Oligodeoxynucleotides Containing Fluorophore / Quencher Pairs

Volume 1 of 1

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ABSTRACT

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Oligodeoxynucleotides Containing Fluorophore / Quencher Pairs

by Jamie Nicol

Four phosphoramidite monomers were prepared for use in solid phase oligodeoxynucleotide synthesis. Each monomer held the fluorescence quencher molecule 2-(4-dimethylamino-phenylazo)-benzoic acid (methyl red) with attachment by aminoalkyl pendant arms. The monomer 1'-α-O-[(2-(dimethylamino-phenylazo)-benzoyl)-6-aminohexyl]-5'-O-(5-bis-(4-methoxyphenyl)-phenylmethyl)-2'-deoxy-D-ribose-3'-(2-cyanoethyl-N,N-diisopropyl-phosphoramidite) was prepared in 9 steps with an overall yield of 10% and was tested in solid phase oligodeoxynucleotide synthesis giving coupling efficiencies of 96%. The monomer 4-(di-*n*-butylamino-methyleneamino)-7-(O-(5-bis-(4-methoxyphenyl)-phenylmethyl)-O-[3-(2-cyanoethyl-N,N-diisopropyl)-phosphoramidite)]-2-deoxy-β-D-ribosyl)-5-(3-N-(4-dimethylamino-phenylazo-benzamido)-prop-2-ynyl)-pyrrolo[2,3-d]pyrimidine was synthesised in 14 steps with an overall yield of 0.9%. Molecular beacon studies using oligodeoxynucleotides containing these monomers showed the required fluorogenic responses.

Two further major groove monomers were synthesised. 4-(Di-n-butylamino-methyleneamino)-7-(O-5-bis-(4-methoxyphenyl)-phenylmethyl)-2-deoxy-β-D-ribosyl-O-3-(2-cyanoethyl-N,N-di-isopropyl-phosphoramidite)-5-(6-(2-(dimethylamino-phenylazo)-benzoyl)-prop-2-ynyl-hexanamido)-pyrrolo[2,3-d]pyrimidine was synthesised in 0.73% yield over 12 steps. 7-[O-(5-Bis-(4-methoxyphenyl)-phenylmethyl)-2-deoxy-β-D-ribosyl-O-3-(2-cyanoethyl-N,N-diisopropyl-phosphoramidite)]-5-(6-(2-(dimethylamino-phenylazo)-benzoyl)-prop-2-ynyl-hexanamido)-pyrrolo[2,3-d]pyrimidin-5-one was synthesised in 0.50% yield over 12 steps.

List of Contents

List of Illustrations

Acknowledgements

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Finally I wish to thank my wife, parents, brother, sisters, kids, friends and my colleagues for providing the entertaining backdrop that carried me through these postgraduate studies.

Abbreviations

Bracketed Arabic numerals refer to molecular structures illustrated in the text and Arabic superscripts indicate literature references. The following abbreviations appear within the text and diagrams:

A	adenine, adenosine
AcOH	acetic acid
APCI	atmospheric pressure chemical ionisation
aq.	aqueous, dissolved in water
B	nucleobase
b.	broad
Boc	*t*-butyloxycarbonyl
b.s.	broad singlet
C	cytosine, cytidine
$\underline{C}_n$ and C-n	this refers to carbon (n) in a molecule. Where not otherwise specified this relates to IUPAC numbering (eg. of the ribose ring)
ca.	approximately
calc.	calculated
cat.	catalyst, catalytic quantity
c.	concentrated
CPG	controlled pore glass
DBU	diazabicyclo[5.4.0]undec-7-ene
DCCI	dicyclohexylcarbodiimide
DCM	dichloromethane
d.d.	double doublet
d.e.	diastereoisomeric excess
decomp.	decomposition
def	deformation
DIPEA	N,N-diisopropylethylamine

DMF	N,N-dimethylformamide
DMT	4,4-dimethoxytrityl
DMAP	4-N,N-dimethylaminopyridine
DNA	deoxyribonucleic acid
DNP	dinitrophenyl
DTT	dithiothreitol
EDC	1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride
eq.	equivalents
ES+, ES-	electrospray ionisation (positive or negative)
Et_3N	triethylamine
EtOAc	ethyl acetate
EtOH	ethanol
FAB	fast atom bombardment
FAM	fluorescein addition monomer
FRET	fluorescence resonance energy transfer
G	guanine, guanidine
$\underline{H}_n$	the proton attached to carbon (n) in a molecule. Where not otherwise specified this relates to IUPAC numbering of the ribose ring.
Hobt	1-hydroxybenzotriazole
HPLC	high performance liquid chromatography
HRMS	high resolution mass spectrometry
i-PrOH	isopropanol
kb	kilo-bases
lit.	literature
m.	multiplet
M	molecular ion
MeCN	acetonitrile
m.p.	melting point
mRNA	messenger RNA

MeOH	methanol
NaOEt	sodium ethoxide
NaOMe	sodium methoxide
NHS	N-hydroxysuccinimide
NMR	nuclear magnetic resonance
PCR	the polymerase chain reaction
RNA	ribonucleic acid
rRNA	ribosomal RNA
r.t.	room temperature
s.	singlet
sat.	saturated
str	stretch
t.	triplet
T	thymine, thymine
TCA	trichloroacetic acid
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin layer chromatography
T_m	melting temperature
Trityl	triphenylmethyl
U	uracil, uridine
u.v.	ultraviolet
W:W	weight : weight
"X"-mer	an oligonucleotide containing "X" units

Section 1 Introduction

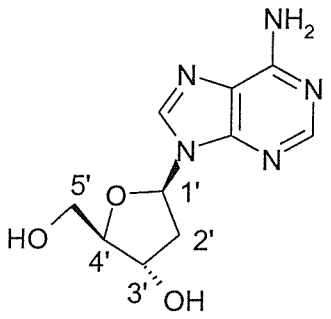
1.0.0 Overview of Objectives

The primary objective of this research has been to chemically synthesise novel phosphoramidite monomers that allow fluorescence quenchers to be positioned within oligodeoxynucleotides prepared on the solid phase and to test and demonstrate fluorescence quenching of suitable oligodeoxynucleotides in molecular beacon experiments.

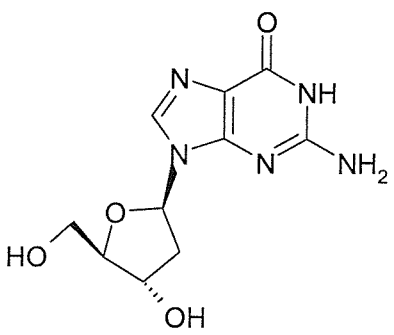
Four phosphoramidite monomers were synthesised over the course of this research project. Chemical synthesis is discussed in Sections 2.1, 2.2, 2.3 and 2.4. Two monomers were tested in solid phase synthesis and used to prepare oligonucleotides that were used in molecular beacon studies (Section 2.5). Two monomers are still to be tested. This introductory section covers some basic chemical and biological properties of DNA in Section 1.1. Much of the material in the first section is covered more extensively by Blackburn and Gait.[1] Sections 1.2, 1.3 and 1.4 deal with the chemical synthesis of oligonucleotides and existing strategies for functionalising nucleic acids. The background for molecular beacon experiments is covered in Sections 1.5, 1.6 and 1.7. The chemistry of oligonucleotide synthesis has been comprehensively reviewed by Beaucage and Iyer.[2] Beaucage and Iyer have also reviewed the techniques for synthesising functionalised oligonucleotides.[3] Attaching fluorescent groups to oligonucleotides has been reviewed more recently by Wojczewski *et al.*[4]

1.1.0 DNA and RNA

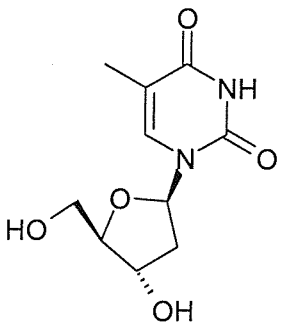
DNA is a high molecular weight linear polymer constructed from monomeric nucleotides. There are two types of nucleic acid: DNA (deoxyribonucleic acid) and RNA (ribonucleic acid). The primary structure of DNA results from the sequential ordering of the four nucleotides **(1-4)**. Nucleotides are the 5'-phosphate esters of nucleosides that in turn are N-glycosides formed between ribose and certain nitrogenous bases.



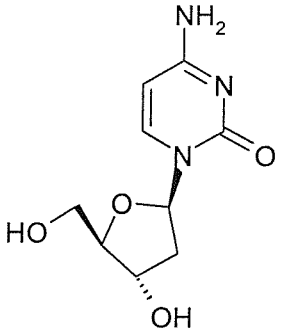
2'-deoxyadenosine **(1)**



2'-deoxyguanosine **(2)**



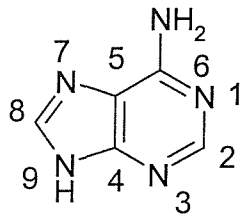
2'-deoxythymidine **(3)**



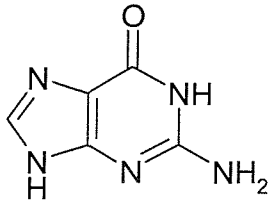
2'-deoxycytidine **(4)**

Figure 1.1-1: Structures of the Four Major 2'-Deoxyribonucleosides Showing IUPAC Numbering for the Furanose Ring

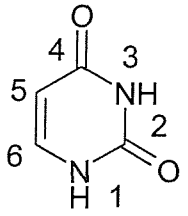
Nucleic acids are formed from nucleosides joined by phosphate diesters that link the 5' hydroxyl unit of one nucleoside to the 3' hydroxyl of the next. The four possible nucleobases are two bicyclic purines [adenine **(5)** and guanine **(6)**] and two smaller pyrimidine structures [cytosine **(7)** and thymine **(8)**]. The primary structuring of RNA is similar to that of DNA, except the sugar component is ribose rather than deoxyribose, and uracil **(9)** replaces thymine as one of the bases.



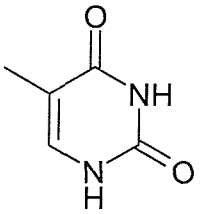
adenosine **(5)**



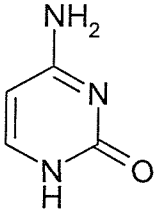
guanosine **(6)**



uracil **(7)**



thymine **(8)**



cytosine **(9)**

Figure 1.1-2: Structures of the Five Principle Nucleobases Showing IUPAC Numbering for the Purine and Pyrimidine Rings

Genes are regions of DNA that code for the primary structures (*ie.* the sequence of amino acids) of proteins. Each gene corresponds to a single protein in the organism and therefore each protein has a corresponding gene. In the entire DNA complement (genome) of each human cell there are approximately 3×10^9 pairs of cross-linked nucleotides (base pairs). This would give an extended

length of about 2m. The DNA is packed into 46 cylindrical chromosomes of total length 200μm.

