.30-3.45 (2H, m, **H5'**) 3.78 (6H, s, **$OCH_3$**) 3.88 (1H, t, *J* = 5.2 Hz, **H27**) 4.09 (2H, d, *J* = 2.9 Hz, **H18**) 4.22 (1H, d, *J* = 5.9 Hz, **H4'**) 4.38 (2H, d, *J* = 6.6 Hz, **H26**) 4.53 (1H, m, **H3'**) 5.43 (1H, m, **H1'**) 6.84 (4H, d, *J* = 8.1 Hz, **H9**) 7.20-7.44 (13H, m, **H8**+**H13**+**H14**+**H15**+**H30**+**H31**) 7.59 (2H, d, *J* = 7.4 Hz, **H29**) 7.76 (2H, d, *J* = 7.4 Hz, **H32**) 8.20 (1H, s, **H6**).

$\delta_C(CDCl_3)$ 25.1 (**C22**) 26.7 (**C21**) 30.1 (**C23**) 30.3 (**C20**) 31.1 (**C18**) 36.2 (**C26**) 39.2 (**C24**) 41.3 (**C2'**) 55.4 (**0CH$_3$**) 64.2 (**C5'**) 66.8 (**C4'**) 72.7 (**C11**) 85.7 (**C3'**) 86.6 (**C1'**) 87.7 (**C5**) 89.1 (**C16**) 99.5 (**C17**) 113.8 (**C8**) 120.2 (**C32**) 125.6 (**C30**) 127.4 (**C15**) 128.0 (**C13**) 128.3 (**C14**) 130.8 (**C9**) 135.8 (**C10**) 141.6 (**C33**) 141.9 (**C6**) 144.4 (**C12**) 144.8 (**C28**) 159.0 (**C7**) 163.7 (**C25**).



**5-[6-(9-Fluorenylmethoxycarbonylamino)hexanoyl-3-propargylamino]-2'-deoxyuridine-5'-triphosphate-3'-O-succinamide resins [35-38]**

The nucleoside PS (0.21g, 65μmol), TG (0.36g, 0.14mmol), HG (0.24g, 84μmol) and CPG (0.57g, 30μmol) resins **[24-27]** were treated using general method 6.1.2.2. An aliquot of each resin was treated with conc. aqueous ammonia at r.t. for 30 minutes, releasing the triphosphate into solution. This was concentrated *in vacuo*, the residue dissolved in water and purified by anion exchange HPLC (Buffer system 1) to give the following yields and loading of pure triphosphate respectively: 14%, 24μmol $g^{-1}$, 26%, 68μmol $g^{-1}$, 30%, 105μmol $g^{-1}$, 69%, 21μmol $g^{-1}$.



$C_{58}H_{58}N_4O_{13}$
Mol. Wt.: 1019.10
C, 68.36; H, 5.74; N, 5.50; O, 20.41

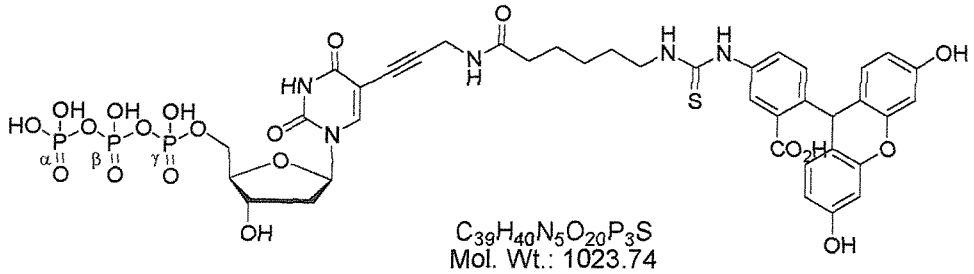
**5-[6-(9-Fluorenylmethoxycarbonylamino)hexanoyl-3-propargylamino]-5'-O-(4,4'-dimethoxytrityl) -2'-deoxyuridine-3'-O-succinate [39]**

To a solution of compound **[34]** (465mg, 0.51mmol) in anhydrous pyridine (5mL) was added succinic anhydride (153mg, 1.53mmol) and DMAP (31mg, 0.26mmol). The reaction mixture was stirred under an argon atmosphere for 16 hours before being concentrated *in vacuo*. The residue was dissolved in DCM (100mL) and washed with sat. KCl (3 x 50mL) followed by brine (50mL). The organic layer was separated, dried ($Na_2SO_4$) and the solvent removed *in vacuo*. The crude product was purified by wet-flash column chromatography eluting with a gradient of methanol in DCM (0-10%) to give the title compound as a pale yellow foam which was dried in a vacuum desiccator over $P_2O_5$ for 24 hours (478mg, 0.47mmol, 92%).

$R_f$(G) 0.29; LRMS ($ES^+$ mode): *m/z* 1020 $[MH]^+$; $\lambda_{max}$ (MeOH): 301, 281nm.

$\delta_H$($CDCl_3$) 1.85 (2H, m, **H22**) 1.41 (4H, ddd, *J*= 29.1, 15.5, 7.5 Hz, **H21**+**H23**) 1.82 (2H, t, *J* = 7.0 Hz, **H24**) 2.29 (1H, dt, *J* = 14.1, 7.3 Hz, **H2'**[a]) 2.49-2.58 (5H, m, **H2'**[b]+**H35**+**H36**) 3.08 (2H, m, **H20**) 3.32 (2H, m, **H5'**) 3.67 (6H, s, **$OCH_3$**) 3.88 (1H, t, *J* = 5.3 Hz, **H27**) 4.10 (2H, d, *J* = 1.5 Hz, **H18**) 4.30 (2H, d, *J* = 7.0 Hz, **H26**) 4.98 (1H, m, **H4'**) 5.34 (1H, m, **H3'**) 6.22 (1H, dd, *J* = 8.5, 5.5 Hz, **H1'**) 6.76 (4H, d, *J* = 8.0 Hz, **H9**) 7.11-7.25 (11H, m, **H8**+**H14**+**H15**+**H30**+**H31**) 7.50 (2H, d, *J* = 7.5 Hz, **H13**) 7.66 (2H, d, *J* = 8.0 Hz, **H29**) 8.10 (1H, s, **H6**) 8.55 (2H, d, *J* = 4.0 Hz, **H32**).

$\delta_C(CDCl_3)$ 25.3 (**C22**) 26.7 (**C21**) 30.0 (**C23**) 30.3 (**C20**) 31.1 (**C18**) 31.2 (**C35**) 31.8 (**C36**) 36.2 (**C26**) 39.2 (**C24**) 41.2 (**C2'**) 55.4 (**0CH$_3$**) 64.2 (**C5'**) 66.9 (**C4'**) 72.7 (**C11**) 85.8 (**C3'**) 86.8 (**C1'**) 87.7 (**C5**) 89.2 (**C16**) 99.5 (**C17**) 113.8 (**C8**) 120.3 (**C32**) 125.5 (**C30**) 127.4 (**C15**) 128.0 (**C13**) 128.3 (**C14**) 130.4 (**C9**) 135.8 (**C10**) 141.5 (**C33**) 141.7 (**C6**) 144.4 (**C12**) 144.9 (**C28**) 159.0 (**C7**) 163.7 (**C25**) 172.6 (**C37**).



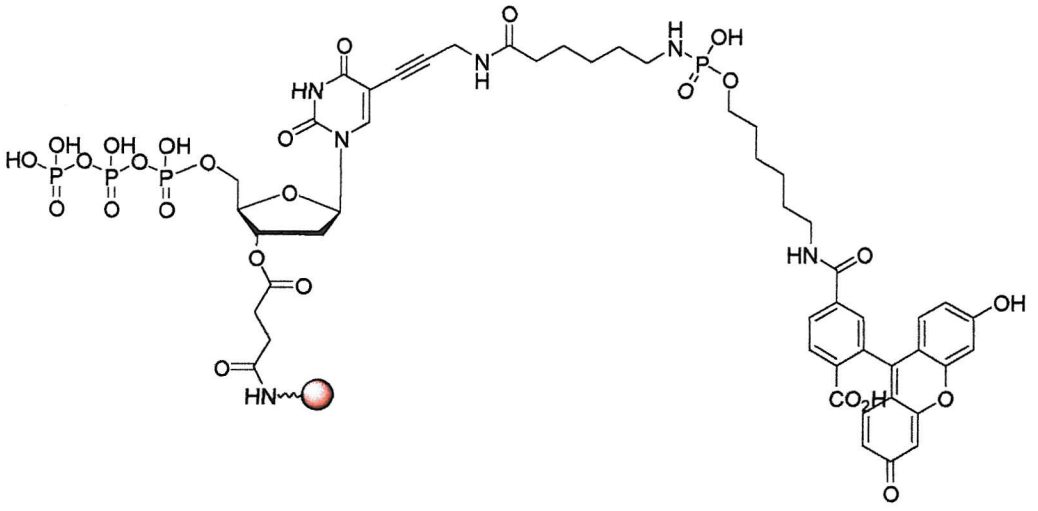
$C_{39}H_{40}N_5O_{20}P_3S$
Mol. Wt.: 1023.74
C, 45.76; H, 3.94; N, 6.84; O, 31.26; P, 9.08; S, 3.13

**5-[6-(Fluoresceinurea)hexanoyl-3-propargylamino]-2'-deoxyuridine-5'-triphosphate [40]**

The FAM-labelled triphosphate CPG-resin **[121]** (414mg, 8.7μmol) was cleaved using general method 6.1.2.5 and purified by anion exchange HPLC (Buffer system 2). The appropriate fraction was lyophilised repeatedly to give the title compound as a yellow lyophilised solid (6mg, 6μmol, 69%).

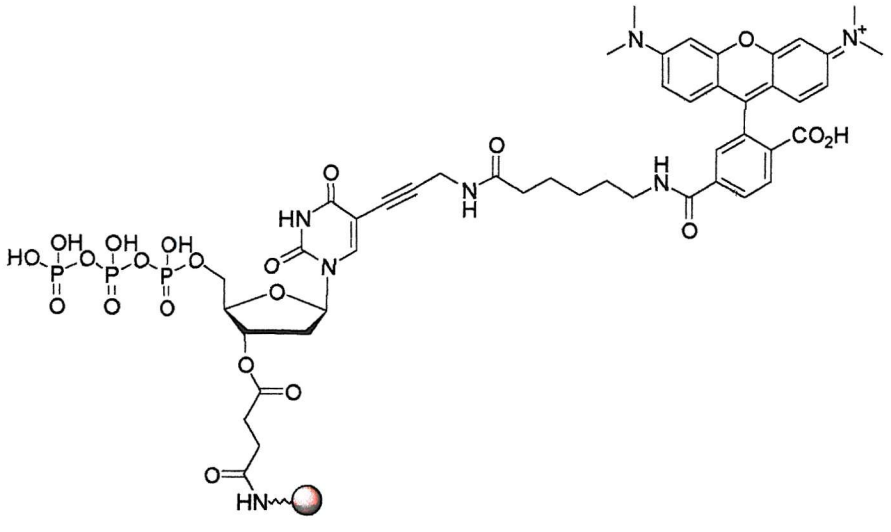
*HPLC retention time (Buffer system 2) 19.54 minutes; LRMS (ES$^+$ mode): m/z* 1062.1 $[MK]^+$; $\lambda_{max}$ ($H_2O$): 493, 243nm.

$\delta_P(D_2O)$ 2.4 (1P, γ) -10.0 (1P, α) –18.6 (1P, β).



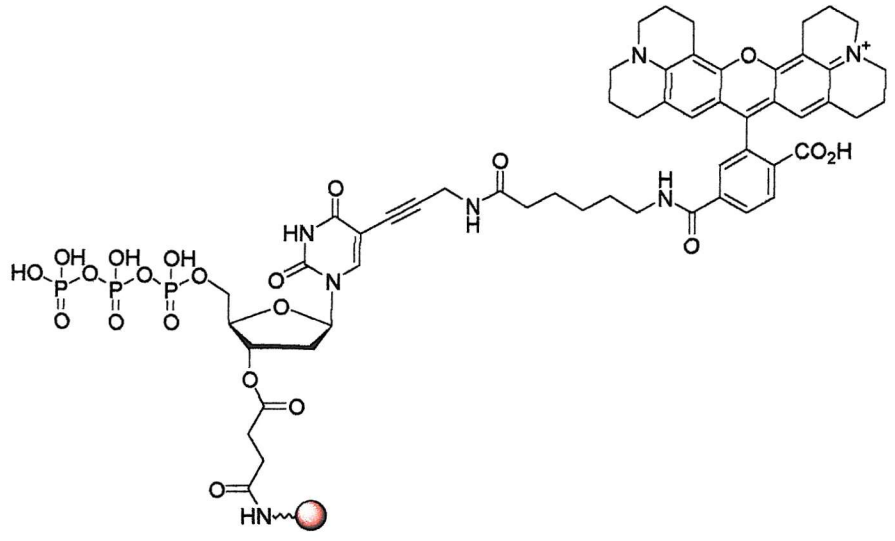
**5-[6-(Hexanoylphosphayl-fluoresceinamido)hexanoyl-3-propargylamino]-2'-deoxyuridine-5'-triphosphate-3'-O-succinamide resin [41]**

The triphosphate CPG-resin **[38]** (48mg, 1.0μmol) was treated using general method 6.1.2.4



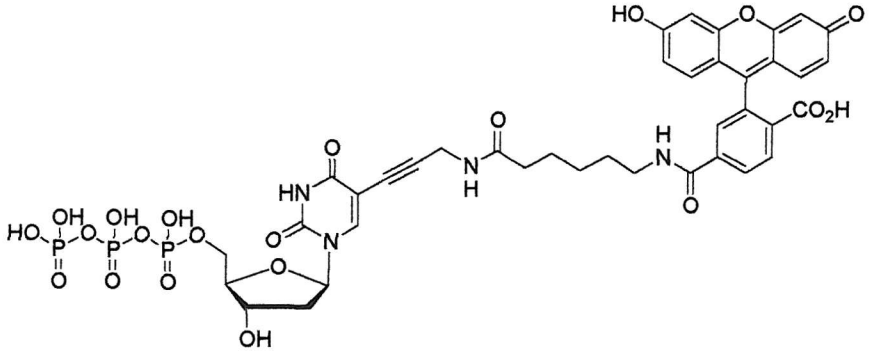
**5-[6-(Tetramethylrhodamido)hexanoyl-3-propargylamino]-2'-deoxyuridine-5'-triphosphate-3'-O-succinamide resins [42]**

*The triphosphate CPG-resin* ***[38]*** *(134mg, 2.8μmol) was treated using general method* 6.1.2.3.



**5-[6-(ROXamido)hexanoyl-3-propargylamino]-2'-deoxyuridine-5'-triphosphate-3'-O-succinamide resins [43]**

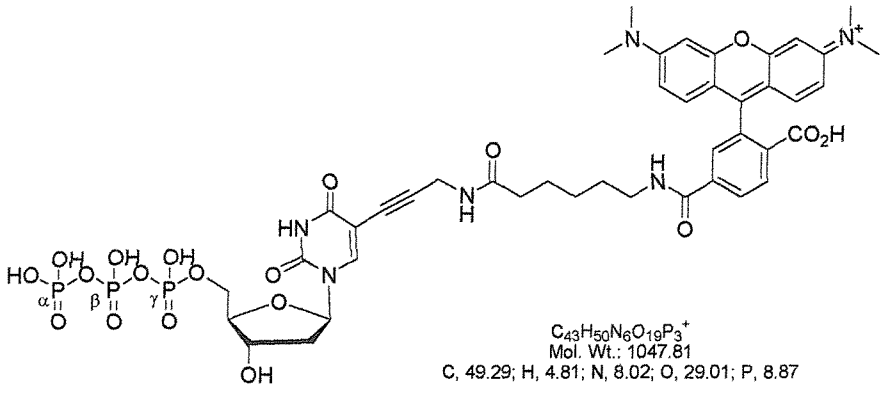
*The triphosphate CPG resin* **[38]** *(134mg, 2.8μmol) was treated using general method* 6.1.2.3.



**5-[6-(Fluoresceinamido)hexanoyl-3-propargylamino]-2'-deoxyuridine-5'-triphosphate [44]**

The FAM-labelled triphosphate CPG-resins **[120]** (95mg, 2μmol) was cleaved using general method 6.1.2.5 and purified by anion exchange HPLC (Buffer system 2). The appropriate fraction was lyophilised repeatedly to give the title compound as a yellow lyophilised solid (1.4mg, 1.4μmol, 71%).

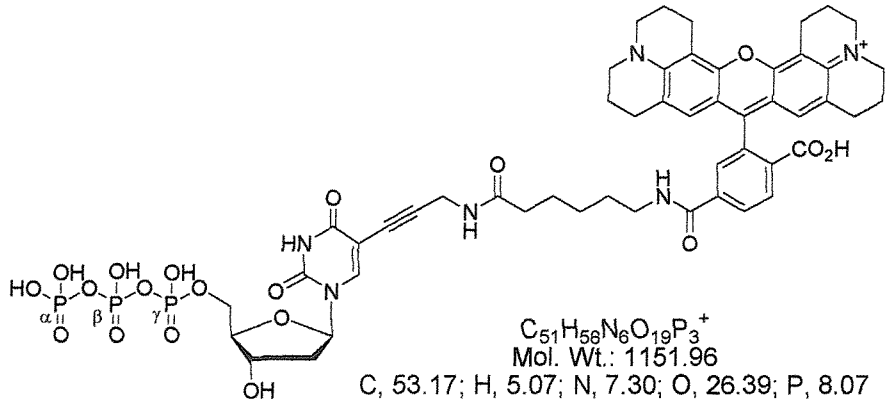
HPLC retention time (Buffer system 2); LRMS ($ES^+$ mode): *m/z* 1015 $[MNa]^+$; $\lambda_{max}$ ($H_2O$): 495, 244nm.




**5-[6-(Tetramethylrhodamido)hexanoyl-3-propargylamino]-2'-deoxyuridine-5'-triphosphate [45]**

*The HEX-labelled triphosphate CPG resin* **[42]** (134mg, 2.8μmol) was cleaved using general method 6.1.2.7 and purified by anion exchange HPLC (Buffer system 2). The appropriate fraction was lyophilised repeatedly to give the title compound as a pink lyophilised solid (1.2mg, 1.1μmol, 41%).

HPLC retention time (Buffer system 2): 14.99 minutes; LRMS ($ES^+$ mode): *m/z* 1065.3 $[MNH_4]^+$; $\lambda_{max}$ ($H_2O$): 557, 294, 229nm.

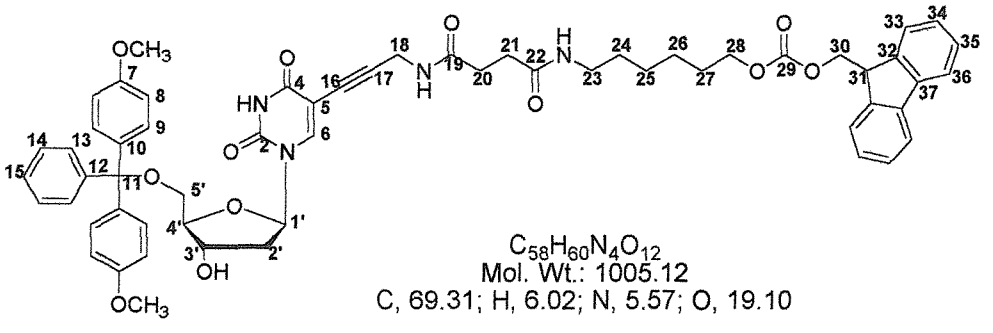


$C_{51}H_{58}N_6O_{19}P_3^+$
Mol. Wt.: 1151.96
C, 53.17; H, 5.07; N, 7.30; O, 26.39; P, 8.07

**5-[6-(ROXamido)hexanoyl-3-propargylamino]-2'-deoxyuridine-5'-triphosphate [46]**

*The ROX-labelled triphosphate CPG resin* **[43]** (134mg, 2.8μmol) was cleaved using general method 6.1.2.7 and purified by anion exchange HPLC (Buffer system 2). The appropriate fraction was lyophilised repeatedly to give the title compound as a purple lyophilised solid (1.5mg, 1.3μmol, 46%).

HPLC retention time (Buffer system 2): 14.60 minutes; LRMS ($ES^+$ mode): *m/z* 1152 $[MH]^+$; $\lambda_{max}$ ($H_2O$): 587, 242nm.



$C_{58}H_{60}N_4O_{12}$
Mol. Wt.: 1005.12
C, 69.31; H, 6.02; N, 5.57; O, 19.10

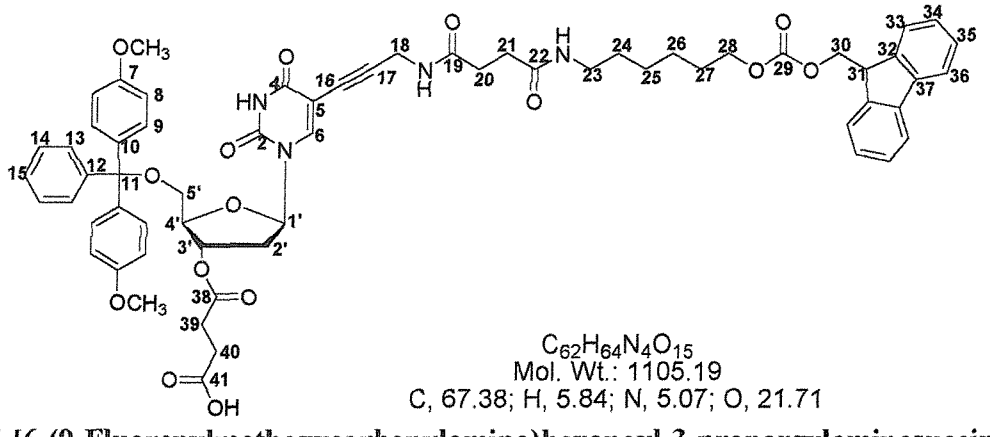
**5-[6-(9-Fluorenylmethoxycarbonylamino)hexanoyl-3-propargylaminosuccinyl] - 5'-O-(4,4'-dimethoxytrityl)-2'-deoxyuridine [47][190]**

Compound **[33]** (1.310g, 2.25mmol) was co-evaporated three times from anhydrous pyridine (20mL) before being dissolved in anhydrous pyridine (10mL) and to this solution was added drop-wise a solution of succinic anhydride (236mg, 2.36mmol) in anhydrous pyridine (2mL). The reaction mixture was stirred under an argon atmosphere for 1 hour before addition of EDC (474mg, 2.48mmol) and Fmoc-

hydroxy amine (839mg, 2.48mmol). The reaction mixture was stirred under an argon atmosphere for 12 hours before being concentrated *in vacuo*. The residue was dissolved in DCM (300mL) and washed with $H_2O$ (2 x 100mL), sat. KCl (2 x 100mL) and brine (100mL). The organic layer was separated, dried ($Na_2SO_4$) and the solvent removed *in vacuo*. The crude product was purified by wet-flash column chromatography eluting with a gradient of methanol in DCM (0-10%) to give the title compound as a cream coloured foam which was dried in a vacuum desiccator over $P_2O_5$ for 24 hours (930mg, 0.93mmol, 41%).

$R_f$(G) 0.82;

$\delta_H(CDCl_3)$ 0.79 (2H, t, $J$ = 6.0 Hz, **H28**) 1.35-1.46 (4H, m, **H24+H26**) 1.59 (2H, dt, $J$ = 14.6, 7.0 Hz, **H23**) 2.18 (2H, dt, $J$ = 14.1, 7.5 Hz, **H2'**) 2.31-2.38 (4H, m, **H25+H27**) 2.47 (4H, t, $J$ = 7.0 Hz, **H20+H21**) 3.26 (2H, m, **H5'**) 3.69 (6H, s, **$OCH_3$**) 3.97 (1H, t, $J$ = 7.0Hz, **H31**) 4.08 (2H, d, $J$ = 5.5 Hz, **H18**) 4.16 (1H, *t*, J = 7.5 Hz, **H4'**) 4.31 (2H, d, $J$ = 7.5 Hz, **H30**) 4.48 (1H, m, **H3'**) 6.57 (1H, m, **H1'**) 6.75 (6H, d, $J$ = 6.8 Hz, **H9+H35**) 7.10-7.35 (11H, m, **H8+H13+H14+H15+H34**) 7.52 (2H, d, $J$ = 7.0Hz, **H33**) 7.67 (2H, d, $J$ = 7.5 Hz, **H36**) 8.07 (1H, s, **H6**).