The secondary structure of DNA contains the key to the function of this molecule. The information stored in our cell nuclei is based on twin antiparallel DNA chains arranged together in double helices of constant diameter. DNA double helices are stabilised by the stacking of the hydrophobic base pairs within the lipophilic core of the structure and location of the hydrophilic sugar-phosphate backbone at the outside of the structure where favourable interactions occur with water.[5,6] The antiparallel chains are held together by selective hydrogen bonds between purine bases and pyrimidines. This process of molecular recognition between the base residues is known as Watson-Crick base pairing; where adenine pairs selectively with thymine via two hydrogen bonds and guanine pairs with cytosine *via* three hydrogen bonds. The complementarity of base pairing provides nature with a very compact system for the storage and transfer of genetic information.

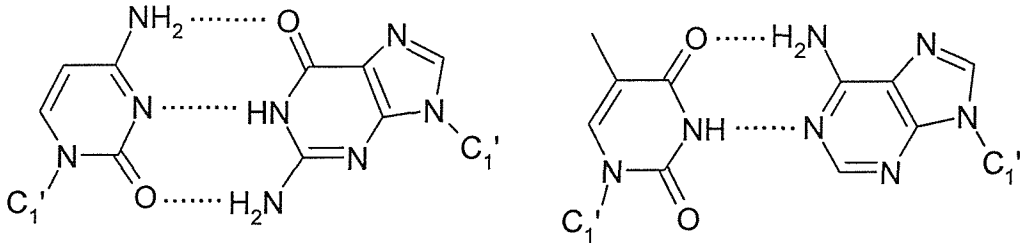


Figure 1.1-3: Watson-Crick Base Pairing

Watson-Crick base-pairing has an additional feature that the four base pair combinations, A-T, T-A, C-G and G-C have isomorphous geometry and can therefore be built into a regular framework regardless of the sequence of base pairs along the double stranded nucleic acid.

When a cell divides it must duplicate its set of chromosomes in a process called replication, so that both daughter cells possess a set of genes identical to those of the parental cell. Replication of DNA is co-ordinated by enzymes known as DNA polymerases. These require a single stranded template DNA (the chromosome being copied), the four deoxynucleoside 5'-triphosphates, and an oligonucleotide primer with a 3'-hydroxyl group at which the polymerase can attach new nucleotide residues. The oligonucleotide primers are made up of short sections of RNA and are later removed by an enzyme called ribonuclease-H (RNase-H) which is specific for regions of DNA-RNA hybridisation.

Elongation is in the direction 5'- to 3'- and the product shows semi-conservative replication (one new strand is laid down against each old strand). The sequence of the new strand is complementary to the sequence of the old strand according to the rules for DNA base pairing.

1.1.1 Polymerase Chain Reaction (PCR)

The polymerase chain reaction was discovered by Kary Mullis in the mid 1980's.[6] The technique uses modified polymerase enzymes to replicate and amplify DNA *in vitro*. Starting with trace amounts (as little as three to five double strands) of a particular nucleic acid sequence from any source, PCR can generate millions of exact copies in a few hours. Most reactions are designed to amplify segments of less than 2kb length, but amplification of up to 12kb is achievable. The technique is used extensively in molecular biology, genetic research, medical diagnostics and forensic science.

The reaction comprises a repetitive series of temperature cycles, with each cycle having three stages; denaturing, annealing and extension. The DNA is first denatured by heating at >91°C to separate the duplex strands of the target DNA molecule. Cooling to *ca.* 50°C then allows synthetic single-stranded oligonucleotide primers to selectively anneal to the template. These primers are

designed to flank the region of DNA that is of interest. Extension of the primers by DNA polymerase on warming to about 70°C then leads to selective replication of the DNA segment of interest. The newly synthesised strands can themselves act as templates in the next round of amplification. This leads to a theoretically exponential growth in the product.

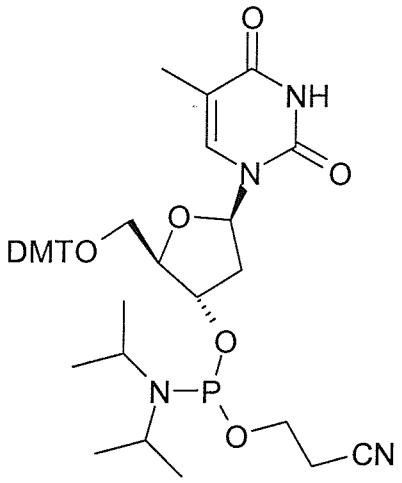
The PCR reaction is catalysed by thermostable DNA polymerases such as Taq polymerase, from the extreme thermophile *Thermus aquaticus*. These evolutionarily specialised enzymes can withstand the repeated exposures to high temperature required for strand separation.

1.2.0 Synthetic Oligodeoxynucleotides

All early work in oligodeoxynucleotide synthesis involved solution-phase reactions. In the mid 1950's Michelson and Todd reported a chemical synthetic route to dinucleotides.[7] Further work in the mid 1950's and early 1960's by a group lead by Khorana, permitted the preparation of a series of short, defined sequences of deoxyribonucleotides which were used in deciphering the genetic code.[8,9] The inherent problem encountered with these linear, homogeneous syntheses was the need to isolate and purify the product at the end of each reaction step. This was time consuming and long syntheses suffered from low cumulative yields. In general only very short oligodeoxynucleotides could be prepared this way.

Similar problems in peptide chemistry at that time prompted the development of solid-phase synthesis. Pioneering work in peptide chemistry by Merrifield in 1962 demonstrated that solid-phase synthesis had many advantages over solution-phase (homogeneous) chemistry for biopolymer synthesis.[10] Covalent attachment of oligomers to insoluble solid supports meant that purification at the end of each monomer addition cycle could be effected by rinsing with a suitable solvent. This facile purification then allowed the use of large excesses of reagents for each monomer addition and consequently gave fast, high-yielding reactions at each step.

Much effort followed in applying this technique to oligonucleotide synthesis, culminating in a method developed by Beaucage and Caruthers which made use of phosphoramidites such as the 2'-deoxy-T monomer (**10**) (Figure 1.2-1) as electrophilic synthons for phosphate.[11-13]



2'-deoxy T **(10)**

Figure 1.2-1: The 2'-Deoxythymidine Phosphoramidite Monomer **(10)**

1.2.1 Automated Solid Phase DNA Synthesis

Polystyrene, glass (CPG) and a variety of specialised resins can be used as solid supports in oligonucleotide synthesis. The automated synthesiser provides the resin with solvents and reagents in the required order for the programmed oligodeoxynucleotide sequence.

The orthogonal protection strategy developed by the Khorana group is still in use. The 5'-hydroxyl groups of nucleosides are protected with trityl derivatives. The exocyclic amino functions of adenine and cytosine are acylated with benzoyl groups and the C-2 amino group of guanine is acylated with the isobutyryl group. These amides are stable to acid and are usually cleaved with ammonium hydroxide.[8,9]

An acceptable overall yield for the synthesis of a 100mer is 22.4% with coupling times of less than 1 minute and cycle times of *ca.* 5 minutes. This requires an average coupling efficiency of 98.5% for each monomer addition.

1.2.2 The Synthesis Cycle

Synthesis starts with removal of the DMT protecting group from the 5'-hydroxyl group of the resin bound monomer. The O-DMT protecting group is rapidly cleaved by exposure to 3% trichloroacetic acid in dichloromethane. The acid lability of this protecting group is due to the resonance stabilisation of the DMT carbocation. This cation has a characteristic u.v. absorption at 495 nm, leading to orange colouration. In subsequent cycles the DMT group attached to the 5' hydroxyl group of each nucleoside is similarly cleaved.

In the next stage of the cycle the free hydroxyl group reacts with the 3'-phosphoramidite of the next nucleoside which is activated by protonation of the phosphoramidite tertiary amine using catalytic quantities of tetrazole in acetonitrile. Unreacted hydroxyl groups are then capped to minimise the length of failure sequences as an aid to HPLC purification. Finally the phosphite linkage is oxidised to the corresponding phosphate by a solution of iodine in aqueous THF.

The cycle is then repeated until the required oligomer has been constructed. An ammonium hydroxide treatment then simultaneously releases the molecule from the support and cleaves the protecting groups from the exocyclic amino functions of the nucleobases.

1.3.0 Oligodeoxynucleotide Probes

A probe is defined as a molecule capable of recognising a specific target, binding strongly to it and being subsequently detected. The detection of the reporter group then confirms the presence of the target sequence. This detection can be a **direct** method such as the measurement of fluorescence intensity, or it can be **indirect** and involve further manipulation.

Nucleic acid base-pairing is very selective and consequently single-stranded oligodeoxynucleotide probes of around 20 units in length contain enough sequence information to be totally selective in binding to complementary single-stranded target sequences.

Probe-target hybridisation is possible for nucleotides modified with single atoms, functional groups or long side chains, depending on the site of attachment and nature of the side chain. This has significance when choosing sites for the attachment of reporter groups.

1.3.1 Direct Detection of Reporter Groups

Reporter groups such as fluorescent dyes and radioisotopes are directly detectable.

1.3.2 Fluorescent Reporter Groups

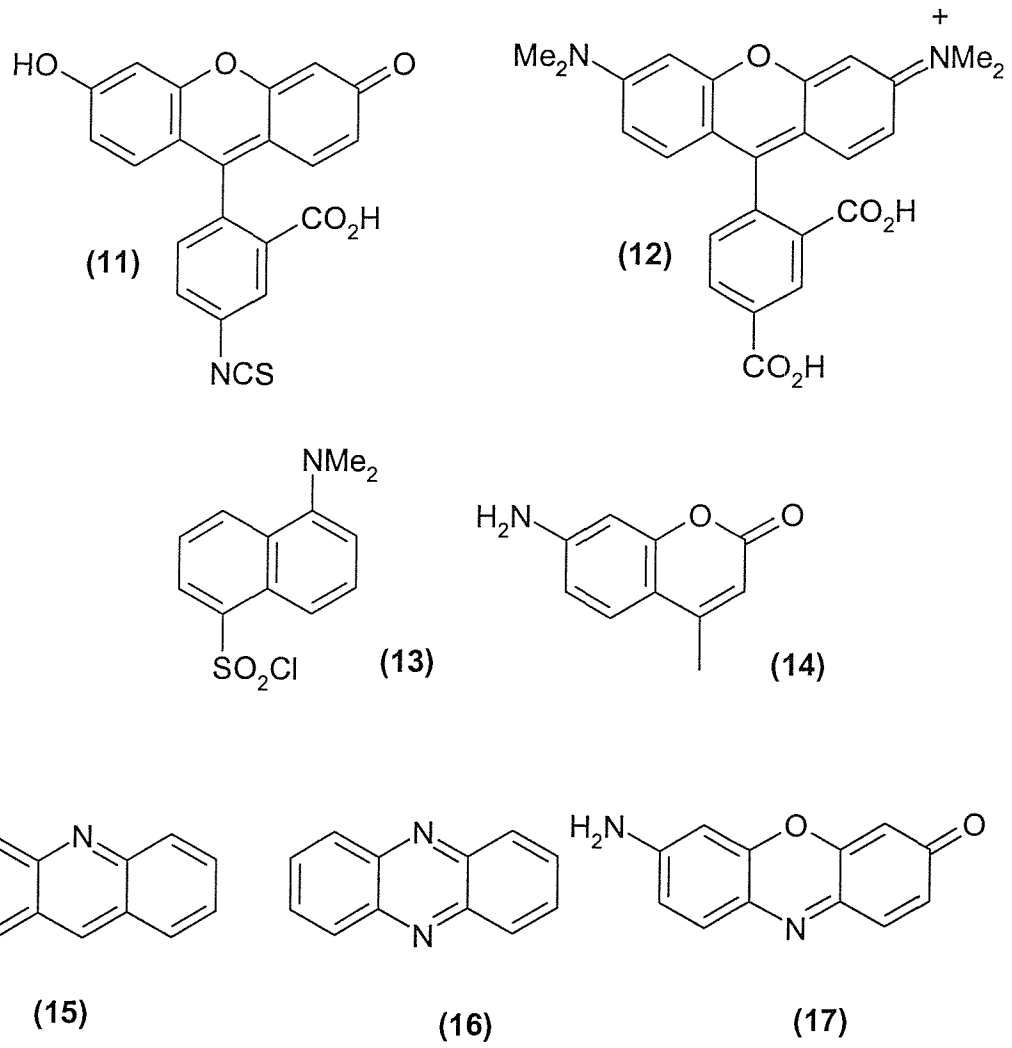


Figure 1.3-1: Structures of Some Common Fluorophores; Fluorescein-5-isothiocyanate **(11)**, 5-Carboxytetramethylrhodamine (5-TAMRA) **(12)**, Dansyl Chloride **(13)**, Parent Structures for; Coumarines **(14)**, Acridines **(15)**, Phenazines **(16)** and Oxazines **(17)**

Due to their ease of detection fluorescent nucleic acid probes are useful tools in quantitative and qualitative analysis. A great number of fluorescent dyes are available for labelling nucleic acids. These include derivatives of fluoroscein,

rhodamine, acridine, dansyl, coumarin, phenazine, oxazine and cyanine dyes as well as polyaromatics such as anthracene and pyrene.[14]

Fluorophores can be detected directly and with high sensitivity. They must be attached to oligonucleotides by relatively long spacer arms to prevent fluorescence quenching by the oligonucleotide bases. Simultaneous detection of different fluorophores, for instance in DNA sequencing requires a range of dyes with distinct spectral properties.[15,16]

Incorporating multiple fluorescent building blocks within an oligodeoxynucleotide may increase detection sensitivity, but in some cases it is also known to quench the fluorescence and increase the extinction coefficients of the dye. Furthermore, since even one dye molecule can perturb the structure of the probe-target duplex, several bulky fluorescent residues can considerably reduce the melting point of the duplex *ie.* decrease duplex stability. New energy transfer dyes have recently come into use. These rely on energy transfer between rhodamine and fluorescein to boost the fluorescence signal and thereby allow detection of nucleic acids at very low concentrations and have found use in DNA sequencing.[17]

1.3.3 Radioactive Labels

Radioactive labels are detected directly by autoradiography which is a highly sensitive, but hazardous technique. Phosphodiester linkages can be modified to contain ^{32}P or ^{35}S. Isotope labelled nucleosides can be incorporated into oligonucleotides using enzymes. For example ^{32}P can be incorporated onto the 5'-end of oligonucleotides by the action of polynucleotide kinase. 3'-Labelling can be brought about by the use of terminal transferase. Nucleosides can be modified to contain ^{3}H or ^{14}C. In addition dU and dC can be modified to contain ^{125}I in the 5-position.

Detection by autoradiography is time consuming and does not lend itself to rapid assays. In addition the fast radioactive decay of labelled molecules, the expense of purchasing radio-labelled substrates and the obvious safety issues encountered in radio-isotope labelling combine to make this technique undesirable in many applications. There is a strong trend towards non-radioactive labelling for oligonucleotide detection, in particular towards the use of fluorescent reporter groups.

1.3.4 Indirect Detection of Reporter Groups

Haptens such as biotin **(19)**, digoxigenin **(20)** and the 2,4-dinitrophenyl group (DNP) **(18)**, are indirectly detectable. Indirect detection involves secondary systems that rely on high affinity non-covalent bonds between the hapten and a suitable secondary molecule labelled with a reporter group.

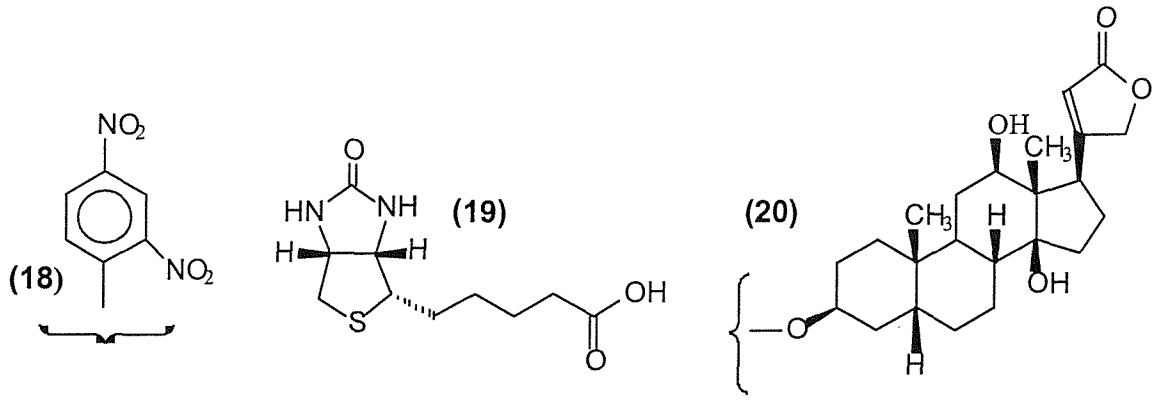


Figure 1.3-2: *Common Hapten Reporter Groups*; 2,4-Dinitrophenyl **(18)**, Biotin **(19)** and Digoxigenin **(20)**

Secondary reporter groups such as alkaline phosphatase and horseradish peroxidase can digest suitable chemical substrates to give a colourimetric or chemiluminescent response. Chemiluminescent detection is especially sensitive.[18]

1.4.0 Building Blocks for Oligodeoxynucleotide Probes

There are many tactics for functionalising oligodeoxynucleotides. The following section illustrates most of the main design features that have been used. These examples show the possibilities for tailoring individual monomers towards specific probe systems.

1.4.1 Building Blocks that Carry Reporter Groups

Reporter groups can be attached directly during automated oligodeoxynucleotide synthesis by using non-standard monomers. The following examples show some of the strategies that have been used.

The phosphoramidite **(21)** has been used to incorporate biotin onto the 5'-termini of oligonucleotides.[19] This monomer has no functional site for further chain extension after attachment and is therefore a single addition monomer.

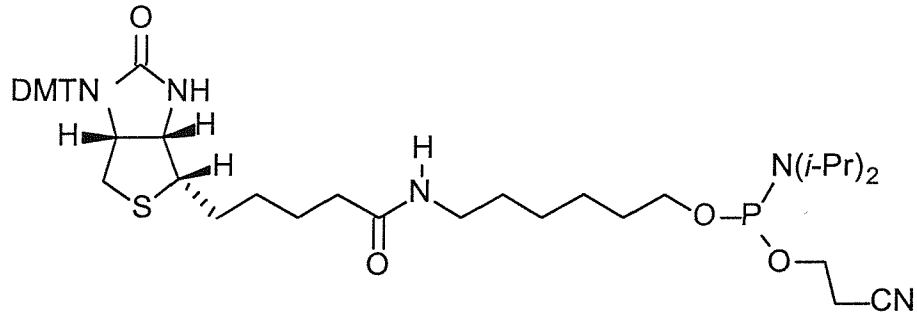


Figure 1.4-1: Single Addition Biotin Phosphoramidite **(21)**

Oligonucleotides have been labelled with up to 8 biotin units at the 5'- end using multi-addition phosphoramidite monomer **(22)**.[20] Multiple addition is a desirable feature because it allow specific incorporation of the building block at any position within an oligonucleotide sequence.