$C_{62}H_{64}N_4O_{15}$
Mol. Wt.: 1105.19
C, 67.38; H, 5.84; N, 5.07; O, 21.71

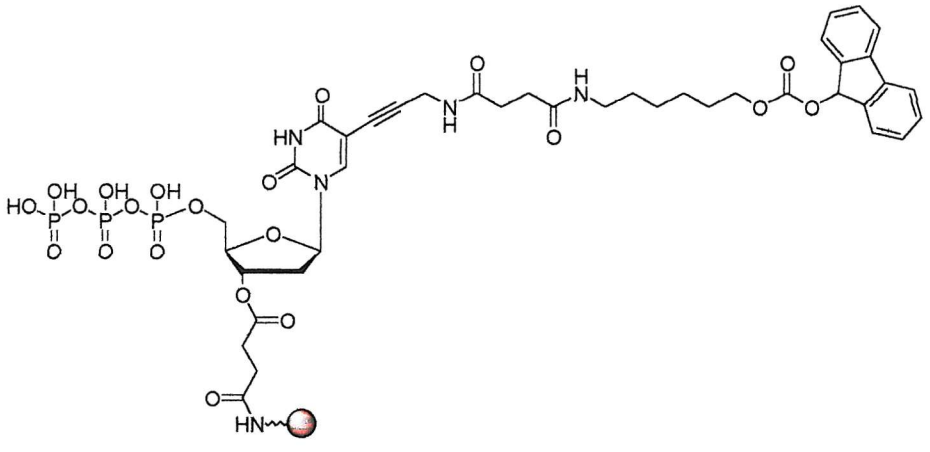
**5-[6-(9-Fluorenylmethoxycarbonylamino)hexanoyl-3-propargylaminosuccinyl] -5'-O-(4,4'-dimethoxytrityl) -2'-deoxyuridine-3'-O-succinate [48]**

Compound **[47]** (870mg, 0.87mmol) was co-evaporated three times from anhydrous pyridine (10mL) before being dissolved in anhydrous pyridine (10mL) and to this

solution was added succinic anhydride (262mg, 2.62mmol) and DMAP (53mg, 0.44mmol). The reaction mixture was stirred under an argon atmosphere for 12 hours before being concentrated *in vacuo*. The residue was dissolved in DCM (200mL) and washed with sat. KCl (2 x 50mL) and brine (50mL). The organic layer was separated, dried ($Na_2SO_4$) and the solvent removed *in vacuo*. The crude product was purified by wet-flash column chromatography eluting with a gradient of methanol in DCM (0-20%) to give the title compound as a cream coloured foam which was dried in a vacuum desiccator over $P_2O_5$ for 24 hours (778mg, 0.70mmol, 81%).

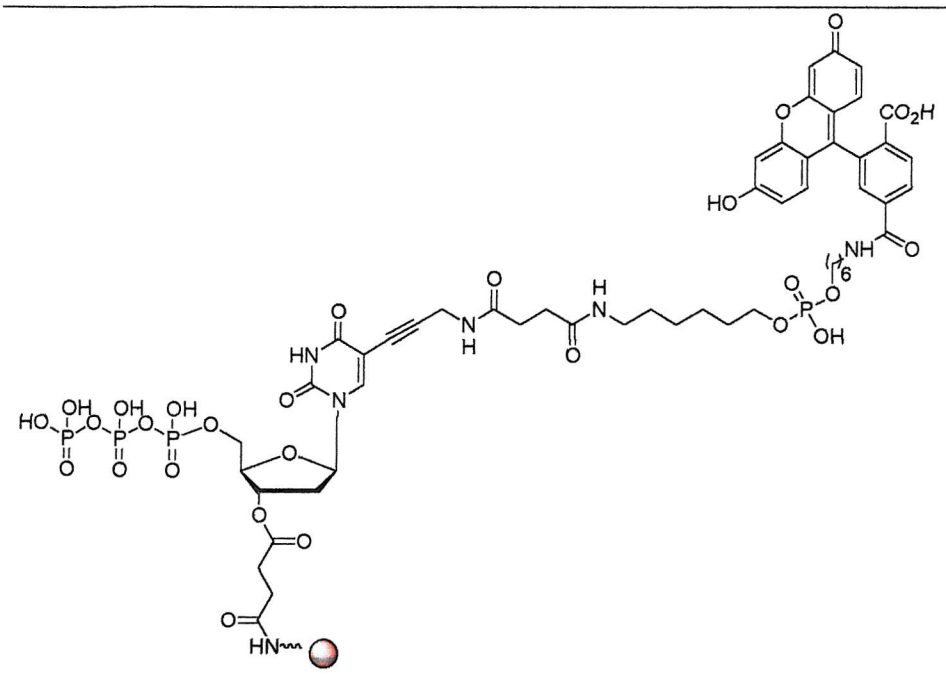
$R_f$(G) 0.24; LRMS ($ES^+$ mode): *m/z* 1106 $[MH]^+$; $\lambda_{max}$ (MeOH): 302, 277nm.

$\delta_H$($CDCl_3$) 1.37 (2H, t, J = 6.5 Hz, **H28**) 1.50 (2H, t, J = 6.8 Hz, **H23**) 2.24-2.35 (6H, m, **H2'+H24+H26**) 2.44-2.54 (10H, m, **H20+H21+H25+H27+H39**) 2.98 (2H, s, **H18**) 3.13 (2H, dd, J = 13.1, 6.4 Hz, **H5'**) 3.33 (2H, m, **H40**) 3.71 (6H, s, **$OCH_3$**) 3.77 (1H, t, J = 4.5 Hz, **H31**) 3.97 (2H, m, **H30**) 4.12 (1H, m, **H4'**) 5.33 (1H, m, **H3'**) 6.23 (1H, m, **H1'**) 6.47 (2H, d, J = 7.0 Hz, **H35**) 6.78 (4H, d, J = 8.5 Hz, **H9**) 7.13-7.31 (13H, m, **H8+H13+H14+H15+H33+H34**) 7.34 (2H, d, J = 7.5 Hz, **H36**) 8.07 (1H, s, **H6**).



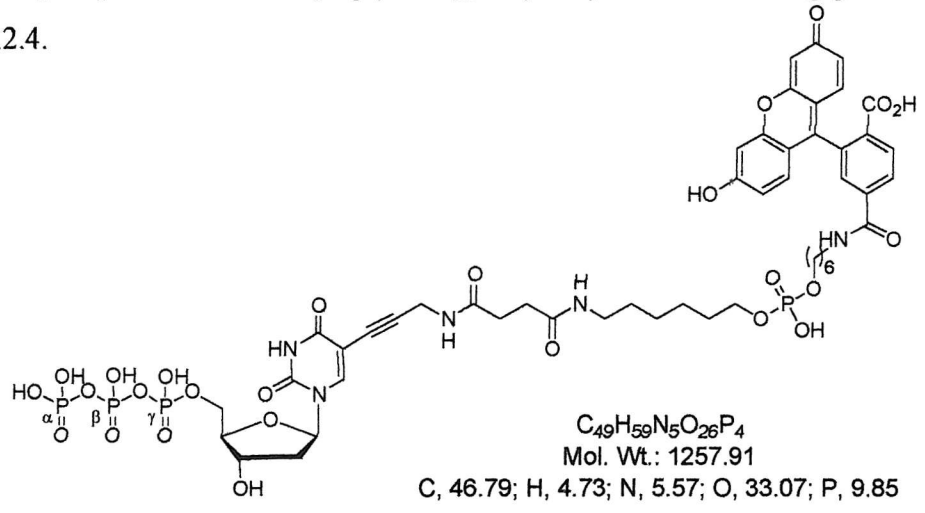
**5-[6-(9-Fluorenylmethoxycarbonylamino)hexanoyl-3-propargylaminosuccinyl]-2'-deoxyuridine-5'-triphosphate-3'-O-succinamide resin [49]**

The nucleoside CPG-resin **[78]** (300mg, 11.7μmol) was phosphorylated using general method 6.1.2.2 to give loading of pure triphosphate of 26μmol $g^{-1}$, 67%.



**5-[6-(Hexanoylphosphatyl-fluoresceinamido)hexanoyl-3-propargylaminosuccinyl]-2'-deoxyuridine-5'-triphosphate-3'-O-succinamide resin [50]**

The triphosphate CPG resin **[49]** (100mg, 2.6μmol) was treated using general method 6.1.2.4.



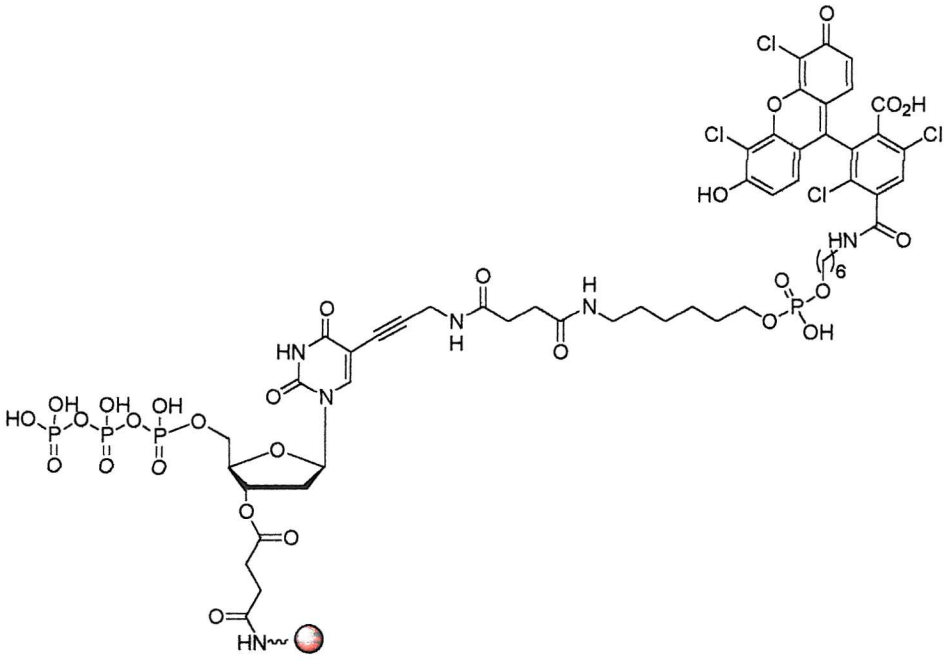
$C_{49}H_{59}N_5O_{26}P_4$
Mol. Wt.: 1257.91
C, 46.79; H, 4.73; N, 5.57; O, 33.07; P, 9.85

**5-[6-(Hexanoylphosphatyl-fluoresceinamido)hexanoyl-3-propargylaminosuccinyl]-2'-deoxyuridine-5'-triphosphate [51]**

The FAM-labelled triphosphate CPG-resin **[50]** (100mg, 2.6μmol) was cleaved by general method 6.1.2.6 and purified by anion exchange HPLC (Buffer system 2). The

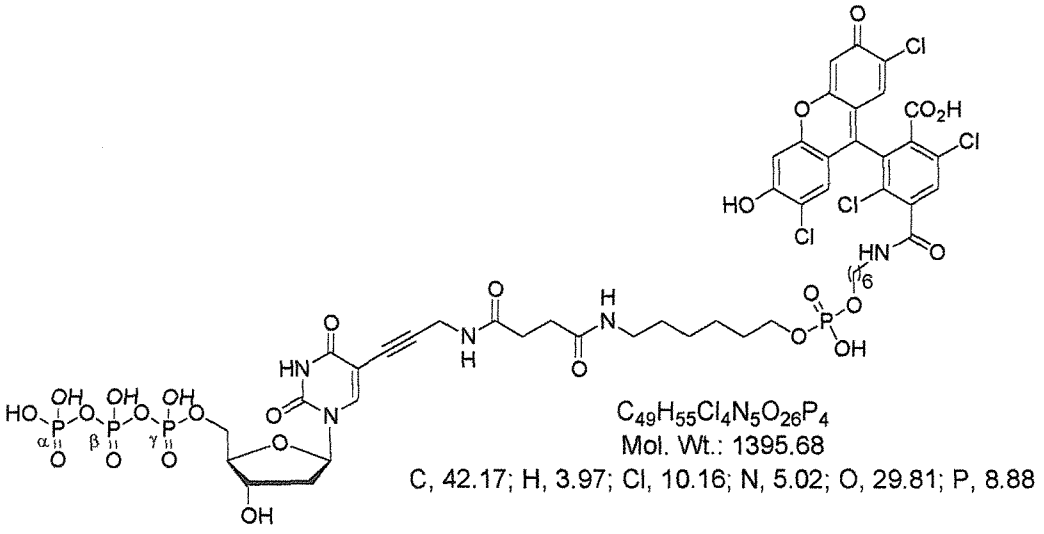
appropriate fraction was lyophilised repeatedly to give the title compound as a yellow lyophilised solid (1.8mg, 1.4μmol, 56%)

HPLC retention time (Buffer system 2): 22.60 minutes; LRMS ($ES^+$ mode): *m/z* 1258 $[MH]^+$; $\lambda_{max}$ ($H_2O$): 494, 251nm.



**5-[6-(Hexanoylphosphatyl-tetrachlorofluoresceinamido)hexanoyl-3-propargylaminosuccinyl]-2'-deoxyuridine-5'-triphosphate-3'-O-succinamide resin [52]**

The triphosphate CPG resin **[49]** (100mg, 2.6μmol) was treated using general method 6.1.2.4.

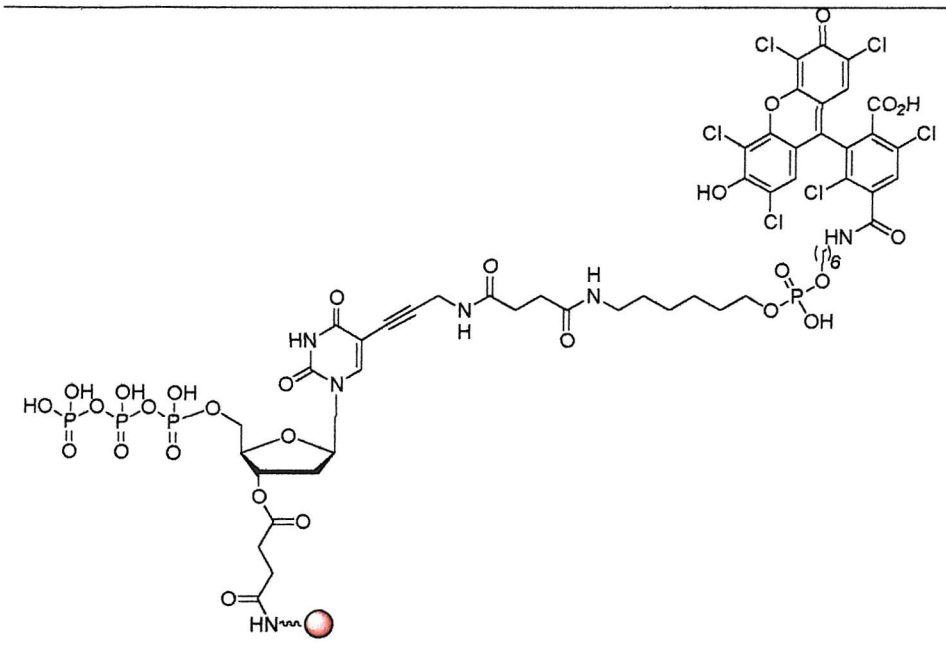


$C_{49}H_{55}Cl_4N_5O_{26}P_4$
Mol. Wt.: 1395.68
C, 42.17; H, 3.97; Cl, 10.16; N, 5.02; O, 29.81; P, 8.88

**5-[6-(Hexanoylphosphatyl-tetrachlorofluoresceinamido)hexanoyl-3-propargylaminosuccinyl]-2'-deoxyuridine-5'-triphosphate [53]**

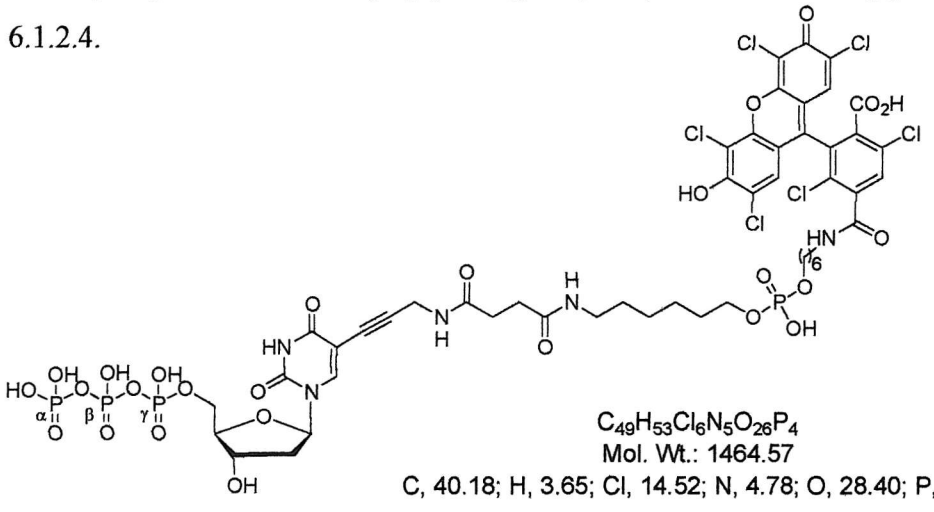
*The TET-labelled triphosphate CPG resin* ***[52]*** *(100mg, 2.6μmol) was cleaved using* general method 6.1.2.6 and purified by anion exchange HPLC (Buffer system 2). The appropriate fraction was lyophilised repeatedly to give the title compound as an orange lyophilised solid (1.1mg, 0.8μmol, 29%)

HPLC retention time (Buffer system 2): 22.26 minutes; LRMS ($ES^+$ mode): *m/z* 1431.8 $[MK]^+$; $\lambda_{max}$ ($H_2O$): 521, 490, 246nm.



**5-[6-(Hexanoylphosphatyl-hexachlorofluoresceinamido)hexanoyl-3-propargylaminosuccinyl]-2'-deoxyuridine-5'-triphosphate-3'-O-succinamide resin [54]**

The triphosphate CPG-resin **[49]** (100mg, 2.6μmol) was treated using general method 6.1.2.4.

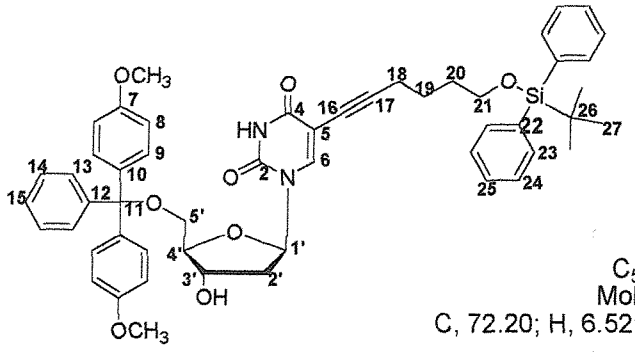


$C_{49}H_{53}Cl_6N_5O_{26}P_4$
Mol. Wt.: 1464.57
C, 40.18; H, 3.65; Cl, 14.52; N, 4.78; O, 28.40; P, 8.46

**5-[6-(Hexanoylphosphatyl-hexachlorofluoresceinamido)hexanoyl-3-propargylaminosuccinyl]-2'-deoxyuridine-5'-triphosphate [55]**

*The HEX-labelled triphosphate CPG resin* ***[54]*** *(100mg, 2.6μmol) was cleaved using* general method 6.1.2.4 and purified by anion exchange HPLC (Buffer system 2) to give the title compound as a pink lyophilised solid (1.3mg, 0.9μmol, 35%)

HPLC retention time (Buffer system 2): 23.80 minutes; LRMS ($ES^+$ mode): *m/z* 1462 $[MH]^+$; $\lambda_{max}$ ($H_2O$): 538, 254nm.




$C_{52}H_{56}N_2O_8Si$
Mol. Wt.: 865.10
C, 72.20; H, 6.52; N, 3.24; O, 14.80; Si, 3.25

**5-Hexyn-1-O-[(1,1-dimethylethyl)diphenyl silyl]- 5'-O-(4,4'-dimethoxytrityl)-2'-deoxyuridine [56]**

*Method 1*:

To a solution of compound **[59]** (198mg, 0.32mmol) in anhydrous DCM (5mL) was added a solution of *t*-butyldiphenylsilyl chloride (106mg, 0.10mL, 0.39mmol), triethylamine (78mg, 1.71mL, 0.77μmol) and DMAP (1mg, 8.2μmol) in anhydrous DCM (3mL) at 0°C. The reaction mixture was allowed to warm to r.t. and stirred under an argon atmosphere for 15 hours before being diluted with DCM (50mL) and washed with sat. KCl (2 x 20mL) and brine (20mL). The organic layer was separated, dried ($Na_2SO_4$) and the solvent removed *in vacuo*. The crude product was purified by wet-flash column chromatography eluting with ethylacetate : hexane (4:1) to give the title compound as a white coloured foam which was dried in a vacuum desiccator over $P_2O_5$ for 24 hours (268mg, 0.31mmol, 97%).

*Method 2*:

To a solution of compound **[13]** (1.975g, 3.01mmol) in anhydrous DMF (10mL) was added a solution of compound **[58]** (3.304g, 9.03mmol), copper (I) iodide 115mg, 0.60mmol) and triethylamine (0.84mL, 0.609g, 6.02mmol) in anhydrous DMF (10ml). To this was then added tetrakistriphenylphosphine palladium (0) (348mg, 0.30mmol) and the solution stirred under an argon atmosphere for 12 hours. The

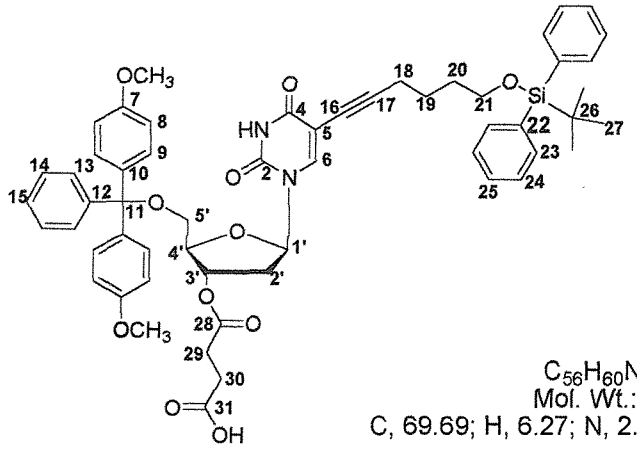
reaction mixture was then reduced in volume, dissolved in DCM (400mL) and washed with a 5% aq. EDTA solution (2 x 50mL), sat. KCl (2 x 50mL) and brine (50mL). The organic phase was separated, dried ($Na_2SO_4$) and the solvent removed *in vacuo*. The crude product was purified by wet-flash column chromatography eluting with ethylacetate : hexane (4:1) to give the title compound as a white foam which was dried in a vacuum dessicator over $P_2O_5$ for 24 hours (2.466g, 1.35mmol, 85%).

$R_f$(K) 0.41; LRMS ($ES^+$ mode): *m/z* 887.3 $[MNa]^+$; $\lambda_{max}$ (MeOH): 283, 232nm; mp: 88°C; *Found C: 71.90, H: 6.47, N: 3.24, $C_{52}H_{56}N_2O_8Si$ requires C: 72.20, H: 6.52, N: 3.24%*

IR: $\nu$ = 2360.2 (m), 1692.6 (s), 1508.0 (s), 1456.6 (m), 1250.3 (s) $cm^{-1}$.

$\delta_H(CDCl_3)$ *0.94 (9H, s,* **H27***) 1.33-1.40 (2H, m,* **H19***) 1.97-2.07 (3H, m,* **H2'$^a$+H20***)* 2.15-2.25 (2H, m, **H18**) 2.38 (1H, ddd, *J* = 13.4, 5.6, 2.6 Hz, **H2'$^b$**) 3.23 (1H, dd, *J* = 10.5, 3.5 Hz, **H5'$^a$**) 3.33 (1H, dd, *J* = 10.7, 3.5 Hz, **H5'$^b$**) 3.41 (2H, t, *J* = 6.3 Hz, **H21**) *3.66 (6H, s,* **OCH$_3$***) 3.98 (1H, m,* **H4'***) 4.42 (1H, m,* **H3'***) 6.23 (1H, dd, J* = 7.5, 6.0 Hz, **H1'**) 6.73 (4H, d, *J* = 9.0 Hz, **H9**) 7.06-7.34 (15H, m, **H8+H13+H14+H15+H23+H25**) 7.56 (4H, d, *J* = 7.5 Hz, **H24**) 7.89 (1H, s, **H6**) 8.57 (1H, br, s, **NH**).

$\delta_C(CDCl_3)$ 19.1 (**C18**) 19.6 (**C26**) 24.6 (**C19**) 27.1 (**C27**) 32.4 (**C20**) 38.8 (**C2'**) 55.5 (**OCH$_3$**) 63.7 (**C21**) 63.9 (**C5'**) 70.6 (**C16**) 75.4 (**C11**) 84.1 (**C4'**) 85.4 (**C3'**) 87.2 (**C1'**) 95.3 (**C17**) 101.7 (**C5**) 113.4 (**C8**) 127.8 (**C15**) 128.0 (**C13**) 128.4 (**C14**) 129.9 (**C9**) 130.0 (**C24**) 130.2 (**C25**) 134.2 (**C22**) 135.7 (**C10**) 135.9 (**C23**) 141.5 (**C6**) 144.7 (**C12**) 158.8 (**C7**).



$C_{56}H_{60}N_2O_{11}Si$
Mol. Wt.: 965.17
C, 69.69; H, 6.27; N, 2.90; O, 18.23; Si, 2.91

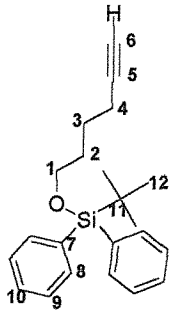
**5-Hexyn-1-O-[(1,1-dimethylethyl)diphenyl silyl]- 5'-O-(4,4'-dimethoxytrityl) -2'-deoxyuridine-3'-O-succinate [57]**

Compound **[56]** (509mg, 0.59mmol) was co-evaporated three times from anhydrous pyridine (10mL) before being dissolved in anhydrous pyridine (5mL) and to this solution was added succinic anhydride (177mg, 1.77mmol) and DMAP (7mg, 0.06mmol). The reaction mixture was stirred under an argon atmosphere for 12 hours before being concentrated *in vacuo*. The residue was dissolved in DCM (200mL) and washed with sat. KCl (2 x 50mL) and brine (50mL). The organic layer was separated, dried ($Na_2SO_4$) and the solvent removed *in vacuo*. The crude product was purified by wet-flash column chromatography eluting ethylacetate to give the title compound as a white coloured foam which was dried in a vacuum desiccator over $P_2O_5$ for 24 hours (537mg, 0.56mmol, 91%).