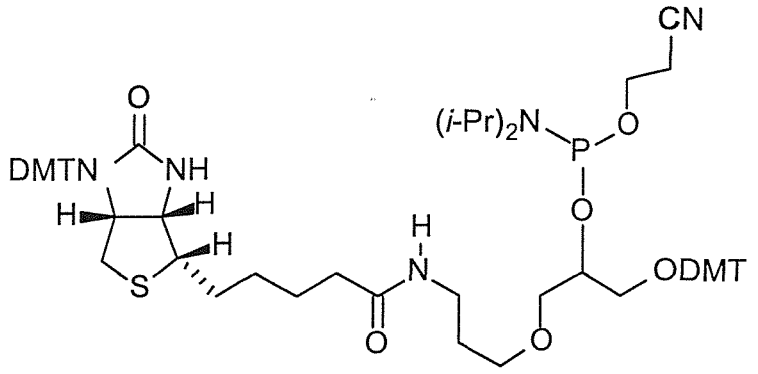


Figure 1.4-2: Multiple Addition Biotin Phosphoramidite **(22)**

Fluorescein phosphoramidite **(23)** is a fluorescent version of the same type of

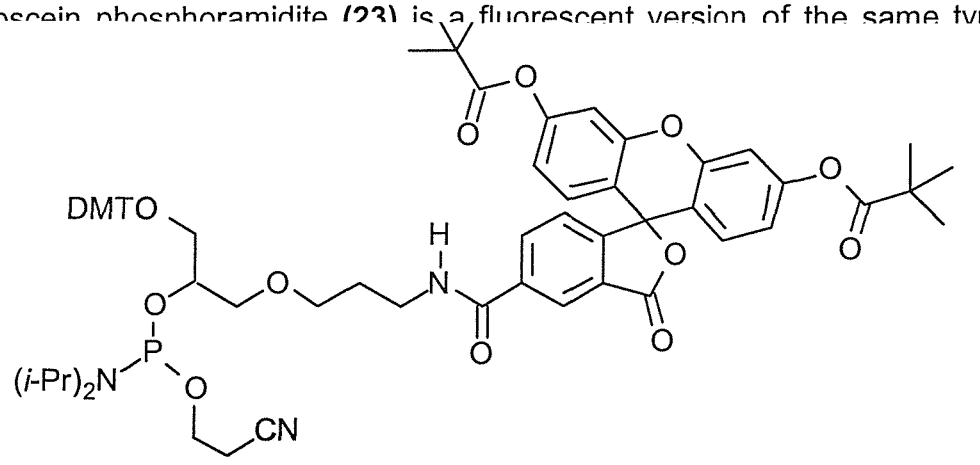


Figure 1.4-3: Fluorescein Multiple Addition Monomer **(23)**

The conjugation of **(24)** with biotinyl, dinitrophenyl, dansyl and pyrenyl groups followed by phosphitylation lead to a range of multi-addition phosphoramidite monomers.[22]

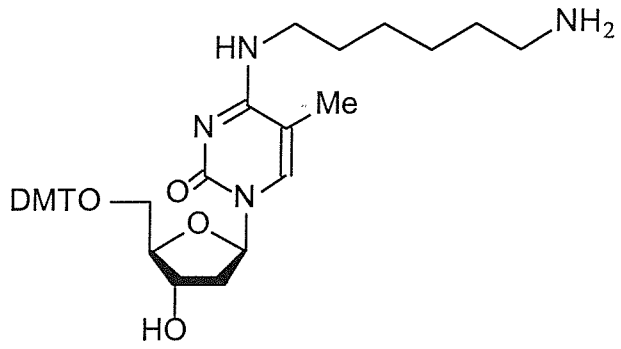


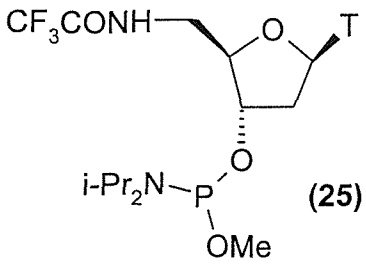
Figure 1.4-4: Intermediate **(24)**: Precursor to a Range of Multiple Addition Monomers

In general these multi-addition monomers are superior over others in terms of flexibility and ease of use. Base-pairing nucleoside analogues have a further advantage that they can be placed in many positions within a probe without causing much duplex destabilisation in the probe-target complex.

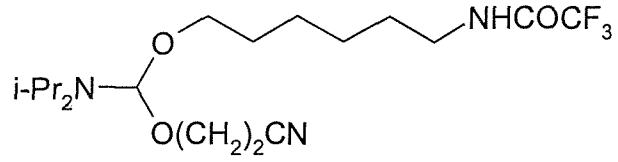
1.4.2 Building Blocks that Allow Post-Synthetic Attachment of Reporter Groups

1.4.2.1 Modified Nucleosides and Replacements for Nucleosides

Oligodeoxynucleotides can be labelled post-synthetically at functional sites that have been incorporated during synthesis. This post-synthetic strategy is characteristically used for the majority of rhodamine dyes as these are sensitive to the conditions encountered in automated DNA synthesis. For example the phosphoramidite monomer **(25)** is based on the modified nucleoside 5'-amino-5'-deoxythymine and was developed by Smith *et al.*[23] This was incorporated onto the 5'-termini of oligonucleotides and after standard deprotection, conjugated to a range of dyes for use in the automated sequencing of DNA.[16]



(25)

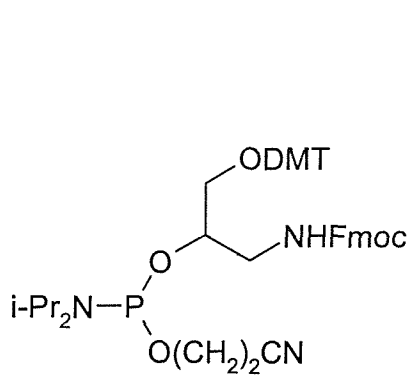


(26)

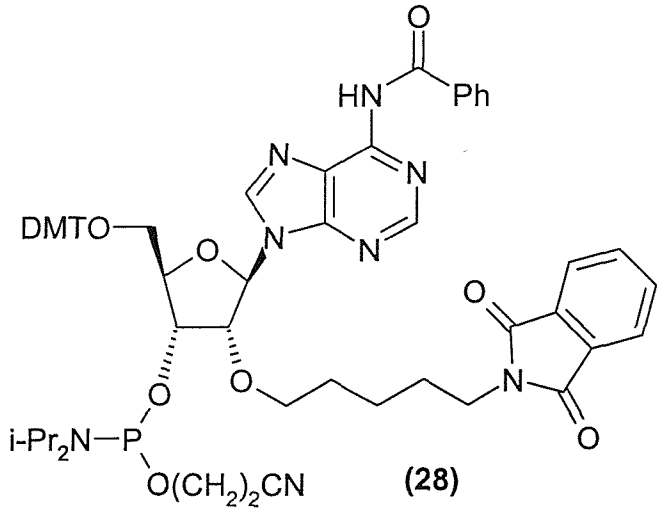
Figure 1.4-5: Single Additon Monomers that Provide Reactive Amino Groups on Deprotection

Applied Biosystems Incorporated developed Aminolink II **(26)**. This design is representative of many similar molecules that have been made, with a variety of aliphatic chains and protecting groups. Murchie *et al* used **(26)** to conjugate fluoroscein and tetramethyl rhodamine to the 5'-ends of selected oligonucleotides to investigate DNA recombination by FRET measurements.[24]

The phosphoramidite **(27)** of Nelson *et al* [25] can be used for multiple additions. The Fmoc protecting group is cleaved during standard (ammonium hydroxide) deprotection to reveal a nucleophilic amino group.



(27)

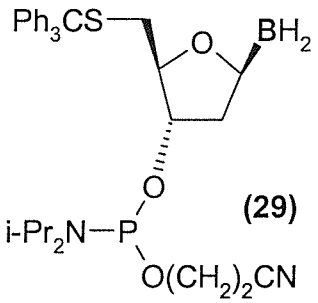


(28)

Figure 1.4-6: Multiple Additon Monomers that Provide Reactive Amino Groups on Deprotection

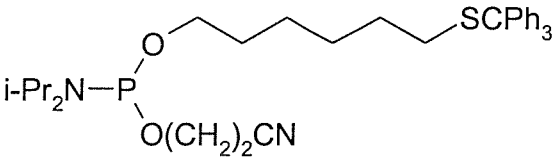
The phosphoramidite monomer **(28)** has also been been prepared by Nelson *et al.*[25] The 2'-alkyl linker arm is designed to position labels into the minor groove of DNA duplexes, where they are less able to interfere with base-pairing and base-stacking interactions.

The range of single addition phosphoramidites **(29)** of Sproat *et al* [26] use standard protecting groups for the exocyclic nucleosidic amine groups of the four DNA nucleosides. The trityl group is cleaved to give 5'-mercapto-oligonucleotides with silver nitrate or with 3-chloromercuri-2-methoxypropylurea.[26] These molecules were used to make 5'-fluoresceinated PCR primers.



(29)

B can be either C A T or G



(30)

Figure1.4-7: Single Addition Monomers that give Reactive Thiols on Deprotection

The less complex single addition mercaptoalkylated phosphoramidite **(30)** was prepared by Sinha and Cook.[27]

1.4.2.2 Attachment at the Internucleosidic Linkage

Functional groups can be attached post-synthetically to the internucleosidic linkage.

Oxidation of the phosphite triesters introduced using monomer **(31)** of Hecht *et al* [28] was followed by displacement of the methoxy group with 1,5-diaminopentane to give an aminoalkyl linker arm **(32)**.[28]

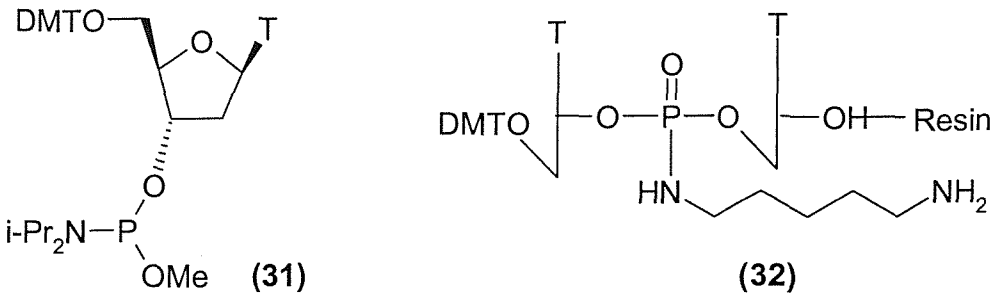


Figure 1.4-8: A Monomer that Leads to Reactive Amino Groups from the Internucleosidic Linkage

Phosphorothioate analogues of oligonucleotides (oligomers with phosphorothioate diester linkages) have nucleophilic thiol groups that can be used for later derivatisation. Oligonucleotides with single, site-specific, internal phosphorothioate diester linkages have been prepared by McLaughlin *et al* [29] through oxidation of the phosphite triester intermediates obtained during standard solid-phase synthesis. This can be done with elemental sulfur to give an intermediate that is converted to the phosphorothioate diester **(33)** by standard oligonucleotide deprotection with ammonium hydroxide.[29]

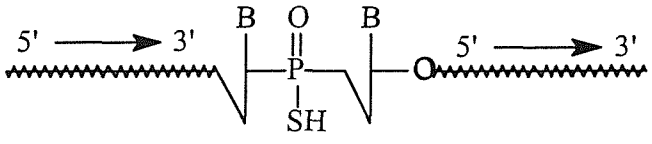


Figure 1.4-9:Phosphorothioate Diester **(33)**

1.4.2.3 Functionalising the 3'-Terminus

Aminoalkyl arms can be attached to the 3'-termini of oligonucleotides using functionalised solid supports such as **(34)**.[30]

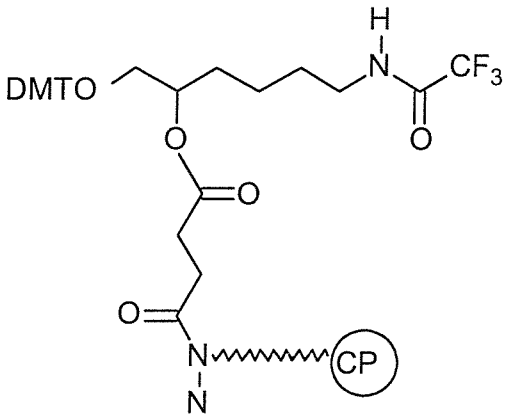


Figure 1.4-10: 3'-Aminoalkyl Solid Support **(34)**

Various methods of attaching thio-alkyl linker arms to the 3'-termini of synthetic oligonucleotides have been reported. Gupta *et al* reported a solid support with a disulfide linkage **(35)**.[31] 3'-Thioalkyl oligonucleotides are released from this resin by the action of dithiothreitol (DTT) on the disulfide linkage.