$R_f$(I) 0.26; LRMS ($ES^+$ mode): *m/z* 303.1 $[DMT]^+$ 987.3 $[MNa]^+$; $\lambda_{max}$ (MeOH): 283, 231*nm*.

$\delta_H(CDCl_3)$ 1.03 (9H, s, **H27**) 1.40-1.45 (2H, m, **H19**) 2.06 (2H, t, $J$ = 6.6 Hz, **H18**) 2.36 (1H, m, **H2'$^a$**) 2.53-2.69 (5H, m, **H2'$^b$**+**H29**+**H30**) 3.36-3.40 (2H, m, **H5'**) 3.47 (2H, t, $J$ = 6.6 Hz, **H21**) 3.73 (6H, s, **$OCH_3$**) 4.19 (1H, m, **H4'**) 5.39 (1H, d, $J$ = 4.4 Hz, **H3'**) 6.29 (1H, m, **H1'**) 6.81 (4H, d, $J$ = 8.1 Hz, **H9**) 7.12-7.42 (15H, m, **H8**+**H13**+**H14**+**H15**+**H23**+**H25**) 7.65 (4H, d, $J$ = 5.9 Hz, **H24**) 8.03 (1H, s, **H6**).

$\delta_C$($CDCl_3$) 19.4 (**C18**) 19.5 (**C26**) 24.9 (**C19**) 27.1 (**C27**) 29.7 (**C29**) 29.9 (**C30**) 32.1 (**C20**) 38.8 (**C2'**) 55.4 (**$OCH_3$**) 63.5 (**C21**) 63.7 (**C5'**) 70.7 (**C16**) 75.6 (**C11**) 84.0 (**C4'**) 85.4 (**C3'**) 87.4 (**C1'**) 95.6 (**C17**) 101.7 (**C5**) 113.5 (**C8**) 127.8 (**C15**) 128.0 (**C13**) 128.3 (**C14**) 129.8 (**C9**) 130.1 (**C24**) 130.2 (**C25**) 134.2 (**C22**) 135.5 (**C10**) 135.8 (**C23**) 141.7 (**C6**) 144.7 (**C12**) 158.8 (**C7**) 162.7 (**C28**) 172.2 (**C31**).




$C_{22}H_{28}OSi$
Mol. Wt.: 336.54
C, 78.51; H, 8.39; O, 4.75; Si, 8.35

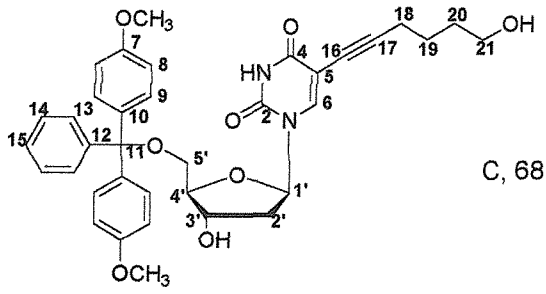
**Hex-5-yn-1-O-(1,1-dimethylethyl)diphenyl silyl [58][263].**

To a solution of *t*-butyldiphenylsilyl chloride (3.18mL, 12.2mmol), triethylamine (1.71mL, 12.2mmol) and DMAP (2.5mg, 0.02mmol) in anhydrous DCM (10mL) was added a solution of 5-hexyn-1-ol (1.12mL, 10.2mmol) in anhydrous DCM (3mL) at 0°C. The reaction mixture was allowed to warm to r.t. and stirred under an argon atmosphere for 12 hours before being diluted with DCM (250mL) and washed with $H_2O$ (100mL). The aqueous layer was re-extracted with DCM (2 x 30mL), the organic phases combined, dried ($Na_2SO_4$) and the solvent removed *in vacuo*. The crude product was purified by wet-flash column chromatography eluting with diethyl ether : petroleum ether (7.5:2.5) to give the title compound as a colourless oil which was dried in a vacuum desiccator over $P_2O_5$ for 24 hours (3.391g, 10.1mmol, 99%).

$R_f$(P) 0.65; LRMS ($ES^-$ mode): *m/z* 335 $[MH]^-$.

$\delta_H$($CDCl_3$) 0.97 (9H, s, **H12**) 1.51-1.63 (5H, m, **H6+H3+H2**) 2.10 (2H, dt, $J$ = 6.8, 2.5 Hz, **H4**) 3.60 (2H, t, $J$ = 6.0 Hz, **H1**) 7.26-7.35 (6H, m, **H9+H10**) 7.59 (4H, dd, $J$ = 7.5, 1.8 Hz, **H8**).

$\delta_C$($CDCl_3$)18.6 (**C3**) 19.7 (**C11**) 25.4 (**C4**) 27.3 (**C12**) 32.0 (**C5**) 63.8 (**C6**) 68.8 (**C1**) 85.0 (**C2**) 128.1 128.3 (**C9**) 130.0 130.3 (**C10**) 134.1 134.4 (**C7**) 136.0 136.3 (**C8**).




$C_{36}H_{38}N_2O_8$
Mol. Wt.: 626.70
C, 68.99; H, 6.11; N, 4.47; O, 20.42

**5-Hexyn-1-olyl-5'-O-(4,4'-dimethoxytrityl)-2'-deoxyuridine [59]**

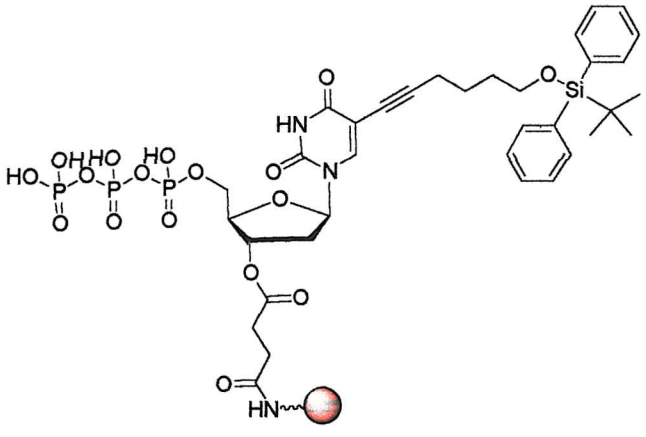
To a solution of compound **[13]** (631mg, 0.96mmol) in anhydrous DMF (5mL) was added a solution of hexyn-1-ol (283mg, 0.32mL, 2.88mmol), copper (I) iodide (37mg, 0.19mmol) and triethylamine (194mg, 0.27mL, 1.92mmol) in anhydrous DMF (5mL). To this solution was added tetrakistriphenylphosphine palladium (0) (111mg, 0.096mmol) and the reaction mixture stirred under an argon atmosphere for 18 hours before being concentrated *in vacuo*. The residue was dissolved in DCM (150mL) and washed with a 5% aq. EDTA solution (2 x 50mL) and brine (50mL). The organic layer was separated, dried ($Na_2SO_4$) and the solvent removed *in vacuo*. The crude product was purified by wet-flash column chromatography eluting with ethylacetate : hexane (4:1) to give the title compound as a white coloured foam which was dried in a vacuum desiccator over $P_2O_5$ for 24 hours (464mg, 0.74mmol, 77%).

$R_f$(K) 0.20; LRMS ($ES^+$ mode): *m/z* 303.0 $[DMT]^+$ 649 $[MNa]^+$; $\lambda_{max}$ (MeOH): 280, 232nm.

$\delta_H$($CDCl_3$) 1.33-1.40 (2H, m, **H19**) 1.99-2.10 (3H, m, **H2'$^a$+H20**) 2.13-2.21 (2H, m, **H18**) 2.39 (1H, ddd, $J$ = 13.4, 5.6, 2.6 Hz, **H2'$^b$**) 3.25 (1H, dd, $J$ = 10.5, 3.5 Hz, **H5'$^a$**) 3.31 (1H, dd, $J$ = 10.7, 3.5 Hz, **H5'$^b$**) 3.40 (2H, t, $J$ = 6.3 Hz, **H21**) 3.68 (6H, s, **$OCH_3$**) 3.99 (1H, m, **H4'**) 4.44 (1H, m, **H3'**) 6.27 (1H, dd, $J$ = 7.5, 6.0 Hz, **H1'**) 6.81

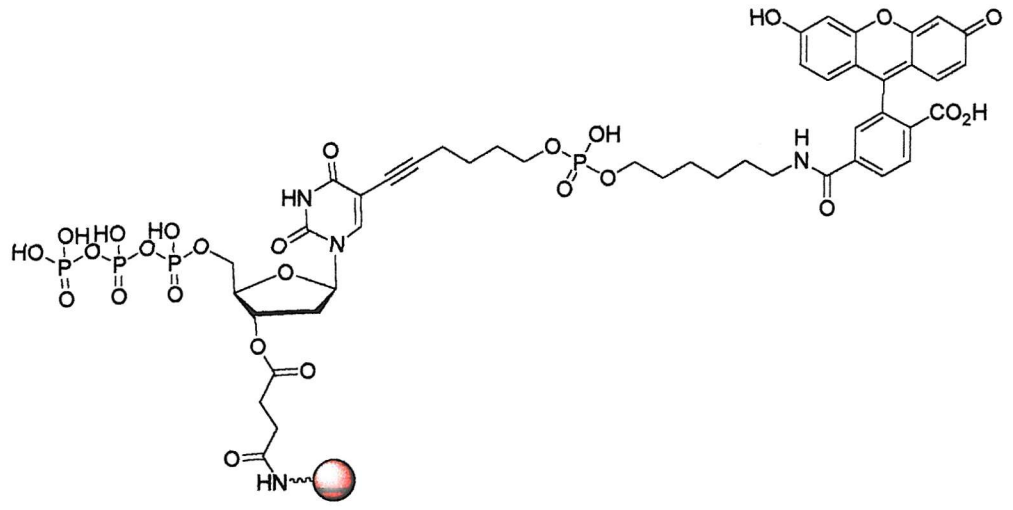
(4H, d, $J$ = 8.0 Hz, **H9**) 7.16-7.24 (7H, m, **H8** +**H14**+**H15**) 7.35 (2H, d, $J$ = 7.1 Hz, **H13**) 8.10 (1H, s, **H6**).

$\delta_C(CDCl_3)$ 19.3 (**C18**) 24.8 (**C19**) 32.1 (**C20**) 38.7 (**C2'**) 55.6 (**$OCH_3$**) 63.7 (**C21**) 63.9 (**C5'**) 70.5 (**C16**) 75.7 (**C11**) 84.3 (**C4'**) 85.4 (**C3'**) 87.4 (**C1'**) 95.6 (**C17**) 101.8 (**C5**) 113.5 (**C8**) 127.8 (**C15**) 128.1 (**C13**) 128.5 (**C14**) 129.9 (**C9**) 135.6 (**C10**) 141.6 (**C6**) 144.7 (**C12**) 158.8 (**C7**).



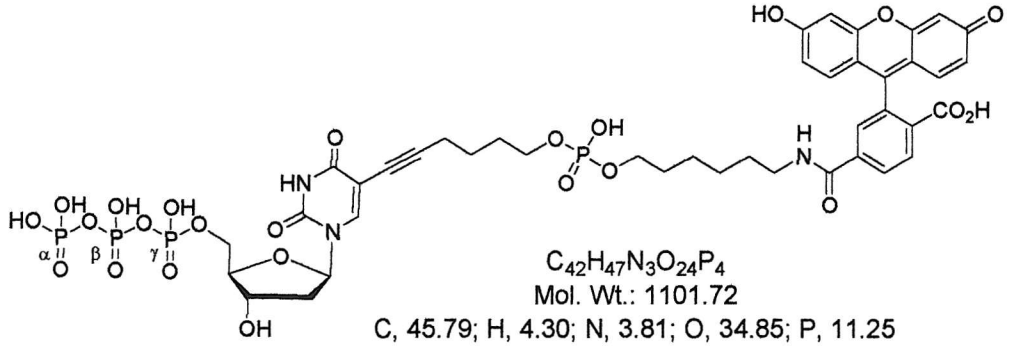

**5-Hexyn-1-O-[(1,1-dimethylethyl)diphenyl silyl]-2'-deoxyuridine- 5'-triphosphate-3'-O-succinamide resin [60]**

*The nucleoside CPG-resin* ***[79]*** *(0.35g, 17μmol) was phosphorylated using general* method 6.1.2.2. An aliquot of the resin was treated with conc. aqueous ammonia at r.t. for 30 minutes, releasing the triphosphate into solution. This was concentrated *in vacuo*, the residue dissolved in water and purified by anion exchange HPLC (Buffer system 1) to give the yield and loading of pure triphosphate (11.0μmol, 65%, 31μmol $g^{-1}$).



**5-Hexyn-1-O-(hexanoylphosphatyl-fluoresceinamido)-2'-deoxyuridine-5'-triphosphate-3'-O-succinamide resin [61]**

The triphosphate resin **[60]** (64.5mg, 2.0μmol) was deprotected with an anhydrous TEA : HF : NMP (1.4M HF concentration) solution for 40 minutes, at 55°C, the resin washed with pyridine (x 3) and DCM (x 5) and dried *in vacuo*. The dye was attached using general method 6.1.2.4 with the exclusion of the piperidine treatment.

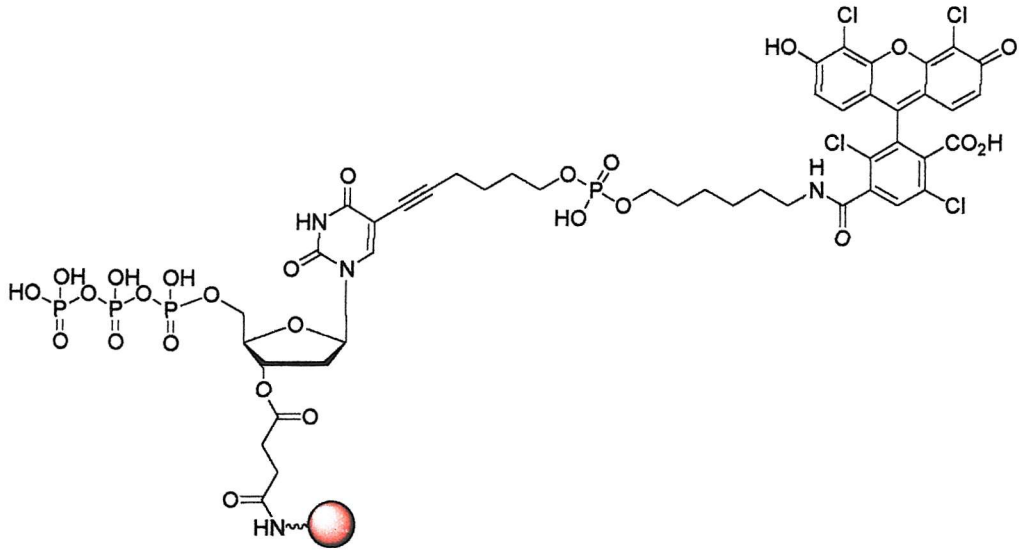


$C_{42}H_{47}N_3O_{24}P_4$
Mol. Wt.: 1101.72
C, 45.79; H, 4.30; N, 3.81; O, 34.85; P, 11.25

**5-Hexyn-1-O-(hexanoylphosphatyl-fluoresceinamido)-2'-deoxyuridine-5'-triphosphate [62]**

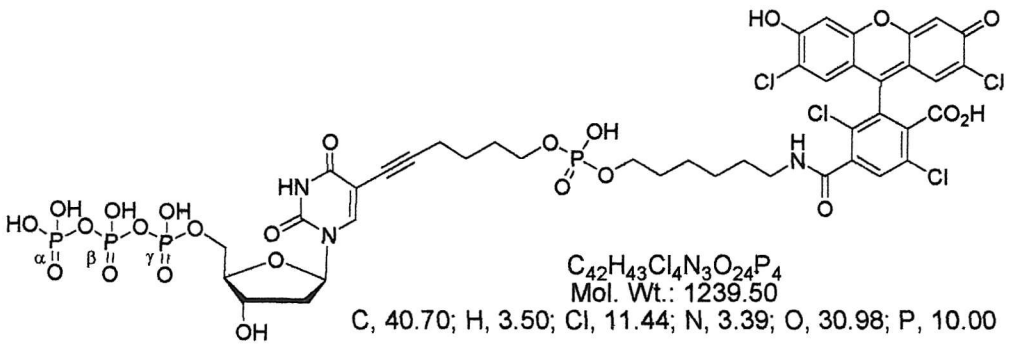
The FAM-labelled triphosphate resin **[61]** (64.5mg, 2.0μmol) was cleaved using general method 6.1.2.6 and purified by anion exchange HPLC (Buffer system 2). The appropriate fraction was repeatedly lyophilised to give the title compound as a yellow lyophilised solid (1.3mg, 1.2μmol, 60%)

HPLC retention time (Buffer system 2): 20.49 minutes; LRMS ($ES^+$ mode): *m/z* 1124 $[MNa]^+$; $\lambda_{max}$ ($H_2O$): 494, 251nm.



**5-Hexyn-1-O-(hexanoylphosphatyl-tetrafluoresceinamido)-2'-deoxyuridine-5'-triphosphate-3'-O-succinamide resin [63]**

The triphosphate CPG-resin **[60]** (64.5mg, 2.0μmol) was treated with an anhydrous TEA : HF : NMP (1.4M HF concentration) solution for 40 minutes, at 55°C, the resin washed with pyridine (x 3) and DCM (x 5) and dried *in vacuo*. The dye was attached using general method 6.1.2.4 with the exclusion of the piperidine treatment.

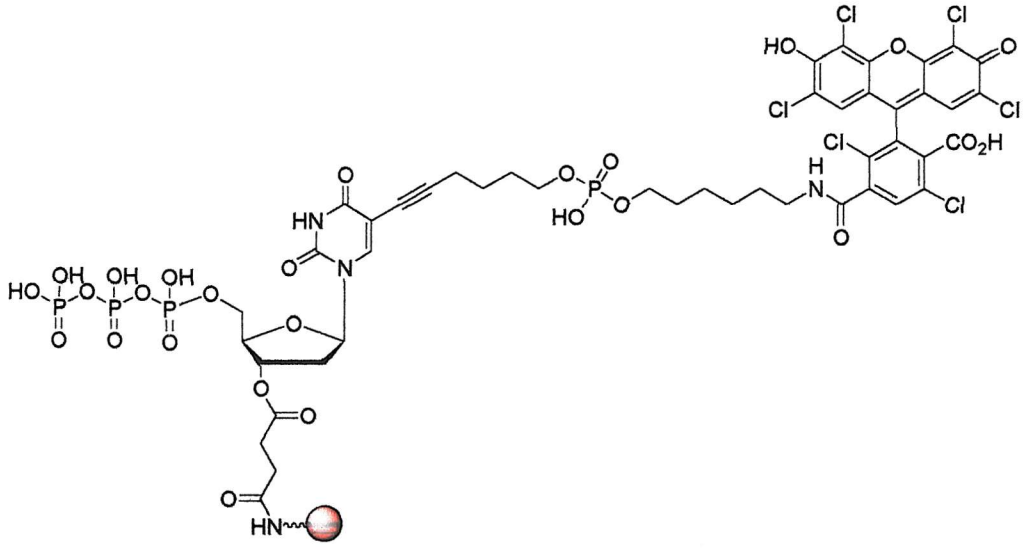


$C_{42}H_{43}Cl_4N_3O_{24}P_4$
Mol. Wt.: 1239.50
C, 40.70; H, 3.50; Cl, 11.44; N, 3.39; O, 30.98; P, 10.00

**5-Hexyn-1-O-(hexanoylphosphatyl-tetrafluoresceinamido)-2'-deoxyuridine-5'-triphosphate [64]**

The TET-labelled triphosphate CPG resin **[63]** (64.5mg, 2.0μmol) was cleaved using general method 6.1.2.6 and purified by anion exchange HPLC (Buffer system 2). The appropriate fraction was lyophilised repeatedly to give the title compound as an orange lyophilised solid (0.92mg, 0.74μmol, 37%).

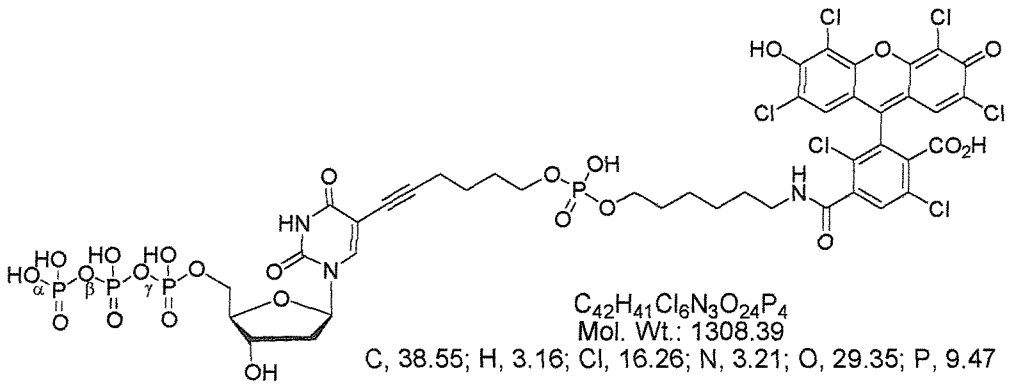
HPLC retention time (Buffer system 2): 24.63 minutes; LRMS ($ES^+$ mode): *m/z* 1260.6 $[MNa]^+$; $\lambda_{max}$ ($H_2O$): 525, 245nm.



**5-Hexyn-1-O-(hexanoylphosphatyl-hexafluoresceinamido)-2'-deoxyuridine-5'-triphosphate-3'-O-succinamide resin [65]**

The triphosphate CPG-resin **[60]** (64.5mg, 2.0μmol) was treated with an anhydrous TEA : HF : NMP (1.4M HF concentration) solution for 40 minutes, at 55°C, the resin

washed with pyridine (x 3) and DCM (x 5) and dried *in vacuo*. The dye was attached using general method 6.1.2.4 with the exclusion of the piperidine treatment.

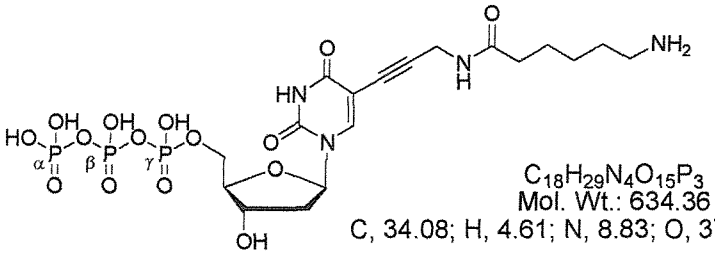


$C_{42}H_{41}Cl_6N_3O_{24}P_4$
Mol. Wt.: 1308.39
C, 38.55; H, 3.16; Cl, 16.26; N, 3.21; O, 29.35; P, 9.47

**5-Hexyn-1-O-(hexanoylphosphatyl-hexafluoresceinamido)-2'-deoxyuridine-5'-triphosphate [66]**

*The HEX-labelled triphosphate CPG resin* **[65]** *(64.5mg, 2.0μmol) was cleaved using* general method 6.1.2.6 and purified by anion exchange HPLC (Buffer system 2). The appropriate fraction was lyophilised repeatedly to give the title compound as a pink lyophilised solid (1.1mg, 0.88μmol, 44%)

HPLC retention time (Buffer system 2): 24.43 minutes; LRMS ($ES^+$ mode): *m/z* 1306 $[MH]^+$; $\lambda_{max}$ ($H_2O$): 538, 254nm.