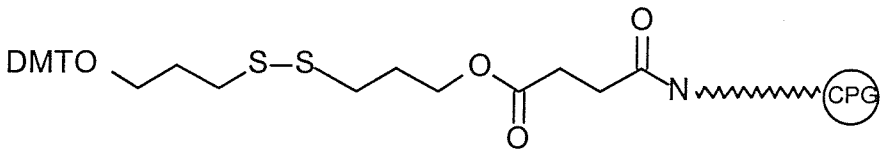
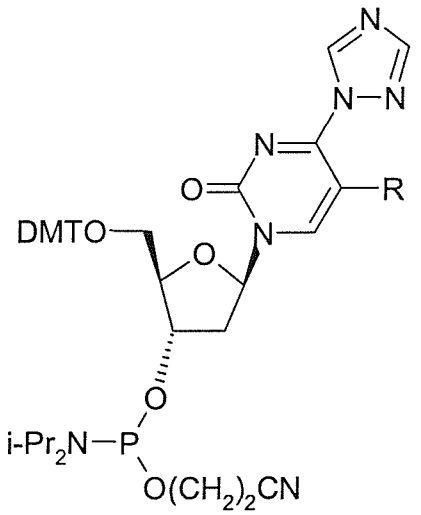


Figure 1.4-11: Disulfide linker **(35)**

1.4.2.4 Phosphoramidite Monomers with Functionalised Nucleobases

The phosphoramidites **(36)** and **(37)** of Xu *et al* have been incorporated into synthetic oligonucleotides to give 4-triazolopyrimidone nucleotide residues in

defined positions.[32] The triazolo functions were displaced post-synthetically with a variety of nucleophiles.



Structure **(36)** [R = H] and Structure **(37)** [R = CH_3]

Figure 1.4-12: 4-Triazolopyrimidone Monomer

The phosphoramidite **(38)** has an amino-linker arm attached to the C-5 of the pyrimidine residue. Telser *et al* studied fluorescence quenching in hybrids between two complementary 8mer strands of DNA using this monomer to tether anthraquinone and pyrene butyrate.[33]

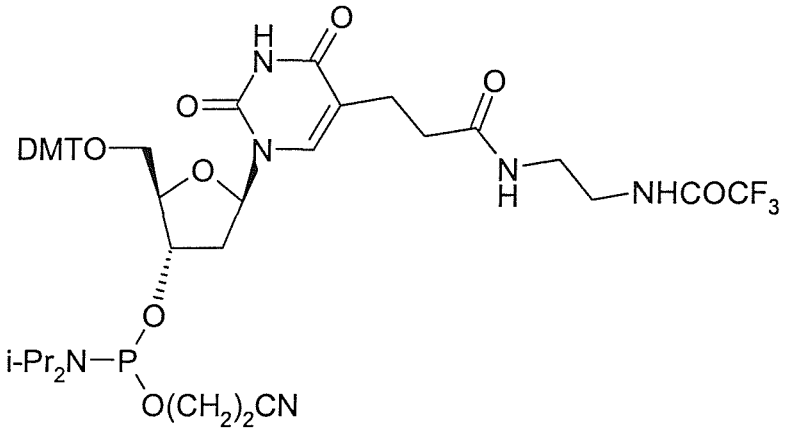
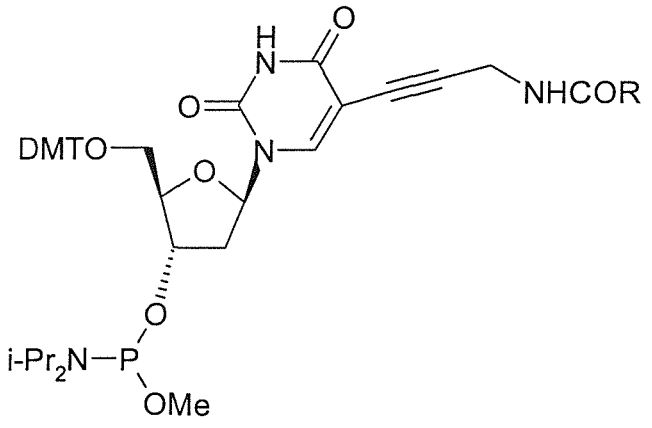


Figure 1.4-13: Base-Functionalised 3'-Deoxythymidine Monomer **(38)**

The phosphoramidite monomers **(39)**, **(40)** and **(41)** were synthesised by coupling protected derivatives of 5-iodo-2'-deoxyuridine with various alkynes *via* a palladium coupling reaction. The monomers were incorporated into synthetic oligonucleotides and labelled post-synthetically with fluorescein. 5'-Singly labelled probes were found to hybridise better than 5'-multi-labelled probes. Probes labelled with **(40)**, which has a long linker arm, hybridised better than those labelled with **(39)**.[34,35] This is a good illustration of the influence that chemical embellishment can have on the physical behaviour of DNA and highlights the importance of developing monomers that have the minimum impact on the systems that are to be studied.



Structure **(39)** R = OMe

Structure **(40)** R=-$(CH_2)_5$NHBoc

Structure **(41)** R = CF_3

Figure 1.4-14: Alkynyl Nucleoside Phosphoramidites

1.5.0 Fluorescence Resonance Energy Transfer (FRET)

FRET is a spectroscopic process by which energy is passed nonradiatively (by Forster resonance energy transfer) between molecules over distances of the order of common biomolecular dimensions (10-100Å). The **donor** molecule, which is a fluorophore, absorbs a photon and transfers the energy nonradiatively to the **acceptor** molecule. Measurements of fluorescence intensity and lifetime can be used to calculate the distance between the donor and acceptor. Structural information from FRET can be obtained *in vitro*, in contrast to information obtained through X-ray crystallography. Consequently dynamic processes can be monitored such as enzyme activity on nucleic acids.[36-40]

1.6.0 Applications of Probe Technology in PCR

Specific nucleic acid sequences can be amplified by the polymerase chain reaction (PCR). This technique has contributed to the understanding of genetic diseases and shown a wide range of practical applications, for example in the fields of DNA diagnostics and forensic science. Specific DNA sequences can be amplified and isolated from samples containing large quantities of other genetic material. Following PCR further analysis is required, such as sequencing, gel electrophoresis or hybridisation with nucleic acid probes.

The use of fluorophore-labelled PCR primers allows any amplified material to be visualised by irradiation with UV or visible light. Simultaneous amplification of several DNA segments is possible thanks to the range of fluorescent dye markers available. For example when two DNA sequences A and B are amplified simultaneously using primers labelled with for example fluorescein (green) for A and rhodamine (red) for B the amplified products can be distinguished from each other by colour. If only one of the sequences is present in the initial sample, the amplified product will emit either a red or a green colour. If both are amplified the product will appear yellow. In fact this amplification strategy has been used by

Chehab *et al* to detect gene deletions, chromosome translocations and infectious agents.[41]

1.7.0 Molecular Beacons: Probes that Fluoresce on Hybridisation

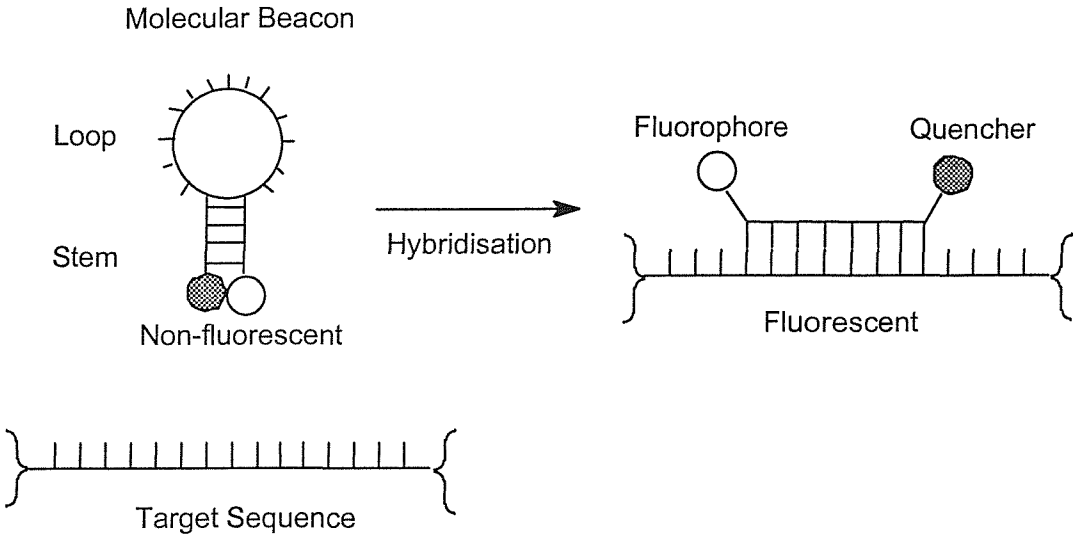


Figure 1.7-1: Molecular Beacons

Molecular beacons are probes that become fluorescent when they encounter their target sequence. These sophisticated molecular devices rely on a combination of internal hybridisation and internal fluorescence quenching to shield fluorescence until the target complex is formed. Molecular beacons can be used to quantitatively detect nucleic acids without the need to wash away unhybridised probes. This removes the need to immobilise the target DNA and therefore broadens the scope for experimentation using oligonucleotide probes. In particular beacons can be used to study dynamic processes. Molecular beacon probes have been used; to detect DNA mismatches,[42] for spectral genotyping of human alleles,[43,44] in the detection of bacterial rRNA[45] and mRNA,[46] and for the real-time monitoring of nucleic acid amplification.[46,47]

Molecular beacon oligonucleotides can form an internal hybrid to give a hairpin structure. This has a double-stranded stem (*ca.* 5 base pairs) and a single-

stranded loop (*ca.* 15 bases). The loop area carries the probe sequence. A fluorophore is attached to one arm of the stem and a quenching moiety is attached to the other. In aqueous solution the molecular beacon holds this hairpin conformation and the internal stem hybrid holds the fluorophore and the quencher in close proximity. Resonance energy transfer and collisional quenching between the dye and the quencher then leads to a diminished fluorescence quantum yield and the beacon can be thought of as 'switched off'.

When the molecular beacon encounters the single-stranded target nucleic acid sequence, the loop sequence can spontaneously form a longer and more stable hybrid than that contained in the stem. This process can be aided by warming the mixture to destabilise the initial hybrid. This process is fluorogenic because as the stem section of the probe melts to accommodate the new duplex region, the level of resonance energy transfer and collisional quenching decreases dramatically. The increase in spatial separation between the fluorophore and the quencher therefore increases the quantum yield for fluorescence and the fluorophore and the beacon can be thought of as being 'switched on'.

It is now necessary to design molecular beacons that give very efficient quenching in the stem and loop conformation (*i.e.* the beacon is very 'dark' when 'switched off') and a high quantum yield for fluorescence in the probe-target complex (*i.e.* the beacon is very 'bright' when 'switched on'). The current beacon design can be developed further by optimising the design of the fluorophore / quencher monomers. This presents a synthetic challenge. Some attempts to optimise a quenching monomer are detailed in Section 2.

1.8.0 Scorpion PCR Primers

Scorpion primers are diagnostic tools for the specific detection of PCR products in real-time. Scorpion PCR primers rely on energy transfer (quenching) between a fluorophore and a proximal quencher molecule to produce a fluorescent 'switch' leading to a fluorescent signal on PCR amplification.[48] The probe unit can be exactly the same as that used in molecular beacons.

The basic elements of Scorpions are:

(i) a PCR primer
(ii) a PCR stopper to prevent PCR read-through of the probe element
(iii) a specific probe sequence
(iv) a fluorescence detection system containing at least one fluorophore and quencher.

The loop section of a beacon within a scorpion probe can be chosen such that after PCR extension of the Scorpion primer, the resultant amplicon contains a sequence that is complementary to the probe. The complete amplicon, including the probe section is rendered single-stranded during the denaturation stage of the PCR cycle. On cooling the probe is free to bind to the complementary sequence, producing an increase in fluorescence, as the quencher is no longer in the vicinity of the fluorophore.

This leads to a unimolecular fluorogenic binding process within the amplified nucleic acid strand (see figure 1.8-1). This has obvious advantages over a bimolecular assay of the amplification reaction: Scorpions technology can be used in allelic discrimination.[49,50] The intramolecular probing mechanism of Scorpions offers significant advantages over other genotyping systems such as Taqman™,[51] molecular beacons [52] and hybridisation probes [53] that all rely on bimolecular probing. Unlike Taqman™ probes, Scorpions do not depend upon

enzymic cleavage, and therefore rapid PCR cycling is possible. The probe / target interaction is kinetically favoured over the re-annealing of the PCR strands or alternative intra-strand folding as unimolecular binding processes are effectively instantaneous in this system. In addition there is a direct proportionality between the intensity of the signal and the amount of amplicon as each amplicon yields one fluorescent signal.

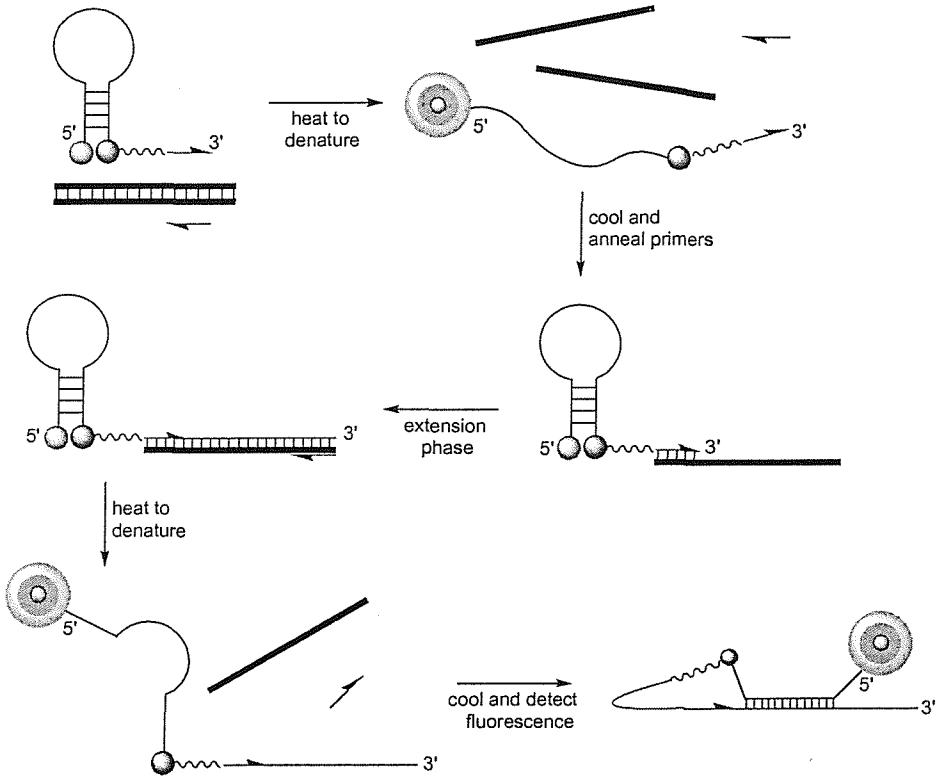


Figure 1.8-1: Sequence of Events During one Cycle of PCR Using a Scorpion Primer in Real-time Fluorescence Detection

As with molecular beacons, it is desirable to design scorpion PCR primers that give strong quenching in the stem and loop conformation (*i.e.* the scorpion is totally 'dark' when 'switched off' prior to PCR assembly). If this can be combined with a high quantum yield for fluorescence in the PCR product then there will be a strong fluorescent signal on amplification.

Section 2 Results and Discussion

2.1 Quencher Attachment *via* an Alkyl Glycoside

2.1.0 Overview

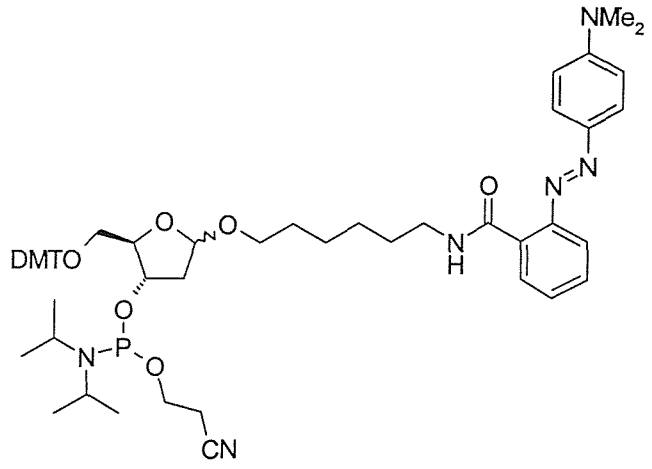


Figure 2.1-1: Aminoalkyl Deoxyribose Monomer (α-**52**)

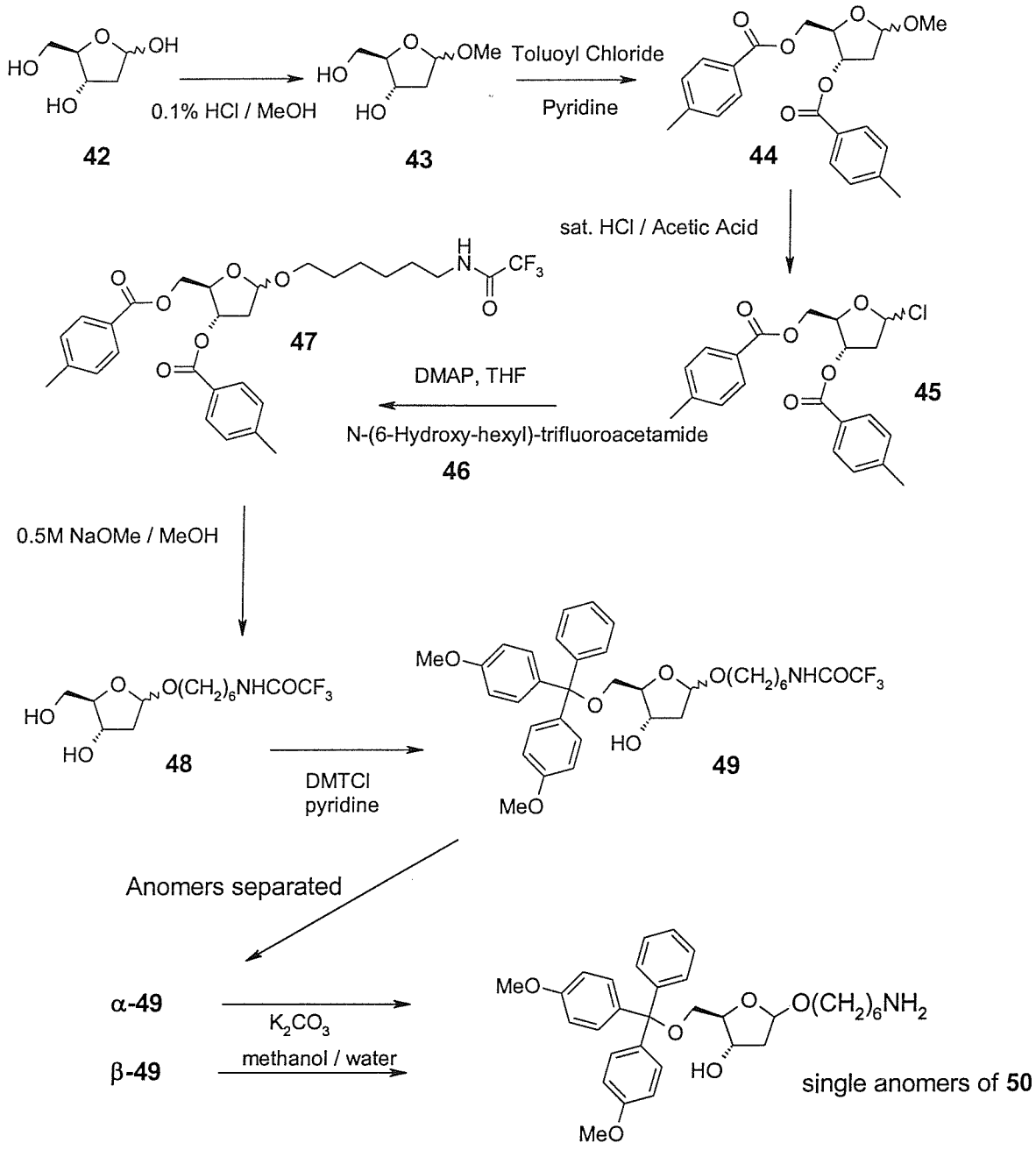
Compound (α-**52**) is a building block for incorporating the quencher methyl red within oligodeoxynucleotides. The flexible aminoalkyl linker arm was designed to allow the relatively mobile quencher to sit in a suitable spatial position for fluorescence quenching in molecular beacon and scorpion PCR experiments. Quenching was expected by both Forster energy transfer and direct collisional quenching.