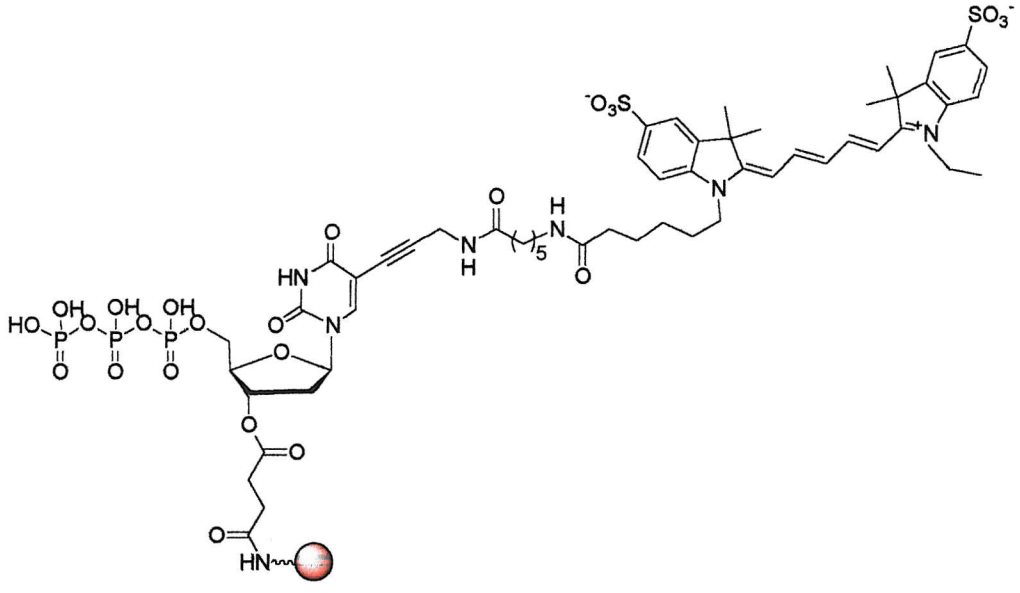
$C_{18}H_{29}N_4O_{15}P_3$
Mol. Wt.: 634.36
C, 34.08; H, 4.61; N, 8.83; O, 37.83; P, 14.65

**5-[6-Hexanoyl-3-propargylamino]-2'-deoxyuridine-5'-triphosphate [67]**

The triphosphate CPG resin **[38]** (352mg, 7.4μmol) was treated with 20% piperidine in DMF for 10 minutes, the resin washed with DMF (x3), DCM (x5) and dried *in vacuo*. The triphosphate was released into solution using general method 6.1.2.5 and

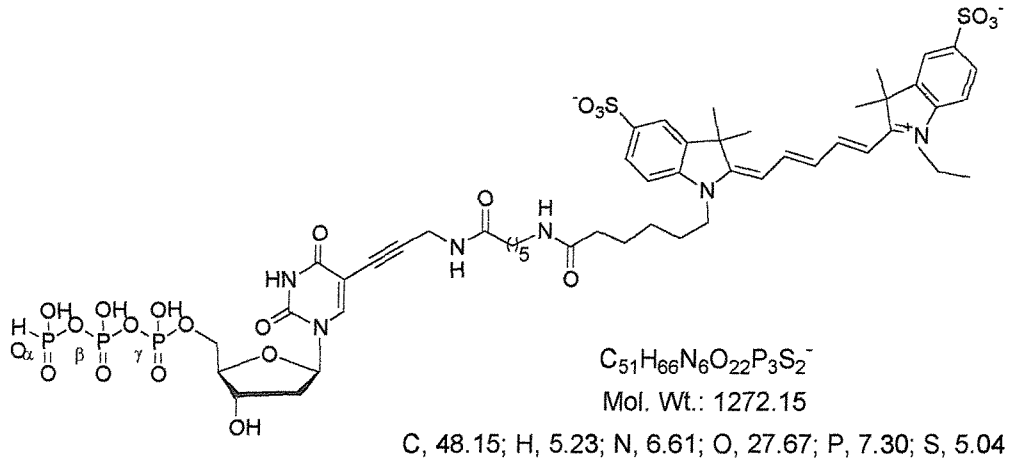
purified by anion exchange HPLC (Buffer system 1). The appropriate fraction was lyophilised repeatedly to give the title compound as a white lyophilised solid (6mg, 6μmol, 81%).

HPLC retention time (Buffer system 1): 12.74 minutes; LRMS ($ES^-$ mode): *m/z* 633.0 $[MH]^-$; $E_{max}$ ($H_2O$): 297nm (6,180), 233nm (7,792).



**5-[6-(Hexanoyl-Cy5-amido)hexanoyl-3-propargylamino]-2'-deoxyuridine-5'-triphosphate-3'-O-succinamide resin [68]**

The triphosphate CPG-resin **[38]** (20mg, 0.56μmol) was treated with 20% piperidine in DMF for 10 minutes, the resin washed with DMF (x 3) and DCM (x 5) and dried *in vacuo*. The resin was then soaked in a 0.5M solution of potassium hydrogen phosphate (250μL) and to the resin was added a solution of Cye 5 *N*-hydroxysuccinimide active ester (1mg) in DMF (0.5mL). The resin suspension was agitated gently with a stream of argon gas, for 30 minutes and allowed to soak, protected from the light for 12 hours before being washed extensively with DMF (x 3), DCM (x 5) and dried over $P_2O_5$ *in vacuo*.



$C_{51}H_{66}N_6O_{22}P_3S_2^-$

Mol. Wt.: 1272.15

C, 48.15; H, 5.23; N, 6.61; O, 27.67; P, 7.30; S, 5.04

**5-[6-(Hexanoyl-Cy5-amido)hexanoyl-3-propargylamino]-2'-deoxyuridine-5'-triphosphate [69]**

*Method 1:*

To a solution of compound **[67]** (125nmol) in a 0.5M aqueous solution of potassium hydrogen phosphate (25μL) was added a solution of Cye 5 *N*-hydroxysuccinimide active ester (1mg) in DMF (500μL) and the reaction mixture stirred, protected from the light for 12 hours. The reaction mixture was then diluted with water (1mL) and washed with diethylether. The aqueous layer was separated and passed through a column of Dowex $Li^+$ eluting with water (1mL) and purified by reverse phase HPLC (Buffer system 3). The appropriate fraction was lyophilised repeatedly to give the title compound as a blue lyophilised solid (65μg, 51nmol, 41%).

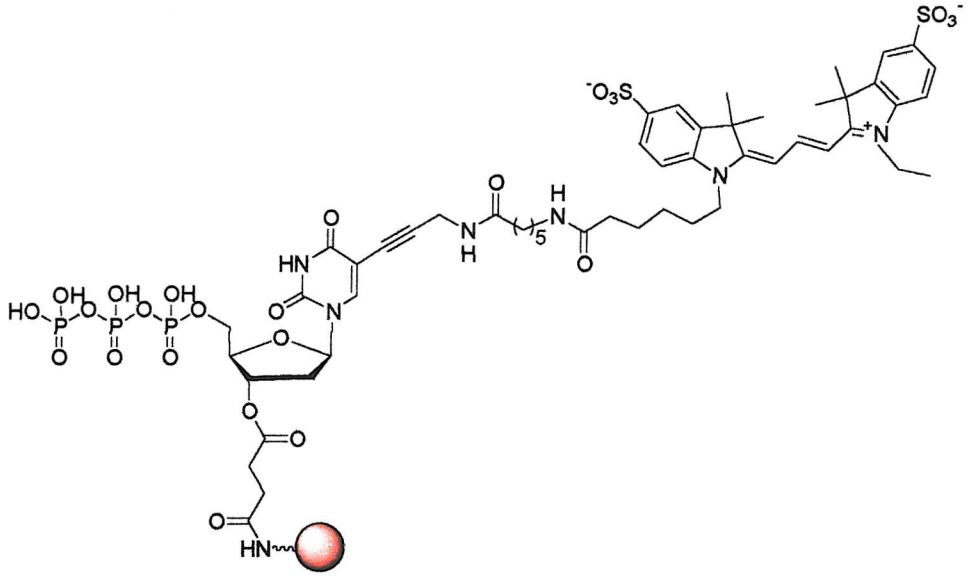
*Method 2:*

The Cy5-labelled triphosphate CPG-resin **[68]** (10mg, 0.28μmol) was cleaved using general method 6.1.2.5 and purified by reverse phase HPLC (Buffer system 3). The appropriate fraction was lyophilised repeatedly to give the title compound as a blue lyophilised solid (280μg, 0.22μmol, 78%).

Method 3:

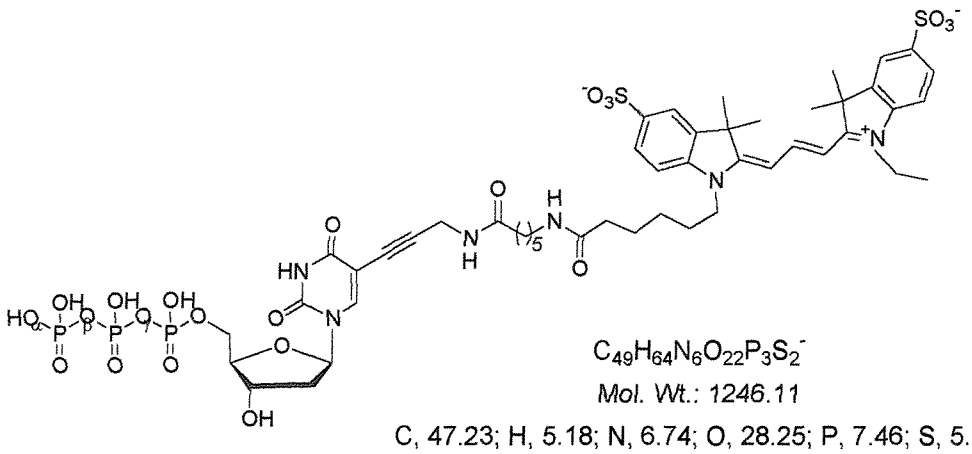
The Cy5-labelled triphosphate CPG-resin **[68]** (10mg, 0.28μmol) was cleaved using general method 6.1.2.7 and purified by reverse phase HPLC (Buffer system 3). The appropriate fraction was lyophilised repeatedly to give the title compound as a blue lyophilised solid (305μg, 0.24μmol, 86%).

HPLC retention time (Buffer system 3): 24.89 minutes; LRMS ($ES^-$ mode): *m/z* 1270 $[MH]^-$; $\lambda_{max}$ ($H_2O$): 647, 604, 255nm.



**5-[6-(Hexanoyl-Cy3-amido)hexanoyl-3-propargylamino]-2'-deoxyuridine-5'-triphosphate-3'-O-succinamide resins [70]**

The triphosphate CPG-resin **[38]** (20mg, 0.56μmol) was treated as described for resin **[68]** using Cy 3 *N*-hydroxysuccinimide active ester.



$C_{49}H_{64}N_6O_{22}P_3S_2^-$

*Mol. Wt.: 1246.11*

C, 47.23; H, 5.18; N, 6.74; O, 28.25; P, 7.46; S, 5.15

**5-[6-(Hexanoyl-Cy3-amido)hexanoyl-3-propargylamino]-2'-deoxyuridine-5'-triphosphate [71]**

*Method 1:*

As described for compound ***[69]*** using Cye 3 *N*-hydroxysuccinimide active ester to give the title compound as a blue lyophilised solid (137μg, 0.11μmol, 40%).

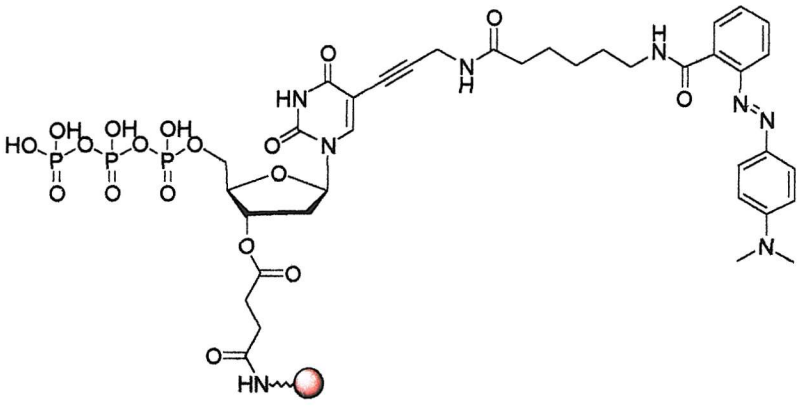
*Method 2:*

The Cy3-labelled triphosphate CPG-resin **[70]** was cleaved using general method 6.1.2.5 and purified by reverse phase HPLC (Buffer system 3). The appropriate fraction was lyophilised repeatedly to give the title compound as a blue lyophilised solid (80%).

Method 3:

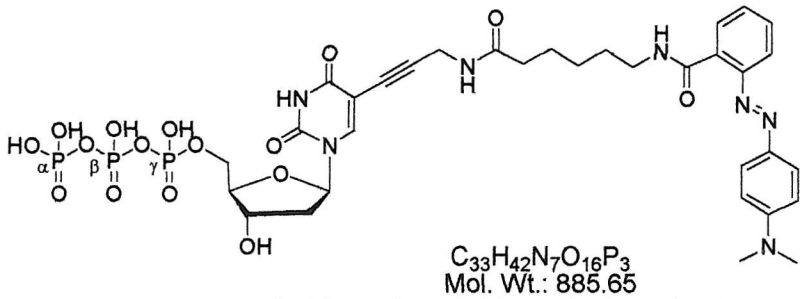
The Cy3-labelled triphosphate CPG-resin **[70]** was cleaved using general method 6.1.2.7 and purified by reverse phase HPLC (Buffer system 3). The appropriate fraction was lyophilised repeatedly to give the title compound as a blue lyophilised solid (88%).

HPLC retention time (Buffer system 3): 22.93 minutes; LRMS ($ES^+$ mode): *m/z* 1219.8 $[MH]^+$; $\lambda_{max}$ ($H_2O$): 549, 516, 279nm.



**5-[6-(2-{4-dimethylamino-phenylazo}-benzoic acid)hexanoyl-3-propargylamino]-2'-deoxyuridine-5'-triphosphate-3'-O-succinamide resins [72]**

The triphosphate CPG-resin **[38]** (60mg, 1.3μmol) was treated with 20% piperidine in DMF for 10 minutes, the resin washed with DMF (x 3) and DCM (x 5) and dried *in vacuo*. To a solution of methyl red (10mg, 27μmol) in anhydrous pyridine (1mL) was added EDC (5mg, 26.1μmol) and the solution stirred under an argon atmosphere for 10 minutes. This was then transferred to the resin that was soaked in anhydrous pyridine, and the resin mixture allowed to stand with occasional mixing for 12 hours. The resin was then washed extensively with pyridine (x 3), ether (x 5) and dried over $P_2O_5$ *in vacuo*.



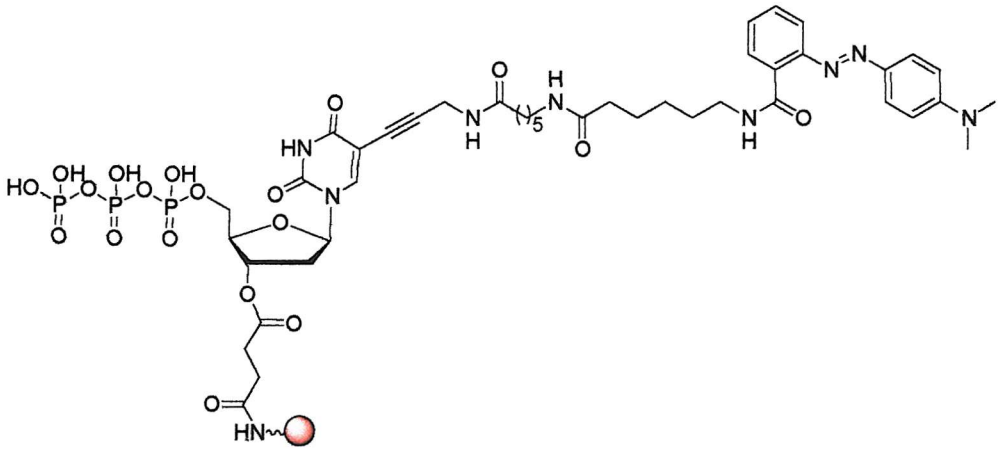
$C_{33}H_{42}N_7O_{16}P_3$
Mol. Wt.: 885.65
C, 44.75; H, 4.78; N, 11.07; O, 28.90; P, 10.49

**5-[6-(2-{4-dimethylamino-phenylazo}-benzoic acid)hexanoyl-3-propargylamino]-2'-deoxyuridine-5'-triphosphate [73]**

*The labelled triphosphate CPG-resin* **[72]** *(60mg, 1.3μmol) was cleaved using general* method 6.1.2.5 and purified by anion-exchange HPLC (Buffer system 1). The

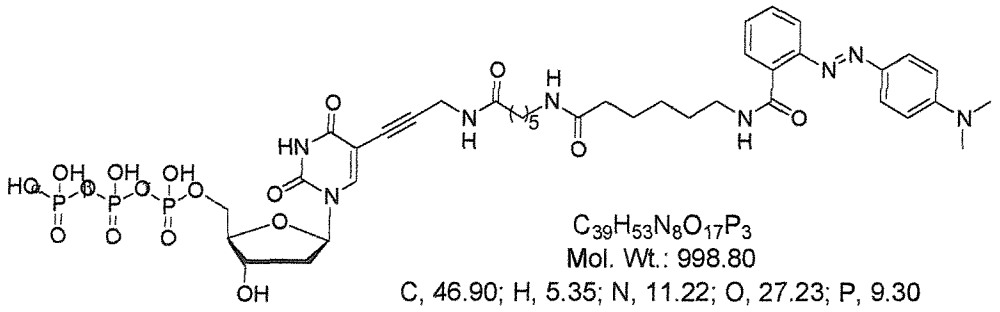
appropriate fraction was lyophilised repeatedly to give the title compound as a red lyophilised solid (451μg, 0.51μmol, 39%).

HPLC retention time (Buffer system 1): 16.23 minutes; LRMS ($ES^+$ mode): *m/z* 822.3 $[MH]^+$; $\lambda_{max}$ ($H_2O$): 494, 312, 233nm.



**5-[6-(6-{2-(4-dimethylamino-phenylazo)-benzoylamino}-hexanoic acid)hexanoyl-3-propargylamino]-2'-deoxyuridine-5'-triphosphate-3'-O-succinamide resins [74]**

The triphosphate CPG-resin **[38]** (50mg, 1.1μmol) was treated with 20% piperidine in DMF for 10 minutes, the resin washed with DMF (x 3) and DCM (x 5) and dried *in vacuo*. A solution of compound **[76]** (4mg, 10.5μmol,) and HOBt (2mg, 14.8μmol) in anhydrous pyridine (1mL) was stirred for 10 minutes before being added to the resin with DIC (2μL). The resin suspension was agitated gently with a stream of argon gas for 2 hours before being washed extensively with pyridine (x 3), DMF (x 3), DCM (x 5) and dried over $P_2O_5$ *in vacuo*.

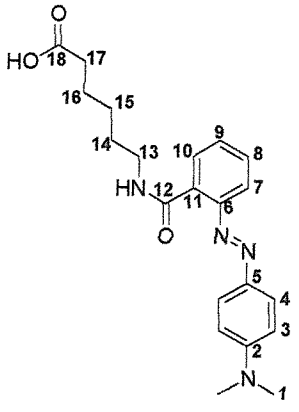


$C_{39}H_{53}N_8O_{17}P_3$
Mol. Wt.: 998.80
C, 46.90; H, 5.35; N, 11.22; O, 27.23; P, 9.30

**5-[6-(6-{2-(4-dimethylamino-phenylazo)-benzoylamino}-hexanoic acid) hexanoyl-3-propargylamino]-2'-deoxyuridine-5'-triphosphate [75]**

The labelled triphosphate CPG resin **[74]** (50mg, 1.1μmol) was cleaved using general method 6.1.2.7 and purified by anion-exchange HPLC (Buffer system 2). The appropriate fraction was lyophilised repeatedly to give the title compound as a red lyophilised solid (629μg, 0.63μmol, 58%).

HPLC *retention time (Buffer system 2): 21.68 minutes*; LRMS ($ES^+$ mode): *m/z 999.3* $[MH]^+$; $\lambda_{max}$ ($H_2O$): 494, 312, 229nm.



$C_{21}H_{26}N_4O_3$
Mol. Wt.: 382.46
C, 65.95; H, 6.85; N, 14.65; O, 12.55

**6-[2-(-Dimethylamino-phenylazo)-benzoylamino]-hexanoic acid [76]**

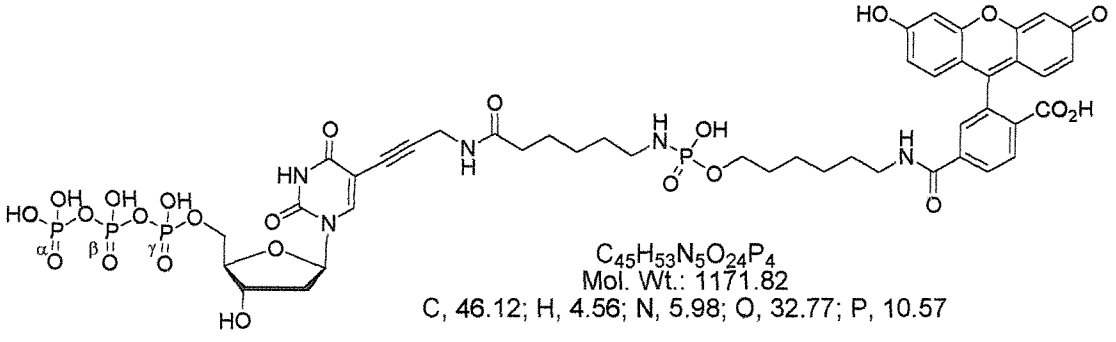
To a solution of 6-aminohexanoic acid (1.471g, 11.2mmol), methylred (3.615g, 13.4mmol, 1.2eq), HOBt (2.270g, 16.8mmol, 1.5eq) and DCC (3.466g, 16.8mmol, 1.5eq) in anhydrous DCM (50mL) was added anhydrous triethylamine (3.12mL, 22.4mmol, 2eq). The reaction mixture was stirred under an argon atmosphere for 12 hours before being diluted with DCM (350mL) and washed with sat. KCl (2 x

100mL) and $H_2O$ (100mL). The organic layer was separated, dried ($Na_2SO_4$) and the solvent removed *in vacuo*. The crude product was purified by wet-flash column chromatography eluting with a gradient of methanol in ethylacetate (0-10%) to give the title compound as a dark red microcrystalline solid which was dried in a vacuum desiccator over $P_2O_5$ for 24 hours (2.609g, 6.83mmol, 61%).

$R_f$(G) 0.69; LRMS ($ES^+$ mode): *m/z* 765.8 $[2MH]^+$; mp: 165°C; $\lambda_{max}$(MeOH): 494, 284nm; Found C: 67.58, H: 7.11, N: 15.23, $C_{21}H_{26}N_4O_3$ requires C: 65.95, H: 6.85, N: 14.64%.

$\delta_H(CDCl_3)$ 0.97-1.14 (2H, M, **H15**) 1.15-1.32 (2H, m, **H16**) 1.50-1.64 (4H, m, **H14**+**H17**) 2.82-2.92 (2H, m, **H13**) 3.09 (6H, s, **H1**) 6.70 (2H, d, $J$ = 9.0 Hz, **H3**) 7.44 (1H, m, **H9**) 7.55 (1H, m, **H8**) 7.74 (2H, d, $J$ = 9.0 Hz, **H4**) 7.93 (1H, d, $J$ = 8.0 Hz, **H7**) 8.30 (1H, dd, $J$ = 7.5, 1.7 Hz, **H10**).

$\delta_C(CDCl_3)$ *24.7 (**C15**) 25.4 (**C14**) 33.7 (**C17**) 38.6 (**C13**) 40.0 (**C1**) 111.6 (**C3**) 115.3* (**C7**) 125.0 (**C4**) 126.7 (**C11**) 129.7 (**C10**) 132.4 (**C9**) 133.3 (**C8**) 141.9 (**C5**) 150.1 (**C2**) 153.8 (**C6**) 156.6 (**C12**) 167.1 (**C18**).

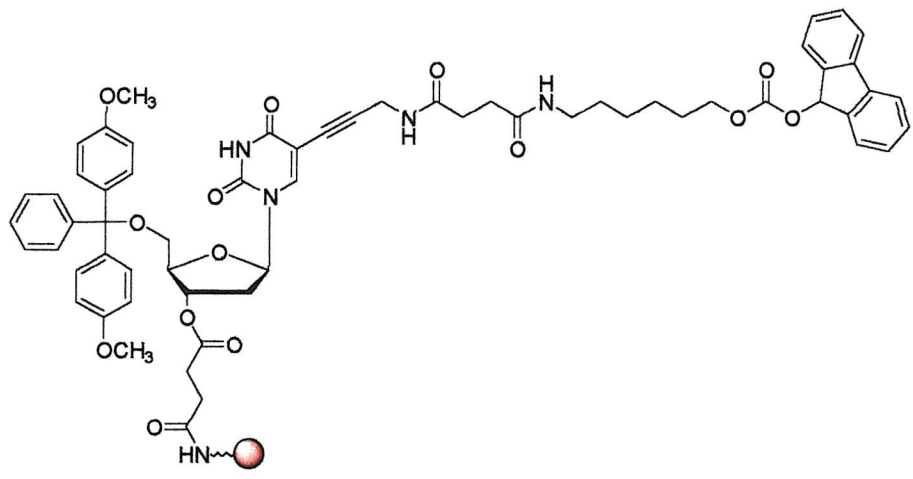



$C_{45}H_{53}N_5O_{24}P_4$
Mol. Wt.: 1171.82
C, 46.12; H, 4.56; N, 5.98; O, 32.77; P, 10.57

**5-[6-(Hexanoylphosphatyl-fluoresceinamido)hexanoyl-3-propargylamino]-2'-deoxyuridine-5'-triphosphate [77]**

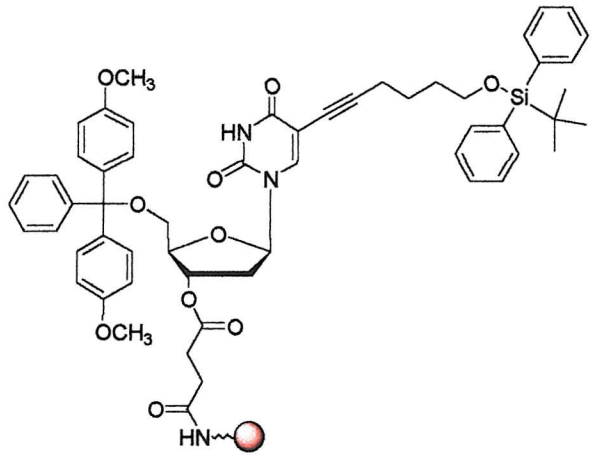
The FAM –labelled triphosphate CPG-resin **[41]** (48mg, 1.0μmol) was cleaved using general method 6.1.2.6 and purified by anion exchange HPLC (Buffer system 2). The appropriate fraction was lyophilised repeatedly to give the title compound as a yellow lyophilised solid (70μg, 0.6μmol, 56%).

HPLC retention time (Buffer system 2); LRMS ($ES^+$ mode): *m/z* 1172 $[MH]^+$; $\lambda_{max}$ ($H_2O$): 492, 251nm.



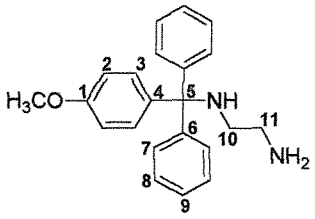
**5-[6-(9-Fluorenylmethoxycarbonylamino)hexanoyl-3-propargylaminosuccinyl]-5'-O-(4,4'-dimethoxytrityl)-2'-deoxyuridine-3'-O-succinamide resin [78]**

Compound **[48]** was attached to the CPG solid support (310mg, 25μmol) using general method 6.1.2.1 with a loading of 39μmol $g^{-1}$, 49%.



**5-Hexyn-1-O-[(1,1-dimethylethyl)diphenyl silyl]- 5'-(4,4'-dimethoxytrityl)-2'-deoxyuridine-3'-O-succinamide resin [79]**

*Compound* **[57]** *was attached to the CPG solid support (0.35g, 28μmol) using general* method 6.1.2.1 to give a loading of 48μmol $g^{-1}$, 60%.

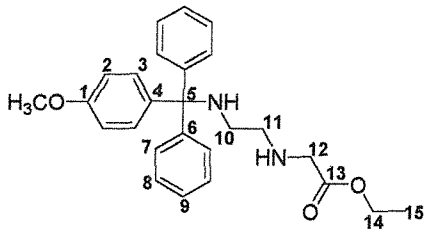



$C_{22}H_{24}N_2O$
Mol. Wt.: 332.44
C, 79.48; H, 7.28; N, 8.43; O, 4.81

***N1*-(4-monomethoxytrityl)-ethylenediamine *[80]***

To a solution of ethylenediamine (11.0mL, 9.889g, 0.16mol, 10eq) in anhydrous DMF (9mL) was added portionwise a solution of 4-monomethoxytritylchloride (5.013g, 16.5mmol) in anhydrous DMF (15mL) followed by diisopropylethylamine (7.19mL, 5.335g, 0.04mol, 2.5eq). The solution was allowed to stir under an argon atmosphere for 5 hours before being reduced in volume. The residue was dissolved in dichloromethane (200mL) and washed with sat. KCl (3 x 40mL). The organic phase was separated, dried ($Na_2SO_4$) and the solvent removed *in vacuo*. The crude product was purified by wet flash column chromatography (silica pre-equilibrated with 1% $Et_3N$) eluting with methanol in dichloromethane (0-3%) to yield the title compound as a colourless oil which was dried in a vacuum desiccator over $P_2O_5$ for 24 hours (4.137g, 12.5mmol, 76%).

$R_f$ (G) 0.31; LRMS ($ES^+$ mode): *m/z* 333.2 $[MH]^+$; $\lambda_{max}$ (MeOH): 271, 230nm.
$\delta_H$(DMSO) 2.01 (2H, s, br, **$NH_2$**) 2.49-2.66 (4H, m, **H10+H11**) 3.70 (3H, s, **$OCH_3$**) 6.85 (2H, d, $J$ = 8.8 Hz, **H3**) 7.16 (2H, t, $J$ = 7.0 Hz, **H9**) 7.25 – 7.33 (6H, m, **H2+H8**) 7.42 (4H, d, $J$ = 7.4 Hz, **H7**).
$\delta_C$ (DMSO) 42.2 (**C11**) 46.9 (**C10**) 55.0 (**$OCH_3$**) 69.7 (**C5**) 113.0 (**C2**) 126.0 (**C9**) 127.7 (**C7**) 128.4 (**C8**) 129.7 (**C3**) 138.3 (**C4**) 146.7 (**C6**) 157.4 (**C1**).



$C_{26}H_{30}N_2O_3$
Mol. Wt.: 418.53
C, 74.61; H, 7.22; N, 6.69; O, 11.47

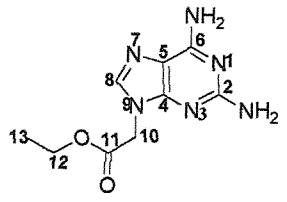
**_N_-(4-monomethoxytrityl)-_N_-(2-aminoethyl)glycine ethyl ester [81]**[258]

To a solution of compound **[80]** (2.098g, 6.32mmol) in anhydrous DMF (10mL) was added diisopropylethylamine (4.4ml, 3.265g, 25.3mmol, 4eq) and ethylbromoacetate (911μL, 1mg, 8.21mmol). The solution was allowed to stir under an argon atmosphere for 3 hours before being reduced in volume. The residue was dissolved in dichloromethane (300mL) and washed with sat. KCl (3 x 50mL) followed by brine (3 x 50mL). The organic phase was separated, dried ($Na_2SO_4$) and the solvent removed *in vacuo*. The crude product was purified by wet flash column chromatography (silica pre-equilibrated with 1% $Et_3N$) eluting with dichloromethane and then further purified by wet flash column chromatography (silica pre-equilibrated with 1% $Et_3N$) eluting with ethylacetate in hexane (25%) to yield the title compound as a pale yellow oil which was dried in a vacuum desiccator over $P_2O_5$ for 24 hours (1.111g, 2.65mmol, 42%).

$R_f$ (G) 0.52; LRMS ($ES^+$ mode): *m/z* 419.3 $[MH]^+$ 441.2 $[MNa]^+$; $\lambda_{max}$ (MeOH): 278, 230nm.

$\delta_H$(DMSO) 1.18 (3H, t, *J* = 7.0Hz **H15**) 2.06 (2H, m, **H11**) 2.63 (2H, t, *J* = 5.9 Hz, **H10**) 3.20 (2H, s, **H12**) 3.72 (3H, s, **$OCH_3$**) 4.06 (2H, q, *J* = 7.0 Hz, **H14**) 6.85 (2H, d, *J* = 8.8 Hz, **H3**) 7.17 (2H, t, *J* = 7.4 Hz, **H9**) 7.25 – 7.31 (6H, m, **H2**+ **H8**) 7.41 (4H, d, *J* = 7.4 Hz, **H7**).

$\delta_C$ (DMSO) 14.2 (**C15**) 43.0 (**C10**) 48.9 (**C11**) 50.2 (**C12**) 55.0 (**$OCH_3$**) 59.9 (**C14**) 69.8 (**C5**) 113.0 (**C2**) 126.0 (**C9**) 127.7 (**C7**) 128.4 (**C8**) 129.7 (**C3**) 138.2 (**C4**) 146.6 (**C6**) 157.4 (**C1**) 172.3 (**C13**).



$C_9H_{12}N_6O_2$
Mol. Wt.: 236.23
C, 45.76; H, 5.12; N, 35.58; O, 13.55

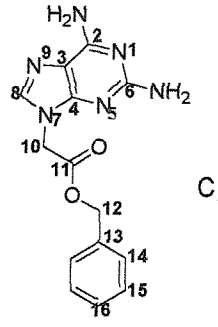
**(2, 6-Diamino-purin-9-yl)-acetic acid ethyl ester [82]**[257].

To a suspension of finely ground 2,6-diaminopurine (1.502g, 9.99mmol) in anhydrous, degassed DMF (30mL) was added portionwise NaH (440mg, 60% in oil, 18.3mmol, 1.1eq) After 2 hours neat ethylbromoacetate (1.44mL, 2.167g, 13.0mmol, 1.3eq) was added and the reaction stirred for a further hour. The reaction was then filtered with the aid of celite and the solvent removed *in vacuo*. The crude product was purified by wet flash column chromatography eluting with methanol in dichloromethane (0-6%) to yield a cream foam which was crystallised from absolute ethanol to yield the title compound as a cream crystalline solid which was dried in a vacuum desiccator over $P_2O_5$ for 24 hours (1.205g, 5.10mmol, 51%).

$R_f$ (F) 0.54; LRMS ($ES^+$ mode): *m/z* 237.1 $[MH]^+$ 339.2 $[2MK]^+$; $\lambda_{max}$ (MeOH): 281, 256nm; mp: 167-169°C.

$\delta_H$(DMSO) 1.20 (3H, t, $J$ = 7.0Hz, **H13**) 4.15 (2H, q, $J$ = 7.1Hz, **H12**) 4.85 (2H, s, **H10**) 5.87 (2H, s, br, $NH_2$) 6.75 (2H, s, br, $NH_2$) 7.67 (1H, s, **H8**).

$\delta_C$ (DMSO) 14.1 (**C13**) 43.5 (**C10**) 61.3 (**C12**) 112.7 (**C5**) 137.9 (**C8**) 152.0 (**C4**) 156.2 (**C2**) 160.5 (**C6**) 168.4 (**C11**).



$C_{14}H_{14}N_6O_2$
Mol. Wt.: 298.30
C, 56.37; H, 4.73; N, 28.17; O, 10.73

**(2, 6-Diamino-purin-9-yl)-acetic acid benzyl ester [83]**

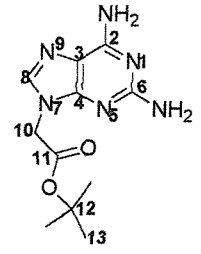
To a suspension of sodium hydride (995mg, 41.5mmol) in anhydrous DMF (150mL) was added portionwise a finely ground suspension of 2,6-diaminopurine (3.392g, 22.6mmol) in anhydrous DMF (170mL) and the reaction mixture stirred under an argon atmosphere. After a period of 2 hours neat benzyl-2-bromoacetate (6.213g, 4.3mL, 27.1mmol) was added and the reaction stirred for a further hour. The reaction mixture was then filtered with the aid of celite and the filtrate reduced in volume. The crude product was purified by wet-flash column chromatography eluting with methanol in dichloromethane (0-10%) producing a pale yellow foam which was re-crystallised from ethanol to yield the title compound as an off-white crystalline solid which was dried in a vacuum desiccator over $P_2O_5$ for 24 hours (4.474g, 15.0mmol, 67%).

$R_f$ (G) 0.25; LRMS ($ES^+$ mode): *m/z* 299.1 $[MH]^+$ 597.1 $[2MH]^+$; $\lambda_{max}$ (MeOH): 281, 256nm; mp: 177-178°C; *Found* C: 56.14, H: 4.50, N: 27.86%, $C_{14}H_{14}N_6O_2$ *requires* C: 56.37, H: 4.73, N: 28.17%.

IR: $\nu$ = 3454.9 (m), 3103.5 (w), 1676.1 (m), 1635.2 (m), 1593.2 (s), 1410.8 (s), 1188.2 (s), 1157.0 (s), 789.8 (s), 739.0 (s) $cm^{-1}$.

$\delta_H$(DMSO) 4.96 (2H, s, **H10**) 5.19 (2H, s, **H12**) 5.91 (2H, br, s, $NH_2$) 6.81 (2H, br, s, $NH_2$) 7.34-7.42 (5H, m, **H14**+**H15**+**H16**) 7.44 (1H, s, **H8**).

$\delta_C$(DMSO) 43.6 (**C10**) 66.6 (**C12**) 112.7 (**C3**) 128.1 (**C14**) 128.3 (**C16**) 128.6 (**C15**) 135.6 (**C13**) 137.9 (**C8**) 152.1 (**C4**) 156.2 (**C6**) 160.5 (**C2**) 168.3 (**C11**).

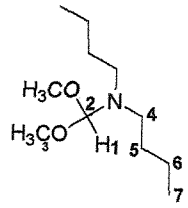


$C_{11}H_{16}N_6O_2$
Mol. Wt.: 264.28
C, 49.99; H, 6.10; N, 31.80; O, 12.11

**(2, 6-Diamino-purin-9-yl)-acetic acid *t*-butyl ester [84]**

To a suspension of sodium hydride (995mg, 6.67mmol) in anhydrous DMF (25mL) was added portion-wise a finely ground suspension of 2,6-diaminopurine (547mg, 3.65mmol) in anhydrous DMF (40mL) and the reaction mixture stirred under an argon atmosphere. After a period of 1 hour neat *t*-butyl-2-bromoacetate (854mg, 0.71mL, 4.38mmol) was added and the reaction stirred for a hour. The reaction mixture was then filtered with the aid of celite and the filtrate reduced in volume. The crude product was purified by crystallisation from ethanol to yield the title compound as an off-white crystalline solid which was dried in a vacuum desiccator over $P_2O_5$ for 24 hours (838mg, 3.18mmol, 87%).

$R_f$ (G) 0.26; LRMS ($ES^+$ mode): *m/z* 265.1 $[MH]^+$; $\lambda_{max}$ (MeOH): 281, 256nm.
IR: $\nu$ = 3401.0 (m), 3193.4 (w), 1739.4 (s), 1639.7 (s), 1591.8 (s), 1413.7 (m), 1238.1 (s), 1152.0 (s), 1022.3 (s) $cm^{-1}$.
$\delta_H$(DMSO) 1.41 (9H, s, **H13**) 4.74 (2H, s, **H10**) 5.88 (2H, br, s, **$NH_2$**) 6.79 (2H, br, s, **$NH_2$**) 7.68 (1H, s, **H8**).
$\delta_C$(DMSO) 27.8 (**C13**) 44.1 (**C10**) 81.9 (**C12**) 112.7 (**C3**) 138.0 (**C8**) 152.0 (**C4**) 156.2 (**C6**) 160.5 (**C2**) 167.4 (**C11**).

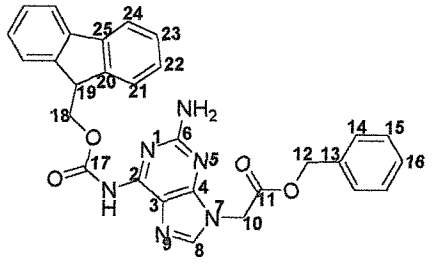


$C_{11}H_{25}NO_2$
Mol. Wt.: 203.32
C, 64.98; H, 12.39; N, 6.89; O, 15.74

**Di-*n*-butylformamide dimethyl acetal [85]**[259]

A solution of di-*n*-butylamine (26.845g, 35mL, 208mmol) and *N,N*-dimethylformamidedimethylacetal (27.447g, 34mL, 229mmol) was heated at 100°C for 3 days. The product was then separated by distillation (14mmHg, 70°C) and further purified by a second distillation (14mmHg, 70°C) producing the title compound as a colourless liquid at 70°C (9.673g, 23%).

$\delta_H(CDCl_3)$; 1.01 (6H, **H7**) 1.25-1.45 (8H, m, **H5**+**H6**) 2.62 (4H, t, $J$ = Hz, **H4**) 3.37 (6H, s, **$OCH_3$**) 4.51 (1H, s, **H1**).
$\delta_C(CDCl_3)$ 13.9 (**C7**) 20.7 (**C6**) 31.2 (**C5**) 47.2 (**C4**) 54.0 (**C3**) 112.8 (**C2**).



$C_{29}H_{24}N_6O_4$
Mol. Wt.: 520.54
C, 66.91; H, 4.65; N, 16.14; O, 12.29

**[2-(9-Fluorenylmethoxycarbonyl)-6-amino-purin-9-yl]-acetic acid benzyl ester [86]**

To a suspension of compound **[83]** (1.001g, 3.36mmol) in anhydrous dioxan (20mL) was added drop-wise a solution of fluorenylmethoxycarbonyl succcinimide (2.491g, 7.38mmol) in anhydrous dioxan (10mL). The reaction mixture was allowed to stir under an argon atmosphere for 12 hours before being concentrated *in vacuo*. The residue was dissolved in DCM (400mL) and washed with sat. KCl (4 x 80mL) followed by $H_2O$ (80mL). The organic phase was separated, dried ($Na_2SO_4$) and the

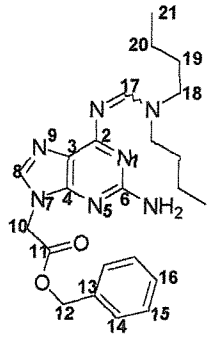
solvent removed *in vacuo*. The crude product was purified by wet-flash column chromatography eluting with methanol in dichloromethane (0-7%) to yield the title compound as pale yellow foam which was dried in a vacuum desiccator over $P_2O_5$ for 24 hours (961mg, 1.85mmol, 55%).

$R_f$ (G) 0.27; LRMS ($ES^+$ mode): *m/z* 521 $[MH]^+$ 875 $[2MH]^+$; $\lambda_{max}$ (MeOH): 301, 256nm.

IR: $\nu$ = 2948.4 (w), 1704.3 (s), 1636.2 (m), 1445.9 (m), 1207.8 (s), 1075.7 (m), 994.7 (m), 726.2 (s)$cm^{-1}$.

$\delta_H$(DMSO) 3.58, 3.59 (4H, s, **H10+H12**) 4.38 (1H, t, *J* = 6.6 Hz, **H19**) 4.65 (2H, d, *J* = 4.7 Hz, **H18**) 7.31-7.47 (7H, m, **H15**+ **H16+H22+H23**) 7.66 (1H, s, **H8**) 7.68 (2H, d, *J* = 7.4 Hz, **H14**) 7.82-7.99 (4H, m, **H21+H24**).

$\delta_C$(DMSO) 46.2 (**C19**) 63.9 (**C10**) 66.7 (**C18**) 70.3 (**C12**) 126.5 (**C23**) 127.3 (**C14**) 127.7 (**C16**) 127.8 (**C24**) 128.0 (**C22**) 128.4 (**C3**) 128.5 (**C21**) 128.6 (**C15**) 129.0 (**C8**) 137.5 (**C25**) 139.5 (**C20**) 140.9 (**C13**) 144.0 (**C2**) 154.0 (**C6**) 162.4 (**C4**) 169.1 (**C17**) 172.4 (**C11**).



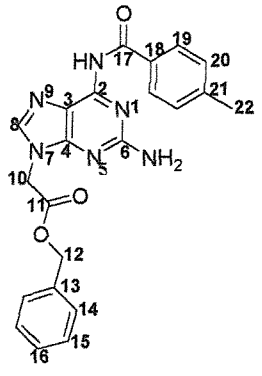

$C_{23}H_{31}N_7O_2$
Mol. Wt.: 437.54
C, 63.14; H, 7.14; N, 22.41; O, 7.31

**(2-*N*-di-*n*-butylformamidine-6-aminopurin-9-yl)-acetic acid benzyl ester [87]**

To a solution of compound **[83]** (148mg, 0.50mmol) in anhydrous DMF (3mL) was added compound **[18]** (122mg, 0.60mmol) and the solution was allowed to stir under an argon atmosphere for 12 hours before being concentrated *in vacuo*. The residue was dissolved in DCM (50mL) and washed with $H_2O$ (10mL) followed by sat. KCl (3 x 10mL). The organic phase was separated, dried ($Na_2SO_4$) and the solvent removed

*in vacuo*. The crude product was purified by wet-flash column chromatography eluting with methanol in dichloromethane (0-5%) to yield the title compound as pale yellow foam which was dried in a vacuum desiccator over $P_2O_5$ for 24 hours (105mg, 0.24mmol, 47%).

$R_f$ (G) 0.78; LRMS ($ES^+$ mode): *m/z* 438. $[MH]^+$ 875 $[2MH]^+$; $\lambda_{max}$ (MeOH): 324, 276, 253nm; mp: 173-174°C.
IR: ν = 3103.4 (w), 2957.2 (w), 2930.4 (w), 1746.6 (s), 1620.4 (m), 1548.1 (s), 1450.4 (m), 1404.2 (s), 1185.2 (s), 1024.7 (s), 746.7 (s), 697.1 (m).
$\delta_H$(DMSO) 0.92 (6H, dt, *J* = 11.8, 3.7 Hz, **H21**) 1.31 (4H, ddd, *J* = 22.4, 15.1, 7.4 Hz, **H20**) 1.57 (4H, dt, *J* = 15.4, 7.4 Hz, **H19**) 3.36 (2H, t, *J* = 7.0 Hz, **H18**) 3.53 (2H, t, *J* = 7.4 Hz, **H18**) 4.99 (2H, s, **H10**) 5.20 (2H, s, **H12**) 6.07 (2H, br, s, **$NH_2$**) 7.32-7.44 (5H, m, **H14+H15+H16**) 7.82 (1H, s, **H8**) 8.85 (1H, s, **H17**).
$\delta_C$(DMSO) 13.7, 13.9 (**C21**) 19.3, 19.8 (**C20**) 28.8, 30.7 (**C19**) 43.6, 44.3 (**C18**) 51.0 (**C10**) 66.6 (**C12**) 118.7 (**C3**) 128.1 (**C14**) 128.3 (**C16**) 128.6 (**C15**) 135.5 (**C13**) 139.7 (**C8**) 153.8 (**C4**) 157.7 (**C6**) 159.9 (**C17**) 160.4 (**C2**) 168.2 (**C11**).




$C_{22}H_{20}N_6O_3$
Mol. Wt.: 416.43
C, 63.45; H, 4.84; N, 20.18; O, 11.53

**(2-*p*-Toluoyl-6-amino-purin-9-yl)-acetic acid benzyl ester [88]**

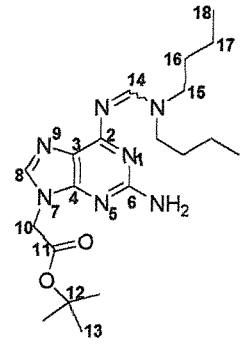
To a solution of compound **[83]** (510mg, 1.71mmol) in anhydrous pyridine (10mL) was added drop-wise at 0°C *p*-toluoyl chloride (344mg, 0.29mL, 2.22mmol). The reaction mixture was allowed to warm to r.t. and stirred under an argon atmosphere

for 2 hours before being concentrated *in vacuo*. The residue was dissolved in DCM (200mL) and washed with sat. KCl (3 x 30mL) followed by brine (30mL). The organic phase was separated, dried ($Na_2SO_4$) and the solvent removed *in vacuo*. The crude product was purified by wet-flash column chromatography eluting with dichloromethane : diethylether (1:1) to yield the title compound as white solid which was dried in a vacuum desiccator over $P_2O_5$ for 24 hours (512mg, 1.23mmol, 72%).

$R_f$ (N) 0.36; LRMS ($ES^+$ mode): *m/z* 417 $[MH]^+$; $\lambda_{max}$ (MeOH): 298, 256nm; mp: 98-99°C; *Found C: 65.81, H: 5.07, N: 15.40%, $C_{22}H_{20}N_6O_3$ requires C: 63.45, H: 4.84,* N: 20.18%.

$\delta_H(CDCl_3)$ 2.42 (3H, s, **H22**) 4.96 (2H, s, **H10**) 5.19 (2H, s, **H12**) 7.13 (2H, d, $J = 8.1$ Hz, **H20**) 7.34-7.42 (5H, m, **H14+H15+H16**) 7.81 (2H, d, $J = 8.1$ Hz, **H10**) 7.94 (1H, s, **H8**).

$\delta_C(CDCl_3)$ 22.0 (**C22**) 44.6 (**C10**) 68.4 (**C12**) 119.5 (**C3**) 128.1 (**C14**) 128.4 (**C16**) 129.0 (**C15**) 129.1 (**C19**) 129.7 (**C20**) 129.9 (**C18**) 135.0 (**C13**) 143.1 (**C8**) 150.3 (**C4**) 153.5 (**C6**) 165.1 (**C2**) 165.5 (**C17**) 167.4 (**C11**).




$C_{20}H_{33}N_7O_2$
Mol. Wt.: 403.52
C, 59.53; H, 8.24; N, 24.30; O, 7.93

**(2-*N*-di-*n*-butylformamidine-6-aminopurin-9-yl)-acetic acid *t*-butyl ester [89]**

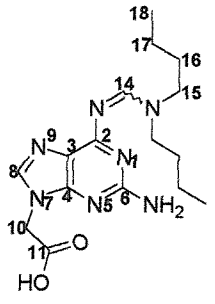
To a solution of compound **[84]** (194mg, 0.73mmol) in anhydrous DMF (3mL) was added compound **[85]** (326mg, 1.61mmol) and the solution was allowed to stir under an argon atmosphere for 12 hours before being concentrated *in vacuo*. The residue was dissolved in DCM (70mL) and washed with sat. KCl (3 x 15mL) followed by

$H_2O$ (15mL). The organic phase was separated, dried ($Na_2SO_4$) and the solvent removed *in vacuo*. The crude product was purified by wet-flash column chromatography eluting with methanol in dichloromethane (0-3%) to yield the title compound as an off-white micro-crystalline solid which was dried in a vacuum desiccator over $P_2O_5$ for 24 hours (136mg, 0.34mmol, 47%).

$R_f$ (G) 0.29; LRMS ($ES^+$ mode): *m/z* 404 $[MH]^+$ 808 $[2MH]^+$; $\lambda_{max}$ (MeOH): 281, 256nm.

$\delta_H$(DMSO) 0.92 (6H, dt, $J$ = 6.6, 4.0 Hz, **H18**) 1.31 (4H, dt, $J$ = 14.7, 7.4 Hz, **H17**) 1.42 (9H, s, **H13**)1.54-1.59 (4H, m, **H16**) 3.35 (4H, m, **H15**) 4.78 (2H, s, **H10**) 6.05 (2H, br, s, $NH_2$) 7.79 (1H, s, **H8**) 8.81 (1H, s, **H14**).