The DMT protected C-5 hydroxyl group allows oligodeoxynucleotide chain extension to continue after attaching the monomer. This is therefore a multiple-addition monomer and can be incorporated at any position within an oligodeoxynucleotide sequence.

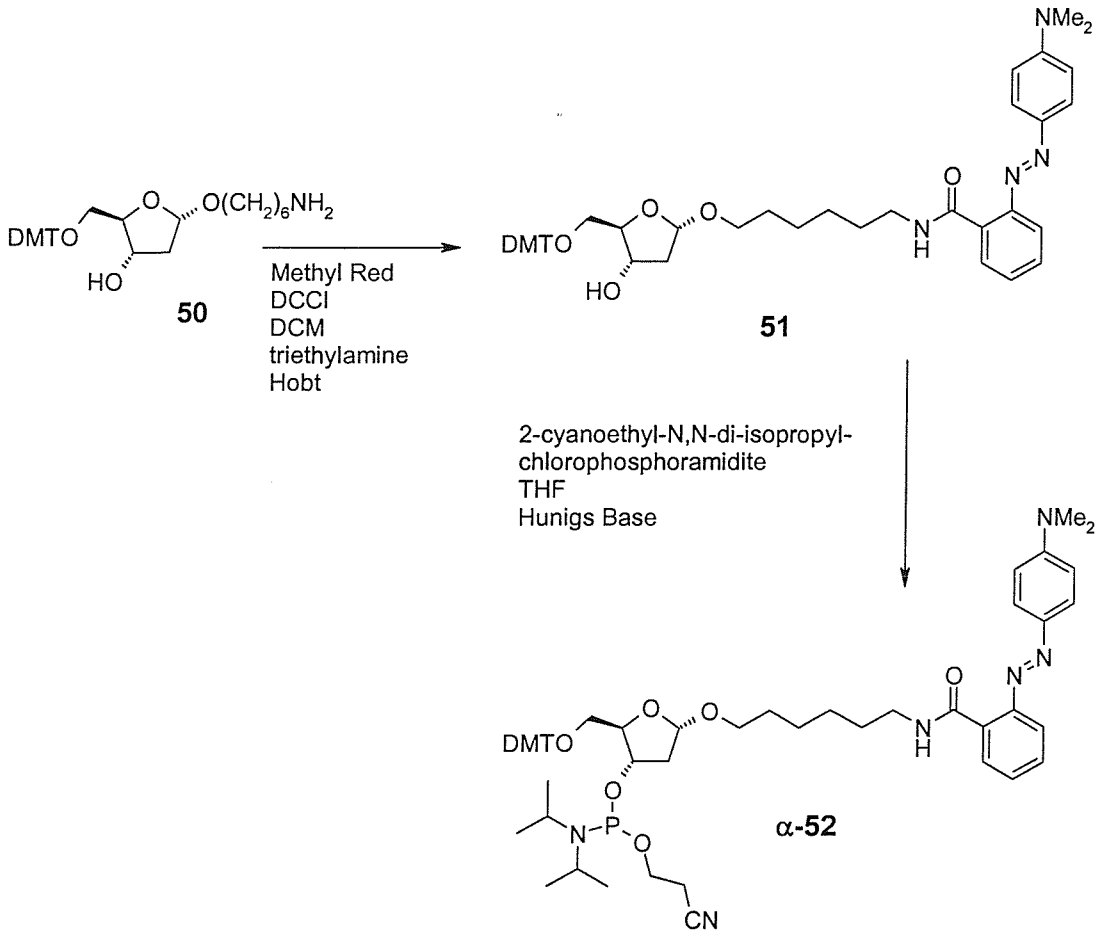
The full synthesis of monomer (α-**52**) is outlined in Schemes 1 and 2. The sugar chloride (**45**) of Hoffer was chosen as a suitable substrate for glycosidation with

the trifluoroacetylamidoalkyl aglycone **(46)**.[54] Intermediate **(45)** was also a potential substrate for asymmetric glycosidation reactions such as those of Paulsen *et al.*[55-57] The advantage of the diastereoselective route would have been a higher overall yield for the synthesis of the β-monomer which has the same anomeric configuration as natural nucleosides. Mimicry of this natural alignment may have implications regarding the suitability of labelled oligodeoxynucleotides as enzyme substrates (e.g. in PCR) and regarding duplex stability.

The synthesis then required cleavage of two ester protecting groups from the sugar hydroxyl functions. This cleavage could also have potentially removed the trifluoroacetyl protecting group from the aminoalkyl pendant arm. If that had happened it was thought that the reactivity of the resulting primary amine would have allowed selective re-protection. The next step in the strategy was to selectively protect the more reactive primary hydroxyl function of the sugar with a DMT ether group, as required for the standard DNA monomer protecting methodology. Cleavage of the amide protecting group at this stage under basic conditions would liberate the highly reactive terminal amino group which would then be selectively coupled to the carboxylic acid dye fragment in the presence of the unreactive sugar 3'-OH group. It is important to avoid phosphitylation until the end of the synthesis because phosphoramidites such as monomer **(α-52)** are air sensitive.

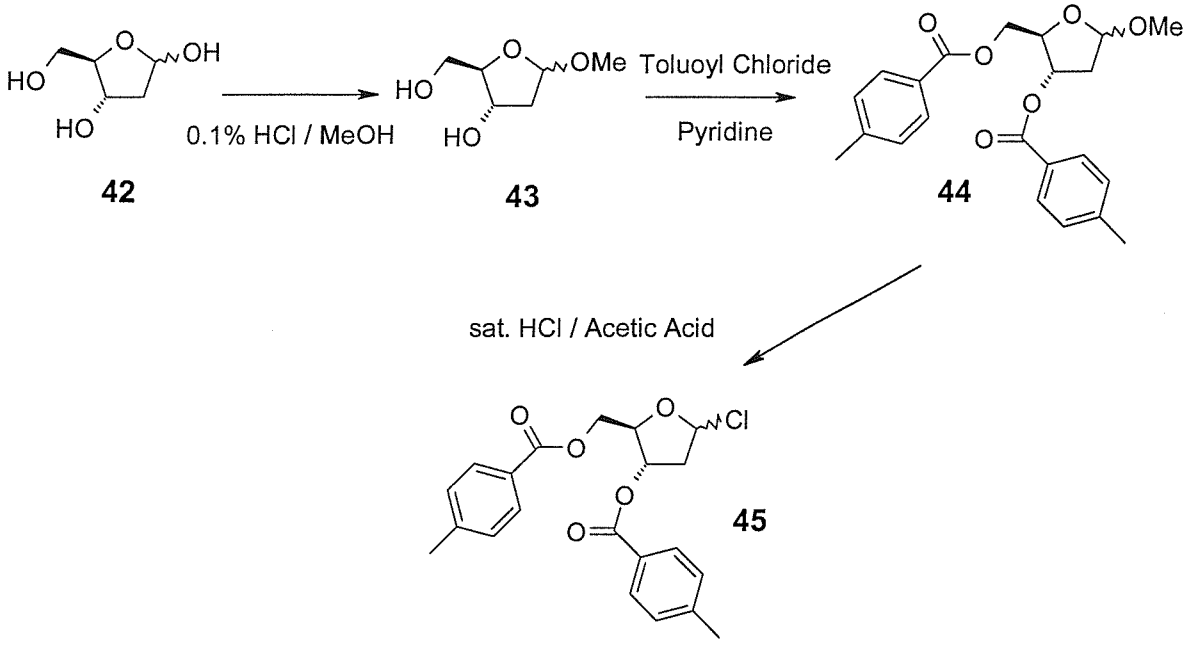


Scheme 1: Towards Aminoalkyl Deoxyribose Monomer (α-52)



Scheme 2: Towards Aminoalkyl Deoxyribose Monomer (α-52)

2.1.1 Preparation of Sugar Chloride (45)



Scheme 1a: Preparation of Sugar Chloride **(45)**

The sugar chloride **(45)** was a key intermediate for all four monomers. This intermediate was derived from deoxyribose which is an expensive starting material. Unfortunately yields from repeating the literature syntheses of the sugar chloride were found to be very variable. A period of time was therefore spent in developing the process to give more repeatable yields and greater product purity. The molecule has been prepared from deoxyribose in three stages in two closely related literature syntheses. The earlier synthesis was described by Hoffer in 1960 in which halogenation of diester **(44)** is effected by passing hydrogen chloride gas through a solution of the substrate in glacial acetic acid at ambient temperature.[54] A variant of the synthesis was developed by Kotera *et al* in response to the poor repeatability of the earlier procedure. In the Kotera method the HCl is produced *in situ* from a solution of acetyl chloride in glacial acetic

acid.[58] This paper reported an improvement both in product purity and in overall yield; also no chromatography was required.