$\delta_C$(DMSO) 13.7, 13.9 (**C18**) 19.3, 19.8 (**C17**) 27.8 (**C13**) 28.8, 30.7 (**C16**) 44.1, 44.3 (**C15**) 50.9 (**C14**) 81.9 (**C12**) 118.6 (**C3**) 139.7 (**C8**) 153.8 (**C4**) 157.6 (**C6**) 159.8 (**C14**) 160.3 (**C2**) 167.3 (**C11**).




$C_{16}H_{25}N_7O_2$
Mol. Wt.: 347.42
C, 55.31; H, 7.25; N, 28.22; O, 9.21

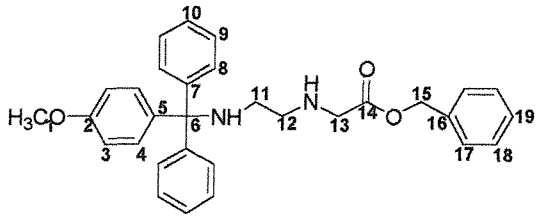
**(2-*N*-di-*n*-butylformamidine-6-aminopurin-9-yl)-acetic acid [90]**

A solution of compound **[89]** (100mg, 0.25mmol) in DCM (2mL) and TFA (1mL) was stirred under an argon atmosphere for 1 hour. The solvents were removed *in vacuo* and the residual oil was triturated with ether (2.5mL) producing the title compound as an off-white micro-crystalline solid which was dried in a vacuum desiccator over $P_2O_5$ for 24 hours (81mg, 0.23mmol, 94%).

$R_f$ (G) 0.36; LRMS ($ES^+$ mode): *m/z* 348 $[MH]^+$; $\lambda_{max}$ (MeOH): 346, 264nm.

$\delta_H$(DMSO) 0.93 (6H, t, $J$ = 7.4 Hz, **H18**) 1.33 (4H, dd, $J$ = 14.0, 6.6 Hz, **H17**) 1.63-1.66 (4H, m, **H16**) 3.60 (4H, m, **H15**) 4.90 (2H, s, **H10**) 5.76 (2H, s, **H8**) 7.46 (2H, br, s, **$NH_2$**) 8.21 (1H, s, **H14**) 9.48 (1H, s, **COOH**).

$\delta_C$(DMSO) 13.6, 13.9 (**C18**) 19.5, 19.8 (**C17**) 28.6, 30.5 (**C16**) 44.1, 44.3 (**C15**) 50.7 (**C14**) 118.6 (**C3**) 139.3 (**C8**) 153.8 (**C4**) 157.5 (**C6**) 159.6 (**C14**) 160.3 (**C2**) 167.5 (**C11**).




$C_{31}H_{32}N_2O_3$
Mol. Wt.: 480.60
C, 77.47; H, 6.71; N, 5.83; O, 9.99

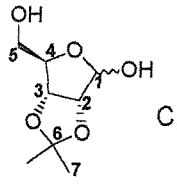
**_N_-(4-monomethoxytrityl)-_N_-(2-aminoethyl)glycine benzyl ester [91]**

To a solution of compound **[80]** (10.306g, 31.0mmol) in anhydrous DMF (45mL) was added DIPEA (6.010g, 8.10mL, 46.5mmol) followed by the portion-wise addition of a solution of benzyl-2-bromoacetate (8.522g, 5.89mL, 37.2mmol) in anhydrous DMF (5mL). The reaction mixture was stirred under an argon atmosphere for 4 hours before being reduced in volume. The residue was dissolved in DCM (700mL) and washed with sat. KCl (4 x 200mL) followed by brine (2 x 100mL). The organic phase was separated, dried ($Na_2SO_4$) and the solvent removed *in vacuo*. The crude product was purified by wet-flash column chromatography (silica pre-equilibrated with 1% $Et_3N$) eluting with hexane : ethyl acetate (1:1) to yield the title compound as a colourless oil which was dried in a vacuum desiccator over $P_2O_5$ for 24 hours (12.113g, 25.2mmol, 81%).

$R_f$ (M) 0.35; LRMS ($ES^+$ mode): *m/z* 273.1 $[MMT]^+$ 481.2 $[MH]^+$; $\lambda_{max}$ (MeOH): 277, 230*nm*.
IR: $\nu$ = 3028.2 (w), 2835.0 (m), 1735.8 (s), 1605.2 (m), 1507.4 (s), 1449.0 (s), 1246.1 (s), 1175.9 (s) $cm^{-1}$.

$\delta_H$(DMSO) 2.02-2.11 (2H, m, **H12**) 2.69-2.70 (2H, m, **H11**) 3.32 (2H, s, **H13**) 3.70 (3H, s, **OCH$_3$**) 5.13 (2H, s, **H15**) 6.85 (2H, d, $J$ = 8.8 Hz, **H4**) 7.16-7.42 (15H, m, **H3+H8+H9+H10+H17+H18+H19**).

$\delta_C$(DMSO) 43.0 (**C12**) 48.9 (**C11**) 50.2 (**C13**) 55.0 (**C1**) 65.5 (**C15**) 69.8 (**C6**) 113.1 (**C3**) 126.0 (**C10**) 127.7 (**C8**) 128.1 (**C19**) 128.4 (**C18**) 128.5 (**C9**) 129.7 (**C4**) 136.2 (**C16**) 138.2 (**C5**) 146.6 (**C7**) 157.4 (**C2**) 172.2 (**C14**).




$C_8H_{14}O_5$
Mol. Wt.: 190.19
C, 50.52; H, 7.42; O, 42.06

**2, 3-O-isopropylidene-D-ribose [98][241].**

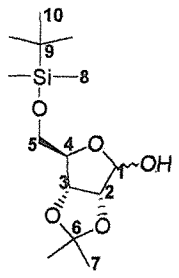
To a solution of D-ribose (2.022g, 13.5mmol) in anhydrous DMF was added PTSA (30mg, 0.16mmol) and 2,2-dimethoxypropane (5mL, 40.4mmol) and the solution stirred under an argon atmosphere for 3 hours. Amberlite IRA-400 ($OH^-$) ion-exchange resin was added and the resin filtered and washed with ethylacetate. The filtrate was diluted with ethylacetate (300mL) and washed with sat. $NaHCO_3$ (50mL) followed by $H_2O$ (2 x 50mL). The organic phase was separated, dried ($Na_2SO_4$) and the solvent removed *in vacuo* to yield the title compound as a pale yellow oil which required no further purification. This was dried in a vacuum desiccator over $P_2O_5$ for 24 hours (630mg, 3.31mmol, 25%).

$R_f$ (G) 0.54; LRMS ($ES^-$ mode): *m/z* 303 $[MTFA]^-$.

IR: $\nu$ = 3350.2 (br), 2939.1 (m), 1713.0 (w), 1375.4 (s), 1209.3 (s), 1031.1 (s) $cm^{-1}$.

$\delta_H$($CDCl_3$) 1.33 (3H, s, **H7**) 1.49 (3H, s, **H7**) 3.64-3.80 (2H, m, **H5**) 3.98 (1H, br, s, **OH**) 4.42 (1H, m, **H4**) 4.59 (1H, d, $J$ = 5.9 Hz, **H2**) 4.84 (1H, d, $J$ = 5.9 Hz, **H3**) 5.42 (1H, s, **H1**).

$\delta_C$($CDCl_3$) 24.8, 26.5 (**C7**) 63.8 (**C5**) 81.8 (**C4**) 87.0 (**C3**) 87.9 (**C2**) 103.1 (**C1**) 112.3 (**C6**).




$C_{14}H_{28}O_5Si$
Mol. Wt.: 304.45
*C*, 55.23; *H*, 9.27; *O*, 26.28; *Si*, 9.22

**5-O-*t*-butyldimethylsilyl-2, 3-O-isopropylidene-D-ribose [99][240]**

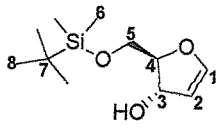
To a solution of compound **[98]** (1.318g, 6.94mmol) in anhydrous DMF (10mL) was added *t*-butyldimethylsilylchloride (1.255g, 8.33mmol) and imidazole (1.181g, 17.3mmol) and the solution stirred under an argon atmosphere for 24 hours before being reduced in volume. The residue was dissolved in DCM (350mL) and washed with sat. KCl (3 x 50mL) followed by sat. $NaHCO_3$ (3 x 50mL). The organic phase was separated, dried ($Na_2SO_4$) and the solvent removed *in vacuo*. The crude product was purified by wet-flash column chromatography eluting with DCM to yield the title compound as an off white waxy solid which was dried in a vacuum desiccator over $P_2O_5$ for 24 hours (1.860g, 6.11mmol, 88%).

$R_f$ (G) 0.81; LRMS ($ES^-$ mode): *m/z* 417 $[MTFA]^-$.

IR: $\nu$ = 3351.0(br), 2935.2(m), 2859.3 (m), 1460.6 (m), 1368.1(m), 1209.1 (s), 1062.6 (s), 984.4 (s) $cm^{-1}$.

$\delta_H(CDCl_3)$ *0.15 (6H, s,* **H8***) 0.94 (9H, s,* **H10***) 1.33 (3H, s,* **H7***) 1.49 (3H, s,* **H7***)* 3.71-3.78 (2H, m, **H5**) 4.36 (1H, m, **H4**) 4.51 (1H, d, *J* = 5.9 Hz, **H2**) 4.72 (1H, d, *J* = 5.9 Hz, **H3**) 4.78 (1H, s, **H1**) 5.42 (1H, br, s, **OH**).

$\delta_C(CDCl_3)$ 18.4 (**C9**) 24.7 (**C7**) 26.0 (**C10**) 26.6 (**C8**) 64.9 (**C5**) 81.9 (**C4**) 87.1 (**C3**) 87.8 (**C2**) 103.6 (**C1**) 112.2 (**C6**).



$C_{11}H_{22}O_3Si$
Mol. Wt.: 230.38
C, 57.35; H, 9.63; O, 20.83; Si, 12.19

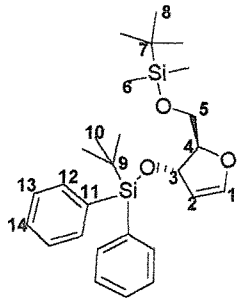
**1, 4-Anhydro-2-deoxy-5-O-[(1, 1-dimethylethyl)dimethylsilyl]-D-erythro-pent-1-enitol [100][239].**

To a solution of lactol **[99]** (4.874g, 16.0mmol) and carbon tetrachloride (1.64mL, 19.2mmol) in anhydrous THF (60mL) at –78°C was added under an argon atmosphere tris(dimethylamino)phosphine (3.05mL, 16.8mmol). After 45 minutes the reaction was allowed to warm to 0°C and then transferred to a stirred solution of lithium (1.332g, 0.19mol) in anhydrous liquid ammonia (300mL) at –78°C. Cooling was then discontinued and after 2.5 hours anhydrous ammonium chloride was added (12g, 0.22mol). The reaction mixture was then diluted slowly with ether (250mL) and the ammonia allowed to evaporate. The ethereal layer was dried ($Na_2SO_4$) and the solvent removed *in vacuo* to yield title compound as a colourless oil which required no further purification. This was dried in a vacuum desiccator over $P_2O_5$ for 24 hours (2.438g, 10.6mmol, 66%).

$R_f$ (P) 0.36; LRMS ($ES^-$ mode): *m/z* 343.3 $[MTFA]^-$.

$\delta_H(C_6D_6)$ 0.15 (6H, s, **H6**) 0.92 (9H, s, **H8**) 1.44 (1H, d, $J$ = 7.4 Hz, **OH**) 3.44 (1H, dd, $J$ = 11.0, 5.2 Hz, **H5**[a]) 3.56 (1H, dd, $J$ = 11.0, 5.2 Hz, **H5**[b]) 4.31 (1H, m, **H4**) 4.69 (1H, m, **H2**) 4.84 (1H, t, $J$ = 2.6 Hz, **H3**) 6.21 (1H, d, $J$ = 1.5 Hz, **H1**).

$\delta_C(C_6D_6)$ 18.6 (**C7**) 26.1 (**C8**) 26.9 (**C6**) 63.5 (**C5**) 75.7 (**C4**) 89.7 (**C3**) 103.8 (**C2**) 149.9 (**C1**).



$C_{27}H_{40}O_3Si_2$
Mol. Wt.: 468.78
C, 69.18; H, 8.60; O, 10.24; Si, 11.98

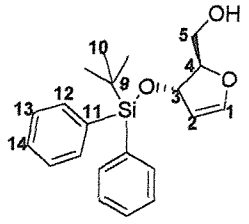
**1, 4-Anhydro-2-deoxy-5-O-[(1, 1-dimethylethyl)dimethylsilyl]-3-O-[(1, 1-dimethylethyl)diphenylsilyl]-D-erythro-pent-1-enitol [101][240].**

To a solution of compound **[100]** (1.496g, 6.51mmol) in anhydrous DMF (20mL) was added imidazole (1.108g, 16.3mmol) and *t*-butyldiphenylsilyl chloride (1.86mL, 7.15mmol) and the solution stirred under an argon atmosphere for 2 hours. The reaction mixture was then poured into ether (250mL) and washed with $H_2O$ (3 x 50mL) followed by brine (50mL). The organic phase was separated, dried ($Na_2SO_4$) and the solvent removed *in vacuo*. The crude product was purified by wet-flash column chromatography eluting with diethylether : petrol (1:10) to yield the title compound as a colourless oil which was dried in a vacuum desiccator over $P_2O_5$ for 24 hours (2.910g, 6.22mmol, 96%).

$R_f$ (P) 0.81.

$\delta_H(C_6D_6)$ -0.06 (6H, s, **H6**) 0.88 (9H, s, **H8**) 1.15 (9H, s, **H10**) 3.28-3.41 (2H, m, **H5**) 4.65 (1H, m, **H4**) 4.83 (1H, t, $J$ = 2.6 Hz, **H2**) 5.01 (1H, t, $J$ = 2.9 Hz, **H3**) 6.23 (1H, d, $J$ = 2.9 Hz, **H1**) 7.22-7.24 (6H, m, **H13+H14**) 7.71-7.81 (4H, m, **H12**).

$\delta_C(C_6D_6)$ 18.6 (**C7**) 19.5 (**C9**) 26.2 (**C8**) 26.9 (**C6**) 27.3 (**C10**) 63.4 (**C5**) 77.6 (**C4**) 89.8 (**C3**) 103.7 (**C2**) 128.1 (**C14**) 129.9 (**C13**) 134.8 (**C11**) 136.2 (**C12**) 149.8 (**C1**).



$C_{21}H_{26}O_3Si$
Mol. Wt.: 354.51
C, 71.15; H, 7.39; O, 13.54; Si, 7.92

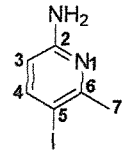
**1, 4-Anhydro-2-deoxy-3-O-[(1, 1- dimethylethyl)diphenylsilyl]-D-erythro-pent-1-enitol [102][240].**

To a stirred solution of compound **[101]** (1.046g, 2.24mmol) in anhydrous THF (10mL) at –22°C was added a 1M solution of tetra-*n*-butylammonium fluoride in THF (2.69mL, 2.69mmol). The resulting mixture was stirred at r.t. for 2 hours before addition of 1,2-dichloroethane (20mL). The volatiles were then removed *in vacuo* and the crude product was purified by wet-flash column chromatography eluting with ethylacetate in hexane (30%), producing the title compound as a colourless oil which was dried in a vacuum desiccator over $P_2O_5$ for 24 hours (0.301g, 0.85mmol, 38%).

$R_f$ (O) 0.81; LRMS (ES$^-$ *mode*): *m/z* 353.2 $[MH]^-$.

$\delta_H(C_6D_6)$ 1.13 (9H, s, **H10**) 3.42 (1H, dd, $J$ = 11.0, 5.5 Hz, **H5**$^a$) 3.54 (1H, dd, $J$ = 11.0, 5.5 Hz, **H5'**$^b$) 4.29 (1H, dt, J = 5.5, 3.0 Hz, **H4**) 4.53 (1H, t, $J$ = 2.6 Hz, **H2**) 4.91 (1H, t, $J$ = 2.9 Hz, **H3**) 6.13 (1H, d, $J$ = 2.9 Hz, **H1**) 7.22-7.24 (6H, m, **H13**+**H14**) 7.71-7.81 (4H, m, **H12**).

$\delta_C(C_6D_6)$ 19.6 (**C9**) 27.4 (**C10**) 63.0 (**C5**) 77.6 (**C4**) 90.2 (**C3**) 104.3 (**C2**) 128.9



$C_6H_7IN_2$
Mol. Wt.: 234.04
C, 30.79; H, 3.01; I, 54.22; N, 11.97

(**C14**) 130.4 (**C13**) 134.8 (**C11**) 136.6 (**C12**) 149.6 (**C1**).

**2-Amino-5-iodo-6-methyl pyridine [103]**

A mixture of 2-amino-6-picoline (5.499g, 50mmol), periodic acid dihydrate (2.281g, 10mmol) and iodine (5.111g, 20mmol) was heated in a mixed solution of acetic acid

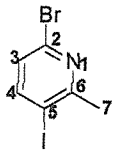
(30mL), $H_2O$ (6mL) and sulfuric acid (0.9mL) at 80°C for 4 hours. After this period, the reaction mixture was poured into a solution of 10% aq. $Na_2S_2O_3$ and extracted with diethylether. The organic extract was washed with 10% aq. NaOH, dried ($Na_2SO_4$) and concentrated *in vacuo*. The crude product was purified by wet flash column chromatography eluting with diethylether : DCM (1:1) producing an off-white waxy solid. This was crystallised from 50% aq. isopropanol producing the title compound as a colourless solid, which was dried in a vacuum desiccator over $P_2O_5$ for 24 hours (3.019g, 12.9mmol, 65%).

$R_f$ (N) 0.11; LRMS ($ES^+$ mode): *m/z* 235.0 $[MH]^+$; $\lambda_{max}$ (MeOH): 311, 247nm; mp: 95-96°C; Found C: 31.12, H: 2.94, N: 11.84, I: 54.39%, $C_6H_7IN_2$ requires C: 30.79, H: 3.01, N: 11.97, I: 54.22%

IR: $\nu$ = 3353.7 (m), 3206.6 (m), 1634.5 (m), 1573.0 (s), 1450.4 (s), 1396.0 (m), 1326.0 (m), 1017.0 (m), 993.0 (m), 805.3 (s) $cm^{-1}$.

$\delta_H(CDCl_3)$ 2.55 (3H, s, **H7**) 4.49 (2H, br, s, $\mathbf{NH_2}$) 6.12 (1H, d, $J$ = 8.8 Hz, **H3**) 7.69 (1H, d, $J$ = 8.8 Hz, **H4**).

$\delta_C(CDCl_3)$ 28.6 (**C7**) 80.8 (**C5**) 108.1 (**C3**) 147.6 (**C4**) 157.6 (**C2**) 158.3 (**C6**).




$C_6H_5BrIN$
*Mol. Wt.*: 297.92
C, 24.19; H, 1.69; Br, 26.82; I, 42.60; N, 4.70

**2-Bromo-5-iodo-6-methyl pyridine [104]**

To a solution of 48% hydrobromic acid (3.1mL, 57.1mmol) at –20°C was added portion-wise compound **[103]** (1.494g, 6.38mmol). While the temperature was maintained at 0°C or below, bromine (1mL, 19.4mmol) was added drop-wise. A solution of sodium nitrite (2.76g, 40.0mmol) in $H_2O$ (1.6mL) was added drop-wise over a period of 1 hour whilst maintaining the temperature of the reaction mixture at 0°C or below. After stirring for an additional 30 minutes a solution of NaOH (5.95g) in $H_2O$ (5.95mL) was added at such a rate that the temperature of the reaction mixture

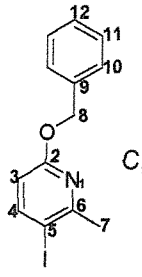
did not exceed 25°C. The reaction mixture was then extracted with diethylether (4 x 20ml), the organic layer separated, dried ($Na_2SO_4$) and concentrated *in vacuo*. The crude product was purified by wet-flash column chromatography eluting with diethylether : DCM (1:1) to yield the title compound as a deep red waxy solid which was dried in a vacuum desiccator over $P_2O_5$ for 24 hours (1.370g, 4.58mmol, 72%).

$R_f$ (N) 0.70; LRMS ($ES^+$ mode): *m/z* 317.3 $[MNH_4]^+$ ($^{81}Br$); $\lambda_{max}$ (MeOH): 283, 238nm; mp: 42-43°C; Found C: 24.26, H: 1.70, N: 4.70, $C_6H_5INBr$ requires C: 24.19, H: 1.69, N: 4.70%

IR: ν = 3074.1 (w), 15447.8 (m), 1525.0 (m), 1407.7 (s), 1234.6 (w), 1201.2 (m), 1135.7 (s), 1117.0 (s), 1006.6 (s), 827.3 (s) $cm^{-1}$.

$\delta_H(CDCl_3)$ 2.70 (3H, s, **H7**) 7.03 (1H, d, *J* = 8.1 Hz, **H3**) 7.84 (1H, d, *J* = 8.1 Hz, **H4**).

$\delta_C(CDCl_3)$ 28.8 (**C7**) 94.9 (**C5**) 126.9 (**C3**) 141.0 (**C4**) 148.4 (**C2**) 161.8 (**C6**).




$C_{13}H_{12}INO$
Mol. Wt.: 325.14
C, 48.02; H, 3.72; I, 39.03; N, 4.31; O, 4.92

**2-(Benzyloxy)-5-iodo-6-methyl pyridine [105]**

To a suspension of sodium hydride (50mg, 60%, 2.08mmol) in anhydrous DMF (1mL) at 0°C was added drop-wise a solution of compound **[104]** (250mg, 0.84mmol) in anhydrous DMF (1mL). Benzyl alcohol (0.104mL, 109mg, 1.00mmol) was then added drop-wise to the reaction mixture over a period of 10 minutes. The reaction mixture was allowed to warm to r.t. over a period of 3 hours and was then heated to 75°C for 16 hours. The resulting mixture was poured into aq. 1M HCl (15mL) and

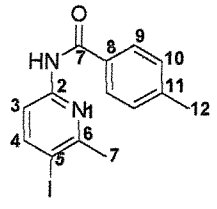
extracted with diethylether (4 x 20mL). The ethereal layers were combined, dried ($Na_2SO_4$) and concentrated *in vacuo* to yield the title compound as a yellow oil which required no further purification. This was dried in a vacuum desiccator over $P_2O_5$ for 24 hours (137mg, 0.42mmol, 50%).

$R_f$ (J) 0.77; LRMS ($ES^+$ mode): *m/z* 326 $[MH]^+$; $\lambda_{max}$ (MeOH): 286, 232nm; mp: 40-41°C; Found C: 77.93, H: 6.54, N: 7.01, $C_{13}H_{13}INO$ requires C: 78.36, H: 6.58, N: 7.03%

IR: ν = 3031.2 (w), 2947.1 (w), 1723.0 (w), 1565.4 (s), 1425.8 (s), 1295.1 (s), 1240.9 (s), 1026.8 (m), 985.9 (m), 815.5 (s), 725.8 (s) $cm^{-1}$.

$\delta_H(CDCl_3)$ 2.67 (3H, s, **H7**) 5.37 (2H, s, **H8**) 6.43 (1H, d, *J* = 8.8 Hz, **H3**) 7.41-7.48 (5H, m, **H10**+**H11**+**H12**) 7.91 (1H, d, *J* = 8.8 Hz, **H4**).

$\delta_C(CDCl_3)$ 28.7 (**C7**) 67.8 (**C8**) 85.0 (**C5**) 110.4 (**C3**) 128.1 (**C10**) 128.4 (**C12**) 128.6 (**C11**) 137.3 (**C9**) 148.4 (**C4**) 163.0 (**C6**) 174.1 (**C2**).




$C_{14}H_{13}IN_2O$
Mol. Wt.: 352.17
C, 47.75; H, 3.72; I, 36.03; N, 7.95; O, 4.54

**2-[*N*-*p*-toluoylamino]-5-iodo-6-methyl pyridine [106]**

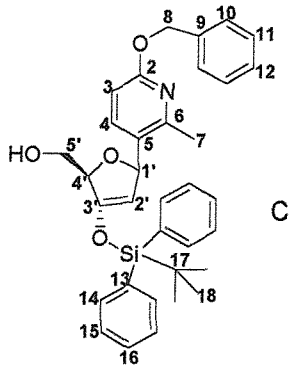
To a solution of compound **[103]** (217mg, 0.93mmol) in anhydrous pyridine (2mL) at 0°C was added slowly with stirring *p*-toluoylchloride (175mg, 0.15mL, 1.13mmol). The reaction mixture was allowed to warm to r.t. and stirred under an argon atmosphere for 18 hours. The resulting mixture was extracted with chloroform (2 x 40mL) and the chloroform solution washed with $H_2O$ (2 x 10mL), 10% aq. citric acid (2 x 10mL), sat. $NaHCO_3$ (2 x 10 mL) and then $H_2O$ (2 x 10mL). The organic phase was separated, dried ($Na_2SO_4$) and concentrated *in vacuo*. The crude product was purified by wet-flash column chromatography eluting with diethylether : DCM (1:1) to yield the title compound as a white solid which was dried in a vacuum desiccator over $P_2O_5$ for 24 hours (71.3mg, 0.20mmol, 22%).

$R_f$ (N) 0.64; LRMS ($ES^+$ mode): *m/z* 353 $[MH]^+$; $\lambda_{max}$ (MeOH): 285, 253nm; mp: 69-70°C; Found C: 58.05, H: 4.25, I: 25.91, N: 4.79%, $C_{14}H_{13}IN_2O$ requires C: 47.75, H: 3.72, I: 36.03, N: 7.95, %

IR: ν = 1773.5 (s), 1710.0 (s), 1607.5 (m), 1495.7 (m), 1290.7 (m), 1220.8 (s), 1207.6 (s), 1034.8 (s), 990.5 (s), 823.9 (m), 746.6 (s) $cm^{-1}$.

$\delta_H(CDCl_3)$ 2.42 (3H, s, **H12**) 2.61 (3H, s, **H7**) 6.94 (1H, d, *J* = 8.8 Hz, **H3**) 7.13 (2H, d, *J* = 8.1 Hz, **H11**) 7.57 (1H, d, *J* = 8.1 Hz, **H4**) 7.81 (2H, d, *J* = 8.1 Hz, **H10**).

$\delta_C(CDCl_3)$ 22.0 (**C13**) 28.5 (**C7**) 88.7 (**C5**) 113.1 (**C3**) 129.2 (**C10**) 129.6 (**C11**) 130.8 (**C9**) 148.5 (**C4**) 158.5 (**C2**) 162.7 (**C6**) 165.7 (**C8**).




$C_{34}H_{37}NO_4Si$
Mol. Wt.: 551.75
C, 74.01; H, 6.76; N, 2.54; O, 11.60; Si, 5.09

**2-Benzyloxy-5-([3'-[{(1,11-dimethylethyl)diphenylsilyl}-oxy]-β- D-glycero-pentofuran-3'-ulos-1'-yl]-6-methylpyridine [107]**

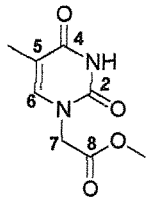
A mixture of bis(dibenzylideneacetone) palladium(0) (32mg, 0.035mmol) and 1, 3-bis (diphenylphosphino) propane (14mg, 0.035mmol) in dry acetonitrile (3mL) was stirred under an argon atmosphere for 25 minutes. This mixture was then transferred to a solution of compound **[102]** (134mg, 0.35mmol), compound **[105]** 228mg, 0.70mmol) and tri-*n*-butylamine (0.25mL, 1.05mmol) in dry acetonitrile (6mL) and stirred at 80°C under an argon atmosphere for 12 hours. The reaction mixture was then filtered with the aid of celite and the filtrate reduced in volume. The crude

residue was purified by wet-flash column chromatography eluting with diethylether : petroleum ether (1:2), producing the title compound as yellow foam which was dried in a vacuum desiccator over $P_2O_5$ for 24 hours (46mg, 0.08mmol, 24%).

$R_f$ (Q) 0.48; LRMS ($ES^-$ mode): *m/z* 550 $[MH]^-$.

$\delta_H(CDCl_3)$ 0.10 (9H, s, **H18**) 1.59 (1H, br, s, **OH**) 2.42 (3H, s, **H7**) 3.28 (2H, dd, *J* = 7.5, 2.5 Hz, H5') 4.78 (1H, t, *J* = 7.5 Hz, H4') 5.25 (2H, s, **H8**) 5.30 (1H, m, **H2'**) 6.55 (1H, d, *J* = 8.5 Hz, **H1'**) 6.62 (1H, d, *J* = 16.1 Hz, **H3**) 7.00-7.64 (15H, m, **H10+H11+H12+H14+H15+H16**) 7.68 (1H, d, *J* = 15.6 Hz, **H4**).

$\delta_C(CDCl_3)$ 19.5 (**C17**) 22.8 (**C18**) 27.0 (**C7**) 41.5 (**C4'**) 47.3 (**C5'**) 67.9 (**C8**) 108.4 (**C3**) 125.8 (**C16**) 128.1 (**C10**) 128.9 (**C12**) 129.4 (**C11**) 131.0 (**C15**) 135.2 (**C13**) 137.6 (**C9**) 143.8 (**C4**) 154.6 (**C6**) 161.7 (**C2**).




$C_8H_{10}N_2O_4$
Mol. Wt.: 198.18
C, 48.48; H, 5.09; N, 14.14; O, 32.29

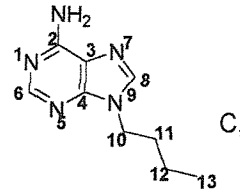
**Thymin-1-ylacetic acid methyl ester [109]**[261]

To a suspension of thymidin-1-yl acetic acid (1.117g, 6.07mmol) in anhydrous methanol (40mL) was added trimethylsilyl chloride (1.54mL, 12.1mmol) and the suspension stirred under an argon atmosphere for 12 hours. Filtration of the reaction mixture gave the title compound as a white solid which required no further purification. This was dried in a vacuum desiccator over $P_2O_5$ for 24 hours (0.986g, 4.98mmol, 82%).

$R_f$ (F) 0.69; LRMS ($ES^+$ mode): *m/z* 397.4 $[2MH]^+$; mp: 190-191°C.

$\delta_H(CDCl_3)$ 1.95 (3H, s, $CH_3$) 3.82 (3H, s, $OCH_3$) 4.46 (2H, s, **H7**) 6.95 (1H, s, **H6**) 8.57 (1H, br, s, **NH**).

$\delta_C(CDCl_3)$ 10.5 (**C**$H_3$) 47.0 (**C7**) 50.9 (**O**$CH_3$) 107.3 (**C5**) 140.2 (**C6**) 149.5 (**C2**) 163.0 (**C4**) 167.4 (**C8**).




$C_9H_{13}N_5$
Mol. Wt.: 191.23
C, 56.53; H, 6.85; N, 36.62

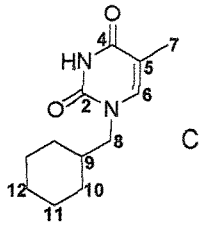
**_N_9-butyl adenine [110]**[262]

To a stirred suspension of adenine (10.374g, 76.8mmol) and sodium hydride (2.212g, 88.3mmol) in anhydrous DMF (200mL) was added 1-bromobutane (9.07mL, 84.5mmol) and the reaction mixture stirred under an argon atmosphere for 5 hours. The reaction mixture was then filtered with the aid of celite and the filtrate reduced in volume. Crystallisation form ethanol afforded the title compound as a colourless crystalline solid which was dried in a vacuum desiccator over $P_2O_5$ for 24 hours (4.616g, 24.2mmol, 32%).

$R_f$ (F) 0.72; LRMS ($ES^+$ mode): *m/z* 192.2 $[MH]^+$; mp: 136°C (Lit. mp; 135-136°C).

$\delta_H(CDCl_3)$ 0.90 (3H, t, $J$ = 7.3 Hz, **H13**) 1.30 (2H, dt, $J$ = 22.6, 7.3 Hz, **H12**) 1.77-1.89 (2H, m, **H11**) 4.13 (2H, t, $J$ = 7.3 Hz, **H10**) 6.31 (2H, br, s, **N**$H_2$) 7.73 (1H, s, **H2**) 8.30 (1H, s, **H8**).

$\delta_C(CDCl_3)$ 14.2 (**C13**) 20.4 (**C12**) 32.8 (**C11**) 44.4 (**C10**) 120.4 (**C3**) 141.0 (**C8**) 150.8 (**C2**) 153.6 (**C6**) 156.5 (**C4**).



$C_{12}H_{18}N_2O_2$
Mol. Wt.: 222.28
C, 64.84; H, 8.16; N, 12.60; O, 14.40

**_N_1-cyclohexylmethylthymine [111]**

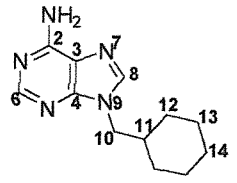
A suspension of thymine (2.645g, 21.0mmol) and potassium carbonate (2.902g, 21.0mmol) in anhydrous DMSO (25mL) was stirred under an argon atmosphere at r.t. for 1 hour before addition of bromomethylcyclohexane (0.97mL, 1.231g, 6.99mmol). The reaction mixture was then heated to 70°C for 3 hours before being allowed to cool to r.t. and stirred under an argon atmosphere for a further 14 hours. The reaction mixture was then filtered, the filtrate diluted with $H_2O$ (100mL) and extracted with DCM (4 x 50mL). The organic layer was then washed with $H_2O$ (3 x 50mL), separated, dried ($Na_2SO_4$) and the solvent removed *in vacuo*. The crude product was purified by wet-flash column chromatography eluting with a gradient of methanol in DCM (0-5%) to give the title compound as a white micro-crystalline solid which was dried in a vacuum desiccator over $P_2O_5$ for 24 hours (1.494g, 6.72mmol, 32%).

$R_f$(G) 0.59; LRMS ($ES^+$ mode): *m/z* 223.1 $[MH]^+$ 445.4 $[2MH]^+$; mp: 236-237°C; Found C: 70.89, H: 8.21, N: 6.41, $C_{13}H_{18}NO_2$ requires C: 70.88, H: 8.24, N: 6.36%

IR: $\nu$ = 2933.5 (m), 2855.2 (w), 2372.8 (w), 1678.7 (s), 1635.2 (s), 1476.4 (m), 1427.1 (m), 1353.9 (m), 1257.2 (m), 1222.8 (m), 949.1 (m) $cm^{-1}$.

$\delta_H$($CDCl_3$) 0.85-0.94 (2H, m, **H10$^a$**) 1.08-1.21 (3H, m, **H10$^e$**+**H12$^a$**) 1.60-1.67 (6H, m, **H9**+**H11**+**H12$^e$**) 1.85 (3H, s, **H7**) 3.45 (2H, d, *J* = 7.5 Hz, **H8**) 6.87 (1H, s, **H6**) 9.10 (1H, br, s, **NH**).

$\delta_C$($CDCl_3$) 20.0 (**C7**) 33.2 (**C10**) 33.8 (**C11**) 38.1 (**C12**) 45.0 (**C9**) 62.2 (**C8**) 117.7 (**C5**) 148.7 (**C6**) 158.8 (**C2**) 172.1 (**C4**).



$C_{12}H_{17}N_5$
Mol. Wt.: 231.30
C, 62.31; H, 7.41; N, 30.28

**N9-cyclohexylmethyladenine [112]**

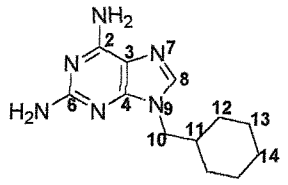
A suspension of adenine (10.134g, 75.0mmol) and sodium hydride (3.546g, 147.8mmol) in anhydrous DMF (300mL) was stirred under an argon atmosphere at r.t. for 1hour before addition of bromomethylcyclohexane (9.51mL, 12.068g, 68.2mmol). The reaction mixture was then heated to 70°C for 3 hours before being allowed to cool to r.t. and stirred under an argon atmosphere for a further 14 hours. The reaction mixture was then filtered through celite, the filtrate diluted with $H_2O$ (200mL) and extracted with DCM (4 x 200mL). The organic layer was then washed with $H_2O$ (2 x 200mL), separated, dried ($Na_2SO_4$) and the solvent removed *in vacuo*. The crude product was purified by wet-flash column chromatography eluting with a gradient of methanol in DCM (0-6%) to give the title compound as a white micro-crystalline solid which was dried in a vacuum desiccator over $P_2O_5$ for 24 hours (7.962g, 34.5mmol, 46%).

$R_f$(G) 0.50; LRMS ($ES^+$ mode): *m/z* 232.1 $[MH]^+$; mp: 215-216°C; Found C: 62.03, H: 7.33, N: 30.03, $C_{12}H_{17}N_5$ requires C: 62.31, H: 7.41, N: 30.26%

IR: $\nu$ = 3143.7 (w), 2902.5 (w), 2358.6 (w), 2325.5 (w), 1735.9 (m), 1650.4 (s), 1597.5 (s), 1573.9 (m), 1483.7 (m), 1415.7 (m), 1311.3 (m), 1237.1 (m) $cm^{-1}$.

$\delta_H$($CDCl_3$) 0.89-0.98 (2H, m, **H12$^a$**) 1.03-1.20 (3H, m, **H12$^e$**+**H14$^a$**) 1.56-1.68 (5H, m, **H13**+**H14$^e$**) 1.84 (1H, ddt, J = 22.3, 11.0, 3.5 Hz, **H11**) 3.96 (2H, d, J = 7.0 Hz, **H10**) 5.75 (2H, br, s, **$NH_2$**) 7.68 (1H, s, **H2**) 8.55 (1H, s, **H8**).

$\delta_C$($CDCl_3$) 33.2 (**C12**) 33.8 (**C13**) 38.3 (**C14**) 45.9 (**C11**) 57.7 (**C10**) 127.3 (**C3**) 148.6 (**C8**) 158.0 (**C4**) 160.7 (**C6**) 163.2 (**C2**).



$C_{12}H_{18}N_6$
*Mol. Wt.*: 246.31
C, 58.51; H, 7.37; N, 34.12

**_N_9-cyclohexylmethyl-2,6-diaminopurine [113]**

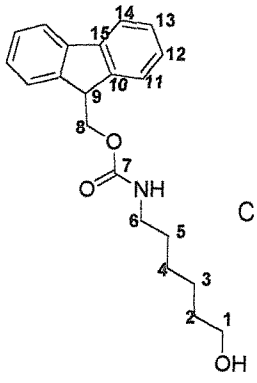
A suspension of 2,6-diaminopurine (6.092g, 40.6mmol) and sodium hydride (1.919g, 79.8mmol) in anhydrous DMF (150mL) was stirred under an argon atmosphere at r.t. for 1 hour before addition of bromomethylcyclohexane (5.15mL, 6.535g, 68.2mmol). The reaction mixture was then heated to 70°C for 3 hours before being allowed to cool to r.t. and stirred under an argon atmosphere for a further 14 hours. The reaction mixture was then filtered through celite, the filtrate diluted with $H_2O$ (200mL) and extracted with DCM (4 x 200mL). The organic layer was then washed with $H_2O$ (2 x 200mL), separated, dried ($Na_2SO_4$) and the solvent removed *in vacuo*. The crude product was purified by wet-flash column chromatography eluting with a gradient of methanol in DCM (0-10%) to give the title compound as a white foam which was dried in a vacuum desiccator over $P_2O_5$ for 24 hours (3.491g, 14.2mmol, 35%).

$R_f$(G) 0.36; LRMS ($ES^+$ mode): *m/z* 247.1 $[MH]^+$; mp: 218°C; Found C: 58.00, H: 7.44, N: 33.55, $C_{12}H_{18}N_6$ requires C: 58.52, H: 7.37, N: 34.10%

IR: ν = 3162.6 (w), 2907.2 (w), 1664.1 (m), 1624.4 (m), 1589.3 (s), 1406.1 (m), 1216.4 (m) $cm^{-1}$.

$\delta_H$($CDCl_3$) 0.86-0.95 (2H, m, **H12$^a$**) 1.03-1.22 (3H, m, **H12$^e$**+**H14$^a$**) 1.56-1.74 (5H, m, **H13**+**H14$^e$**) 1.80 (1H, ddt, J = 22.3, 11.0, 3.5 Hz, **H11**) 3.78 (2H, d, J = 7.5 Hz, **H10**) 4.51 (2H, br, s, **$NH_2$**) 5.23 (2H, br, s, **$NH_2$**) 7.18 (1H, s, **H8**).

$\delta_C$($CDCl_3$) 33.2 (**C12**) 33.9 (**C13**) 38.3 (**C14**) 45.7 (**C11**) 57.2 (**C10**) 127.3 (**C3**) 146.4 (**C8**) 158.5 (**C4**) 161.7 (**C6**) 163.2 (**C2**).




$C_{21}H_{25}NO_3$
Mol. Wt.: 339.43
C, 74.31; H, 7.42; N, 4.13; O, 14.14

**6-(9-Fluorenylmethoxycarbonyl)aminohexanol [114]**

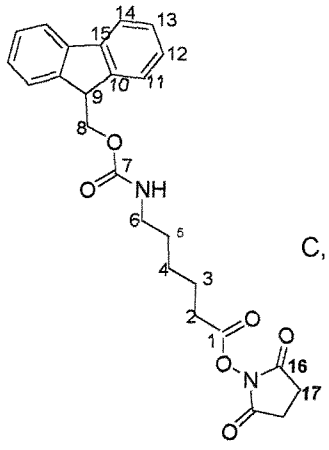
To a solution of 6-amino-1-hexanol (1.007g, 8.61mmol, 1.5eq) in anhydrous dioxan (20mL) was added drop-wise a solution of *N*-(9-fluorenylmethoxycarbonyloxy)succinimide (0.435g, 1.29mmol, 1.0eq) in anhydrous dioxan (10mL). After 5 minutes the reaction mixture was filtered, the filtrate diluted with dichloromethane (100mL), washed with 10% citric acid (2 x 25mL), sat. KCl (2 x 30mL) and sat. $NaHCO_3$ (2 x 30mL). The organic phase was separated, dried ($Na_2SO_4$) and concentrated *in vacuo* to yield a cream coloured solid that required no further purification. The product was dried in a vacuum desiccator over $P_2O_5$ for 24 hours (1.693g, 4.99mmol, 87%).

$R_f$(D) 0.77; LRMS ($ES^+$ mode): *m/z* 340.3 $[MH]^+$ 357.4 $[MNH_4]^+$ 362.4 $[MNa]^+$ 378.3 $[MK]^+$; $\lambda_{max}$ (MeOH): 300, 289, 262nm; mp: 116°C; Found C: 73.94, H: 7.32, N: 3.85, $C_{21}H_{25}NO_3$ requires C: 74.31, H: 7.42, N: 4.12%

IR: ν = 3337.6 (m), 2935.6 (w), 2358.6 (m), 1737.6 (m), 1686.9 (s), 1527.5 (s,) 1248.9 (s) $cm^{-1}$.

$\delta_H$(DMSO) 1.25-1.32 (4H, m, **H3**+**H4**) 1.38-1.43 (4H, m, **H2**+**H5**) 2.92 (2H, d, *J* = 5.2 Hz, **H6**) 3.38 (2H, t, *J* = 6.3 Hz, **H1**) 4.20 (2H, t, *J* = 7.0 Hz, **H10**) 4.39 (2H, d, *J* = 6.6 Hz, **H8**) 7.38 (4H, , ddd, *J* = 22.1, 14.7, 7.3 Hz, **H12**+**H13**) 7.86 (4H, dd, *J* = 14.7, 7.4 Hz, **H11**+**H14**).

$\delta_C$(DMSO) 25.4 (**C3**) 26.3 (**C4**) 29.9 (**C5**) 32.6 (**C2**) 40.3 (**C6**) 60.7 (**C1**) 109.8 (**C8**) 120.1 (**C13**) 121.5 (**C14**) 127.4 (**C12**) 129.0 (**C11**) 137.5 (**C15**) 139.5 (**C9**) 142.7 (**C10**) 157.7 (**C7**).



$C_{26}H_{30}N_2O_6$
Mol. Wt.: 466.53
C, 66.94; H, 6.48; N, 6.00; O, 20.58

**6-(9-Fluorenylmethoxycarbonylamino)hexanoic acid *N*-hydroxysuccinimyl ester [115]**

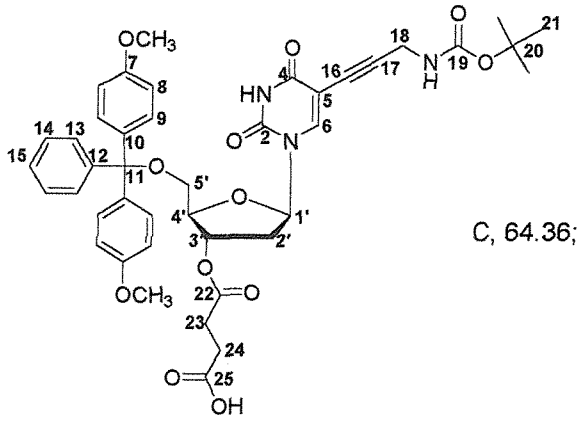
To a solution of compound **[22]** (337mg, 0.95mmol) in anhydrous DMF (8mL) was added *N*-hydroxysuccinimide (131mg, 1.14mmol, 1.2eq) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (274mg, 1.43mmol, 1.5eq) and the solution stirred under an argon atmosphere for 24 hours. The solvent was removed *in vacuo*, the residue dissolved in dichloromethane (200mL) and washed with sat. KCl (3 x 50mL) followed by brine (40mL). The organic phase was separated, dried ($Na_2SO_4$) and concentrated *in vacuo*. The crude product was purified by wet flash column chromatography eluting with ethylacetate in hexane (60%) to yield the title compound as a white solid which was dried in a vacuum desiccator over $P_2O_5$ for 24 hours (232mg, 0.52mmol, 54%).

$R_f$ (L) 0.25; LRMS ($ES^+$ mode): *m/z* 467.3 $[MH]^+$; $\lambda_{max}$ (MeOH): 300, 289, 265nm; mp: 124°C.

*IR*: ν = 2364.2 (m), 1738.9 (s), 1694.0 (m), 1521.3 (m), 1369.0 (m), 1208.9 (s) $cm^{-1}$.

$\delta_H(CDCl_3)$ 1.37-1.59 (4H, m, **H4** + **H3** ) 1.79 (2H, dt, $J$ = 14.7, 7.4 Hz, **H5**) 2.63 (2H, t, $J$ = 7.4 Hz, **H2**) 2.82 (4H, s, **H17**) 3.23 (2H, dd, $J$ = 12.5, 6.6 Hz, **H6**) 4.23 (1H, t, $J$ = 7.0 Hz, **H9**) 4.41 (2H, d, $J$ = 6.6 Hz, **H8**) 4.99 (1H, s, br, **NH**) 7.33 (2H, t, $J$ = 7.4 Hz, **H12**) 7.42 (2H, t, $J$ = 7.4 Hz, **H13**) 7.62 (2H, d, $J$ = 7.4 Hz, **H11**) 7.78 (2H, d, $J$ = 8.1 Hz, **H14**).

$\delta_C(CDCl_3)$ 24.4 (**C4**) 25.7 (**C17**) 25.8 (**C3**) 29.5 (**C5**) 31.0 (**C2**) 40.7 (**C6**) 47.5 (**C9**) 66.6 (**C8**) 120.1 (**C13**) 125.3 (**C14**) 127.2 (**C12**) 127.8 (**C11**) 141.5 (**C15**) 144.2 (**C10**) 156.6 (**C7**) 168.7 (**C16**) 169.4 (**C1**).



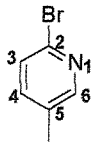

$C_{42}H_{45}N_3O_{12}$
Mol. Wt.: 783.82
C, 64.36; H, 5.79; N, 5.36; O, 24.49

**5-[*N*-(*t*-butoxycarbonyl)propargylamine-5'-O-(4, 4'-dimethoxytrityl)]- 2'-deoxyuridine-3'-O-succinate [116]**

To a solution of compound **[18]** (215mg, 0.31mmol) in anhydrous pyridine (3mL) was added a solution of 4-dimethylaminopyridine (4mg, 0.03mmol, 0.1eq) and succinic anhydride (62mg, 0.62mmol, 2.0eq) in anhydrous pyridine (1mL) and the solution stirred under an argon atmosphere for 24 hours. The solvent was removed *in vacuo*, the residue dissolved in dichloromethane (75ml) and washed with 10% citric acid (20mL) and sat. KCl (2 x 20mL). The organic phase was separated, dried ($Na_2SO_4$) and concentrated *in vacuo*. The crude product was purified by wet flash column chromatography (silica pre-equilibrated with 1% $Et_3N$) eluting with methanol in dichloromethane (0-10%) to yield the title compound as a white foam which was dried in a vacuum desiccator over $P_2O_5$ for 24 hours (214mg, 0.27mmol, 88%).

$R_f$ (G) 0.35; LRMS ($ES^+$ mode): *m/z* 806.5 $[MNa]^+$; $\lambda_{max}$ (MeOH) 280nm.

$\delta_H(CDCl_3)$ 1.39 (9H, s, **H21**) 2.40 (1H, m, **H2'$^b$**) 2.56-2.60 (3H, m, **H2'$^a$+H23+H24**) 3.32 (1H, d, *J* = 9.6 Hz, **H5'$^a$**) 3.46 (1H, d, *J* = 8.1 Hz, **H5'$^b$**) 3.79 (6H, s, **$OCH_3$**) 4.21 (2H, s, **H18**) 4.41 (1H, m, **H4'**) 5.49 (1H, d, *J* = 5.2 Hz, **H3'**) 6.35 (1H, dd, *J* = 8.1, 5.9 Hz, **H1'**) 6.85 (4H, d, *J* = 8.8 Hz, **H9**) 7.19-7.35 (7H, m, **H8+H14+H15**) 7.43 (2H, d, *J* = 7.4 Hz, **H13**) 8.23 (1H, s, **H6**).
$\delta_C(CDCl_3)$ 28.5 (**C21**) 30.9 (**C18**) 31.2 (**C23**) 31.8 (**C24**) 38.9 (**C2'**) 55.4 (**$OCH_3$**) 63.9 (**C5'**) 74.1 (**C11**) 75.0 (**C4'**) 79.8 (**C8**) 84.6 (**C3'**) 85.4 (**C1'**) 87.3 (**C20**) 89.7 (**C17**) 100.1 (**C5**) 113.5 (**C8**) 127.1 (**C15**) 128.0 (**C13**) 128.2 (**C14**) 130.1 (**C9**) 135.5 (**C10**) 142.9 (**C6**) 144.8 (**C12**) 158.7 (**C19**) 173.4 (**C25**).




$C_6H_6BrN$
Mol. Wt.: 172.02
C, 41.89; H, 3.52; Br, 46.45; N, 8.14

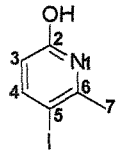
**2-Bromo-5-methyl pyridine [117][260].**

To a solution of 48% hydrobromic acid (16.5mL, 303.8mmol) at –20°C was added portion-wise 2-amino-5-methyl pyridine (7.453g, 69.0mmol). While the temperature was maintained at 0°C or below, bromine (10.7mL, 207.7mmol) was added drop-wise. A solution of sodium nitrite (29.7g, 430.4mmol) in $H_2O$ (25mL) was added drop-wise over a period of 1 hour whilst maintaining the temperature of the reaction mixture at 0°C or below. After stirring for an additional 30 minutes a solution of NaOH (64g) in $H_2O$ (64mL) was added at such a rate that the temperature of the reaction mixture did not exceed 25°C. The reaction mixture was then extracted with diethylether (4 x 100mL), the organic phase separated, dried ($Na_2SO_4$) and concentrated *in vacuo*. The crude product was purified by wet-flash column chromatography eluting with diethylether : DCM (1:1) to yield the title compound as a deep red waxy solid which was dried in a vacuum desiccator over $P_2O_5$ for 24 hours (9.726g, 56.9mmol, 82%).

$R_f$ (N) 0.77; LRMS ($ES^+$ mode): *m/z* 172.0 $[MH]^+$ ($^{79}Br$) 174.0 $[MH]^+$ ($^{81}Br$).

$\delta_H(CDCl_3)$ 2.22 (3H, s, **$CH_3$**) 7.29 (2H, d, $J$ = 1.5 Hz, **H3+H4**) 8.12 (1H, s, **H6**).

$\delta_C(CDCl_3)$ 18.1 (**$CH_3$**) 127.9 (**C3**) 132.9 (**C5**) 139.4 (**C2**) 139.7 (**C4**) 150.8 (**C6**).




$C_6H_6INO$
Mol. Wt.: 235.02
C, 30.66; H, 2.57; I, 54.00; N, 5.96; O, 6.81

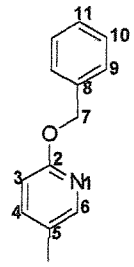
**2-Hydroxy-5-iodo-6-methyl pyridine [118]**

To a solution of 2-hydroxy-6-methylpyridine (2.003g, 18.4mmol) in methanol (50mL) was added sodium iodide (2.758g, 18.4mmol) and sodium hydroxide (0.736g, 18.4mmol). The solution was cooled to between 0-3°C and sodium hypochlorite (9.46mL) was added over a period of 75 minutes. The reaction mixture was stirred at this temperature for 2 hours before being warmed to r.t. and stirred for a further 12 hours. The reaction mixture was then adjusted to pH7 with 2M HCl whereby the product precipitated. The reaction mixture was filtered and the solid washed with diethylether to give the title compound as a yellow solid which was dried in a vacuum desiccator over $P_2O_5$ for 12 hours (3.847g, 16.3mmol, 89%).

$R_f$ (N) 0.46; LRMS ($ES^+$ mode): *m/z* 236.8 $[MH]^+$; mp: 188-189°C; Found C: 30.93, H: 2.62, N: 5.95, $C_6H_6INO$ requires C: 30.66, H: 2.57, N: 5.96%

$\delta_H(CDCl_3)$ 2.22 (3H, s, **$CH_3$**) 7.29 (1H, d, **H3**) 8.12 (1H, d, **H4**).

$\delta_C(CDCl_3)$ 24.2 (**$CH_3$**) 70.3 (**C5**) 118.6 (**C3**) 147.8 (**C6**) 150.7 (**C4**) 165.5 (**C2**).




$C_{13}H_{13}NO$
Mol. Wt.: 199.25
C, 78.36; H, 6.58; N, 7.03; O, 8.03

**2-(Benzyoxy)-5-methyl pyridine [119]**

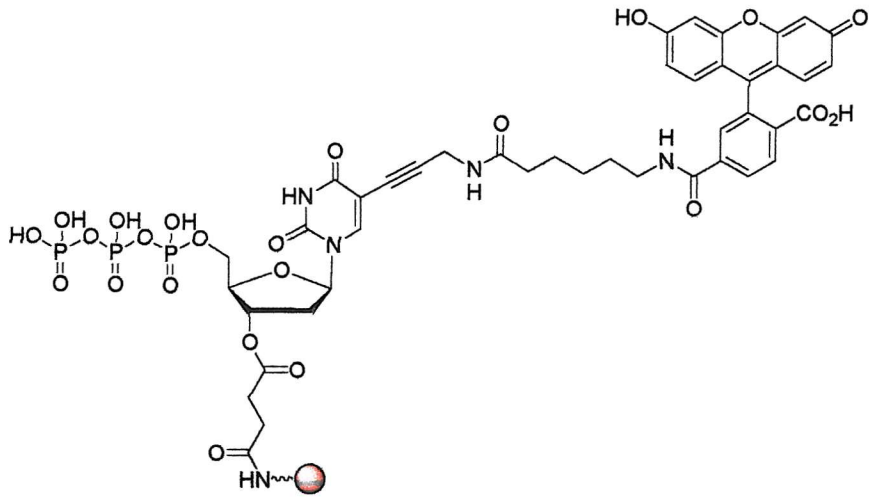
To a suspension of sodium hydride (4.892g, 60%, 122.3mmol) in anhydrous DMF (30mL) at 0°C was added drop-wise a solution of compound **[117]** (9.726g, 48.9mmol) in anhydrous DMF (6mL). Benzyl alcohol (6.07mL, 6.348g, 58.7mmol) was then added drop-wise to the reaction mixture over a period of 10 minutes. The reaction mixture was allowed to warm to r.t. over a period of 3 hours and was then heated to 75°C for 16 hours. The resulting mixture was poured into aq. 1M HCl (150mL) and extracted with diethylether (4 x 200mL). The ethereal layers were combined, dried ($Na_2SO_4$) and concentrated *in vacuo*. The crude product was purified by wet-flash column chromatography eluting with petroleum ether : DCM (1:4) to yield the title compound as a colourless solid which was dried in a vacuum desiccator over $P_2O_5$ for 24 hours (6.033g, 30.3mmol, 62%).

$R_f$ *(J) 0.59; LRMS (ES$^+$ mode): m/z 200.2* $[MH]^+$; $\lambda_{max}$ *(MeOH): 286, 232nm; mp:* 29-30°C; Found C: 77.59, H: 6.52, N: 6.83, $C_{13}H_{13}NO$ requires C: 78.36, H: 6.58, N: 7.03%

IR: ν = 3031.2 (w), 2947.1 (w), 1723.0 (w), 1565.4 (s), 1425.8 (s), 1295.1 (s), 1240.9 (s), 1026.8 (m), 985.9 (m), 815.5 (s), 725.8 (s) $cm^{-1}$.

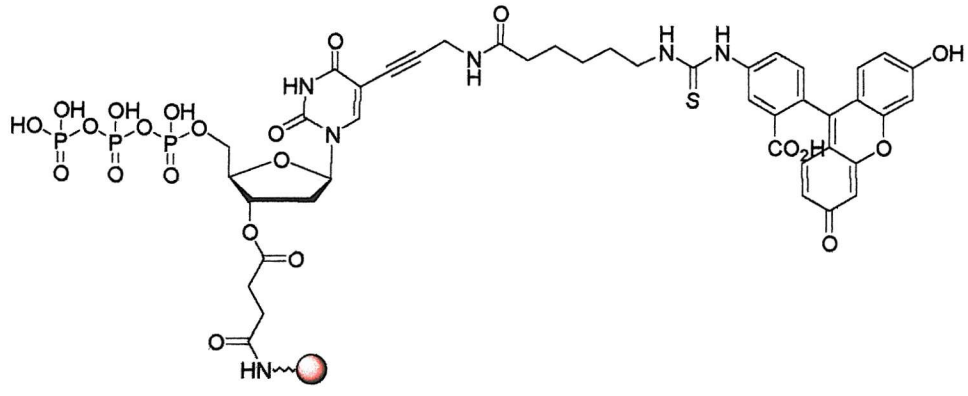
$\delta_H(CDCl_3)$ 2.67 (3H, s, **$CH_3$**) 5.37 (2H, s, **H7**) 6.43 (1H, d, *J* = 8.8 Hz, **H3**) 7.41-7.48 (6H, m, **H6+H9+H10+H11**) 7.91 (1H, d, *J* = 8.8 Hz, **H4**).

$\delta_C(CDCl_3)$ 17.8 (**$CH_3$**) 67.9 (**C7**) 111.1 (**C3**) 126.2 (**C5**) 128.1 (**C9**) 128.3 (**C11**) 128.8 (**C10**) 138.0 (**C8**) 140.1 (**C4**) 146.6 (**C6**) 162.3 (**C2**).



**5-[6-(Fluoresceinamido)hexanoyl-3-propargylamino]-2'-deoxyuridine-5'-triphosphate-3'-O-succinamide resins [120]**

The triphosphate CPG resin [38] (95mg, 2.0μmol) was treated using general method 6.1.2.3.



**5-[6-(Fluoresceinurea)hexanoyl-3-propargylamino]-2'-deoxyuridine-5'-triphosphate-3'-O-succinamide resins [121**

*The triphosphate CPG-resin [38] (100mg, 2.1μmol) was treated with a solution of* 20% piperidine in DMF for 10 minutes before being filtered and washed extensively with DMF (x 3) and DCM (x 5) and dried *in vacuo*. The resin was then treated with a solution of fluoresceinisothiocyanate (10 eq) in the minimum volume of anhydrous pyridine for 2 hours before being filtered and washed with pyridine (x 3), DMF (x 3) and acetonitrile (x 5). The resin was dried over $P_2O_5$ *in vacuo* for 12 hours.

**Tetrakistriphenylphosphine palladium (0)**[265-269]

Palladium chloride (1.014g, 5.72mmol) and triphenylphosphine (7.674g) were suspended in anhydrous DMSO (70mL) and heated to an internal temperature of 140°C for 1 hour. The heat was removed and to the solution was added hydrazine monohydrate (1.2mL, 24.7mmol) causing precipitation to occur. The reaction mixture was protected from the light and allowed to cool to r.t. The reaction mixture was then filtered and the solid washed with methanol and diethyl ether producing the title compound as a yellow crystalline solid which was dried in a vacuum desiccator over $P_2O_5$ for 24 hours (6.444g, 5.58mmol, 97%).

**Salicylphosphochloridite**[169]

Salicylic acid (5.416g, 39.2mmol) was suspended in anhydrous toluene (10mL) and to this was added phosphorus trichloride (3.42mL, 39.2mmol). The reaction mixture was heated at reflux for 3 hours and purified by distillation at reduced pressure (7mm Hg, 123°C) producing a colourless liquid. On cooling the title compound was produced as a waxy solid which was dried in a vacuum desiccator over $P_2O_5$ for 12 hours (6.020g, 29.8mmol, 76%).

$\delta_P(CDCl_3)$ 149.0

## 6.2 Preparation of Synthetic oligonucleotides

### 6.2.1 General methods

All oligonucleotides were synthesised on an Applied Biosystems 394 solid phase DNA/RNA synthesiser using the standard assembly cycle of acid catalysed detritylation, coupling, capping and iodine oxidation procedures. Oligonucleotides

were assembled on a 0.2μmol or 1.0μmol scale, stepwise coupling efficiencies and overall yields were calculated using automated trityl cation conductivity monitoring. DNA phosphoramidites, solid supports and additional reagents were purchased from Applied Biosystems, unless otherwise stated.

Cleavage of the oligonucleotides from the solid support was achieved by exposure to concentrated ammonia, unless otherwise stated. All oligonucleotides requiring base deprotection were placed in concentrated ammonia or ammonia : ethanol (3:1) at 60°C for 4 hours and evaporated to dryness *in vacuo*.

Purification was effected using reversed phase HPLC on a Gilson HPLC system, controlled by Gilson 7.12 software. Elution of oligonucleotides was monitored by UV detection at the appropriate wavelength. Buffer systems consisted of buffer A: 0.1M ammonium acetate, buffer $B_1$: 0.1M ammonium acetate with 30% acetonitrile or buffer $B_2$: 0.1M ammonium acetate with 40% acetonitrile with a typical gradient programme as described in figure 6.2. Analytical injections were monitored at 265nm, 0.1 AUFS, preparative injections were monitored at 280-290nm, 1.0 AUFS. The oligonucleotides were collected manually.

The resins were packaged into Glen research columns suitable for an ABI 394 DNA synthesiser. The amount of resin used was determined by the resin loading and the scale of the synthesis (0.2μmol or 1.0μmol).

| Time (minutes) | % Buffer B | Flow (mL/min) |
|---|---|---|
| 0 | 0 | 4 |
| 2 | 0 | 4 |
| 15 | 70 | 4 |
| 17 | 100 | 4 |
| 20 | 100 | 4 |
| 22 | 0 | 4 |
| 22.9 | 0 | 4 |
| 23 | 0 | 0 |

Figure 6.2 - Typical RP HPLC elution gradient.

### 6.2.2 Oligonucleotide sequences

Modifications:

F = fluorescein phosphoramidite, H = hexaethylene glycol phosphoramidite

| No | Sequence 5'- to 3'- | Molecular Ion | |
|---|---|---|---|
| | | Expected | Found |
| 120 | GGTTATGTGTCACGTGCA | 5545.7 | 5549.3 |
| 121 | GAATCCGAGCATCCTACC | 5443.6 | 5450.1 |
| 122 | GAATTCGAGCATCCTACCCACGATTCCGCTACGAT CAACTAATACACTTGGTATGAAACAATTTCCTCCCA CTCACCCTGGCCCTATGCACGTGACACATAACC | 31654.6 | 31660.1 |
| 123 | *FTACACTTGGTATGAAACAATTH* | 6965.8 | * |
| 124 | CAAGGTCATCCATGACAACTTTG | 7007.3 | 7009.0 |
| 125 | GGGCCATCCACAGTCTTCTG | 6068.7 | 6065.9 |
| 126 | Range of human cDNA | - | - |
| 127 | FCTCATGACCACAGTCCATGCCATCACTH | 8661.8 | - |

Figure 6.3 – Oligonucleotide sequences used in molecular biology experiments (* indicates molecular ion not determined)

## 6.3 Molecular Biology

### 6.3.1 General Methods

Taq DNA polymerase, dATP, dGTP, dCTP, dTTP were purchased from Pharmacia Biotech. Oligonucleotide molecular weight size markers (8-587 base pairs) were purchased from Roche. $Vent_R$ $exo^-$ polymerase was purchased from New England Biolabs Inc. All other molecular biological grade reagents were obtained from Sigma/Aldrich. NAP 10 Sephadex columns for desalting oligonucleotides were purchased from Pharmacia Biotech. Deionised water was used in all experiments.

Primer and template oligonucleotides were synthesised as detailed in section 6.3.2. Human heart total RNA was obtained from Ambion and reversed transcribed using random monomers from a kit supplied from Eurogentec. This produced a pool of cDNA wich was used for PCR experiments on an ABI 7700 Thermal Cycler.

Fluorescent spectra were measured on a Perkin Elmer LS50B Luminescence Spectrometer using quartz glass 0.4mL and quartz suprasil ultra-micro cell 100μL cells.

Irradiation of samples was performed on a transilluminator platform at 254nm and photographed with a Fotodyne UV 'Instant Image' Polaroid HP-4 camera with black and white polaroid film.

Agarose gel electrophoresis was performed using a Hoefer 250 power supply and mini gel equipment.

PCR experiments were performed using either a Perkin Elmer GeneAmp 2400 Thermal Cycler, a Roche Lightcycler or an ABI 7700 Thermal Cycler.

Centrifugation was performed in a microcentrifuge at 13,000rpm.

## 6.3.2 Experimental Procedures

**Preparation of oligonucleotides for electrospray mass spectrometry**

The oligonucleotide sample was dissolved in water (1.0mL), loaded onto a NAP 10 sephadex column and eluted with $H_20$ (1.5mL). The water was removed *in vacuo* and the sample was dissolved in a solution of water : isopropanol (1:1) containing tripropylamine (0.2%) for electrospray mass spectral analysis.

## PCR Reactions on a Perkin Elmer GeneAmp 2400 Thermal Cycler

*General Protocol*

PCR primers, (5μL of 5μM), template (10μL of 12.6nM), $Mg^{2+}$ ions (3μL of 25mM), the DNA polymerase (0.2μL of 5 units $\mu L^{-1}$) and dATP : dCTP : dGTP solution (1μL of 10mM of each dNTP) were combined with PCR buffer (5μL of 10X) and the appropriate dTTP : dXTP solution (10μL of 1mM).

The volume was made up to 50μL by the addition of water (8.8μL). Samples were initially heated to 50°C for 2 minutes, denatured at 95°C for 10 minutes followed by 22 cycles of 95°C for 0.5 minutes, 53°C for 0.5 minutes, 72°C for 0.5 minutes with a final extension period of 72°C for 7 minutes. For negative control experiments the template was replaced with sterile water.

*Reaction Buffers*

Taq Manufacturers Reaction Buffer (10X): 500mM KCL, 100mM Tris base, 12mM $MgCl_2$, 0.1% gelatin, pH 8.3.

Thermopol Reaction Buffer (10X): 100mM KCl, 100mM $(NH_4)_2$ $SO_4$, 200mM Tris-HCl, 20mM $MgSO_4$, 1% Triton X-100, pH 8.8.

*DNA Polymerase Enzymes*

Hot start Taq DNA polymerase 5 units $\mu L^{-1}$ used with manufacturers buffer.

$Vent_R^{®}$ ($exo^-$) DNA polymerase 2 units $\mu L^{-1}$ used with thermopol buffer*.

*dTTP : dXTP solutions*

The dTTP : dXTP solutions used in the PCR reactions were prepared from 1mM solutions of each of the dNTP's to give the following concentrations (Figure 6.4).

* One unit is defined as the amount of enzyme that will incorporate 10nmoles of dNTP into acid insoluble material at 75°C in 30 minutes in 1X buffer.

PCR Reaction

| dNTP conc/μM | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| dTTP | 200 | 180 | 160 | 140 | 120 | 100 | 80 | 60 | 40 | 20 |
| dXTP | 0 | 20 | 40 | 60 | 80 | 100 | 120 | 140 | 160 | 180 |

Figure 6.4 – Various dTTP : dXTP ratios used for PCR.

The following concentrations (figure 6.5) of dTTP only were used for control PCR experiments.

PCR Reaction

| | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| dTTP conc/μM | 200 | 180 | 100 | 60 | 20 | 0 |
| % dTTP | 100 | 90 | 50 | 30 | 10 | 0 |

Figure 6.5 – Various dTTP concentrations used for control reaction.

*PCR product purification*

Method 1: EtOH precipitation. Purification of the whole sample minus a 5μL aliquot (45μL) was carried out by adding 0.1 volumes of 3M sodium acetate and 2 volumes of ice-cold ethanol and cooling the sample to –20°C for 30 minutes. The samples were then centrifuged at 4°C for 15 minutes, the supernatant decanted and the remaining pellet treated with a cold 70% ethanol solution (100μL). The samples were again centrifuged at 4°C for 15 minutes, the supernatant discarded and the pellet dried *in vacuo*. DNA was eluted in water (100μL).

Method 2: PCR purification spin columns (Qiagen). Purification of the whole sample minus a 5μL aliquot (45μl) was carried out according to the manufacturers instructions. DNA was eluted in water (100μL).

Method 3: Gel Extraction Kit (Qiagen). Purification of the sample (50μL) was carried out according to the manufacturers instructions. DNA was eluted in water (100μL).

*Agarose Gel Electrophoresis*

A 2% or 3% agarose gel was prepared by the addition of agarose NA (NuSieve) (1.0g or 1.5g) to TBE (IX, 0.09M Tris borate, 0.002M EDTA, 50mL). The mixture was boiled in a microwave oven until dissolution of the agarose was complete. The resulting solution was cooled to ~60°C and ethidium bromide (5μL, 5mg $mL^{-1}$ aqueous solution) was added immediately prior to pouring the gel into the electrophoresis apparatus. On setting, the gel was covered with TBE buffer (1X) containing 0.5μg $mL^{-1}$ ethidium bromide. The crude reaction mixture (45μL or 5μL) were combined with loading buffer (15% w/v ficoll, 0.25% bromophenol blue, 0.25% xylene cyanol FF) and loaded onto the gel. The gel was subjected to a current of 100mA, 100V for approximately 1 hour. The gel was visualised by irradiation at 254nm on a transilluminator platform and photographed.

**PCR reactions on a Roche LightCycler.**

*General Protocol*

10μL reaction volumes were used, which included 0.5μM unlabelled primers, 200μM of the appropriate dNTP solution, 4-16mM $Mg^{2+}$ ions, 250ng $\mu L^{-1}$ BSA (non-acetylated bovine serum albumin) and 0.5 units of $Vent_R^{®}$ ($exo^-$) DNA polymerase with thermopol buffer. For those reactions requiring SYBR Gold, 1μL was added from a 1/1000 dilution of the stock. Template DNA (5ng) was added to each reaction. This was replaced with sterile water for the negative controls. Cycling conditions were an initial denaturation at 95°C for 3 minutes, 100 cycles of 95°C for 0 seconds,

annealing temperature for 0 seconds and monitoring for 3 seconds. A single fluorescence measurement was made for each cycle during the monitoring step. Melt curves and cooling steps were performed according to the manufacturers instructions. Fluorescence gains (used to set sensitivity of detection) employed for the LightCycler were set at F1-2 in channel 1 using arithmetic mode.

*Reaction Buffers*

Thermopol Reaction Buffer (10X): 100mM KCl, 100mM $(NH_4)_2$ $SO_4$, 200mM Tris-HCl, 20mM $MgSO_4$, 1% Triton X-100, pH 8.8.

PCR buffer concentrations of either 10X, 2X or 1X were used in the PCR reactions.

*$Mg^{2+}$ ions*

PCR reactions were performed using dilutions of magnesium sulphate (100mM) or magnesium chloride (25mM) stock solutions to give the following concentrations (Figure 6.6).

| Experiment No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $MgCl_2$ / mM | 2 | 4 | 6 | 8 | 10 | 12 | 14 | 16 | | | | | | | | |
| $MgSO_4$ / mM | | | | | | | | | 2 | 4 | 6 | 8 | 10 | 12 | 14 | 16 |

Figure 6.6 – Various magnesium ion concentrations used for PCR.

*dNTP solutions*

dNTP solutions used in the PCR reactions were prepared in 20μL volumes from 1mM solutions of each dNTP to give the following concentrations (Figure 6.7).

PCR Reaction

| dNTP conc/μM | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| dTTP | 200 | 180 | 160 | 140 | 120 | 100 | 80 | 60 | 40 | 20 |
| dXTP | 0 | 20 | 40 | 60 | 80 | 100 | 120 | 140 | 160 | 180 |
| dATP | 200 | 200 | 200 | 200 | 200 | 200 | 200 | 200 | 200 | 200 |
| dGTP | 200 | 200 | 200 | 200 | 200 | 200 | 200 | 200 | 200 | 200 |
| dCTP | 200 | 200 | 200 | 200 | 200 | 200 | 200 | 200 | 200 | 200 |

*Figure 6.7 – Various dTTP : dXTP concentrations used for PCR.*

## PCR reactions on an ABI 7700 Genetic Analyser

*General Protocol*

50 or 25μL reaction volumes were used which included, 0.3μM forward primer, 0.9μM reverse primer, 200μM of the appropriate dNTP solution, 4mM $Mg^{2+}$ ions, and 0.04 units $mL^{-1}$ of $Vent_R^{®}$ ($exo^-$) DNA polymerase with thermopol buffer. For those reactions requiring SYBR Green, 1.0μL or 0.5μL was added from a 1/1000 dilution of the stock. Template DNA (5ng) was added to each reaction. This was replaced with sterile water for the negative controls. Cycling conditions were an initial denaturation at 95°C for 3 minutes, 40 cycles of 95°C for 15 seconds, 60°C for 60 seconds and monitoring for 3 seconds. A single fluorescence measurement was made for each cycle during the monitoring step. Melt curves were performed on the Roche LightCycler according to the manufacturers instructions.

*Reaction Buffers*

Thermopol Reaction Buffer (10X): 100mM KCl, 100mM $(NH_4)_2$ $SO_4$, 200mM Tris-HCl, 20mM $MgSO_4$, 1% Triton X-100, pH 8.8.

*dNTP solutions*

dNTP solutions used in the PCR reactions were prepared in 20μL volumes from 1mM solutions of each dNTP to give the following concentrations (Figure 6.8).

PCR Reaction

| dNTP conc/μM | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|
| dTTP | 200 | 180 | 160 | 140 | 90 | 60 | 40 |
| dXTP | 0 | 20 | 40 | 60 | 110 | 140 | 160 |
| dATP | 200 | 200 | 200 | 200 | 200 | 200 | 200 |
| dGTP | 200 | 200 | 200 | 200 | 200 | 200 | 200 |
| dCTP | 200 | 200 | 200 | 200 | 200 | 200 | 200 |

Figure 6.8 – Various dTTP : dXTP concentrations used for PCR.

# Chapter 7

# References

# 7.0 References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