It has been possible to improve the repeatability of this chemistry by prompting a precipitation in the final stage. The methyl glycoside of deoxyribose **(42)** was prepared from deoxyribose by a Fischer glycosidation in quantitative yield by treating with 0.1% HCl / MeOH at ambient temperature for 1 hour using the conditions of Hoffer.[54] The two hydroxyl functions of compound **(43)** were then protected with *p*-toluoyl esters by the action of *p*-toluoyl chloride in pyridine at 65°C for two hours, again using conditions from the Hoffer synthesis. The Kotera synthesis recommended stirring at ambient temperature overnight for this step. In fact it was found that this reaction took three days to reach completion. The crude output from stage 2 could be taken on directly to stage 3 without chromatography, as reported in the Kotera synthesis.[58]

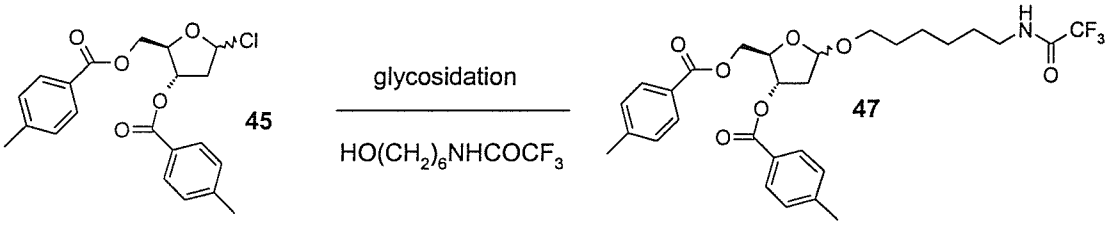
Both the Hoffer and the Kotera conditions for the halogenation at stage 3 were found to give poor repeatability both in terms of chemical purity (by NMR) and with regard to theoretical yield {between 30% and 77% from compound **(44)**}. The furanosyl chloride was seen to be sensitive to chromatography and to crystallisation and routine purification was carried out by simply washing with the minimum of cold diethyl ether.

The chlorination reaction (stage 3) relies on protonation and displacement of the C_1 methoxy group of the glycoside **(44)** by treating with a solution of hydrogen chloride in glacial acetic acid. The less polar furanosyl chloride **(45)** forms a precipitate. The reaction was repeated a number of times and several observations were made. The furanosyl chloride **(45)** was found to be generally unstable in solution, as evinced by very poor recovery from recrystallisations and the rapid darkening of solutions in most solvents. This instability in solution was seen as the most probable cause for the poor repeatability of this step.

In stage 3 the time period before spontaneous precipitation of the product was seen to be variable and the high yielding reactions were those in which the product precipitated quickly. It was thought that perhaps a lack of nucleation sites might impede crystallisation of the product and the yield, the repeatability and the NMR contaminant profile were all improved dramatically by scratching inside the flask with a glass rod within the first few minutes of the reaction.

The HCl source of Kotera was easier to control than the gaseous source of Hoffer. The moisture sensitive furanosyl chloride **(45)** was prepared in 60% yield (lit. 58%)[58] over the three steps by this modification of the Kotera route. The isolated product, an off-white solid, could be taken through without further purification. The NMR spectrum was in agreement with the literature and indicated >95% purity. However the melting point of this precipitate was almost 10°C lower than that of the literature sugar chloride (m.p. 98-105°C *versus* lit.[57] 107-109°C). A sample was purified for analysis by crystallisation from anhydrous diethyl ether. This led to recovery of a white, crystalline solid with m.p.; 104-107°C (crop 1), 106-108°C (crop 2) and 105-109°C (crop 3) with a 65% loss of material. The NMR profile of the material was not obviously changed from that obtained prior to crystallisation but the material recovered by evaporation of the final liquors was very impure. The loss of material during recrystallisation was therefore a further indication of the sensitivity of the molecule to degradation in solution.

2.1.2 Koenigs-Knorr Glycosidation



Scheme 1b: Glycosidation

The Koenigs-Knorr glycosidation can be used to create glycosidic bonds through the reaction between an aglycone unit and a reactive glycosyl halide that acts as a glycosyl donor (the halogen is usually a chlorine or bromine atom). Heterogeneous catalysis can be exploited to give a characteristic inversion at the anomeric centre, presumably by a concerted mechanism. Suitable catalysts include porous silver silicates,[55-57] silver zeolites,[59,60] silver carbonate and silver oxide.[61]

A modest diastereoselectivity was found for the alpha-anomer of glycoside **(47)** using the porous silver silicate promoter of Paulsen *et al*, with aglycone **(46)** in anhydrous DCM at -15°C.[55-57] The selectivity and rate of this reaction were improved by adding a quantity of activated, finely divided 3Å molecular sieves, giving a 49% d.e. for the alpha anomer with 89% yield.

The retention of configuration at the anomeric position was unexpected and was in contrast with analogous reactions in the literature. At this point in the synthesis it was necessary to confirm the stereochemistry of the two glycoside anomers.

All furanose sugars and their derivatives have non-planar ring geometries. As with cyclopentane derivatives, envelope (E) and twist (T) conformations are usually favoured and conformational geometry is less clearly defined than in six-membered rings. Envelope conformations have four atoms in the same plane and one atom removed from the plane. Twist geometries have three adjacent, co-planar atoms, with the other two centres above and below the plane respectively (see Figure 2.1-3). In the neighbourhood of these exoplanar atoms the exocyclic bonds correspond roughly to the axial and equatorial bonds of cyclohexanes. The two orientations are described as *quasi*-axial (a′) and *quasi*-equatorial (e′). Substituents at these points favour e′, as in cyclohexanes.

Envelope Twist

Figure 2.1-2 Envelope and Twist Conformations of 5 Membered Rings

The consequence of these conformational properties is that the Karplus relationship can rarely be used with reliability in furanoid systems and therefore other techniques must be used.[62] Single isomers of samples of compounds **(47)**, **(48)**, **(49)**, **(50)**, and **(51)** were screened in crystal growth using single solvent, mixed solvent and vapour diffusion techniques. These compounds however were seen to be oils or amorphous solids

A number of derivatives were then synthesised in search of a crystalline compound suitable for x-ray analysis. The derivatisation of primary amines as thiourea compounds is historically a reliable way of producing crystalline compounds. The α-anomer of amino compound **(α-50)** was reacted with phenyl isothiocyanate in ethanol, by stirring at room temperature for 1.5 hours to give the thiourea derivative in 57% yield after chromatography. In this case the thiourea could not be induced to crystallise and was recovered as an oil. The β-anomer of compound **(β-50)** was derivatised with an N-2,4-dinitrophenyl group using Sanger's reagent to give an isolated yield of 64%. This compound could not be induced to crystallise. This lack of crystallinity was attributed to the presence of the DMT ether group. The DMT ether group was cleaved from the

compound by treating with 80% acetic acid in water, leading to the diol in an isolated yield of 43%. This compound however was an oil.

Finally the major isomer of glycoside **(47)**, initially an oil, crystallised after standing at ambient temperature for several months and provided several crystals suitable for single crystal x-ray crystallography. An x-ray crystal structure was obtained and the indicated configuration at the anomeric centre was assigned unambiguously. (See Figure 2.1-3)

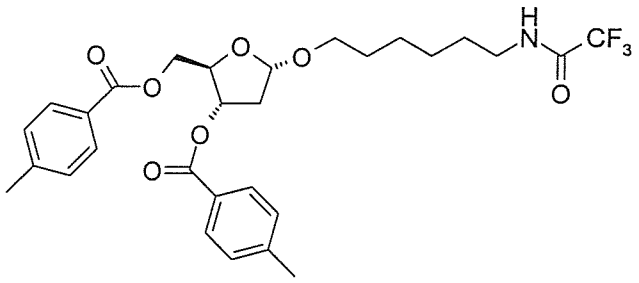
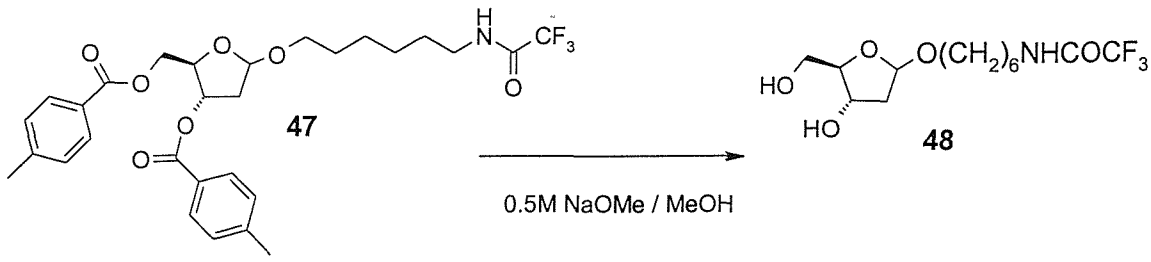


Figure 2.1-3: Glycoside **(α-47)** (Major Isomer) Configuration Determined by X-ray Crystallography

2.1.3 Mixed Diastereomer Glycosidation

The glycosidation step between the furanosyl chloride **(45)** and the aglycone N-(6-hydroxy-hexyl)-trifluoroacetamide **(46)** was carried out under homogeneous conditions to give a 1:1 mixture of diasteromers by ^{1}H-NMR integration. The homogeneous reaction used 0.2 eq. DMAP in THF at ambient temperature to give a 69% yield of a 1:1 mixture of anomers of glycoside **(47)** after chromatography. The diasteromers could be separated at this stage by exhaustive chromatography followed by crystallisation although separation by chromatography was later found to be easier after DMT protection.

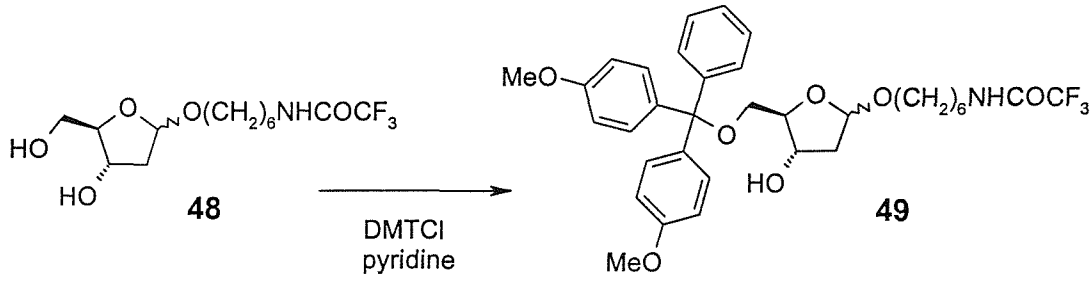
2.1.4 Hydroxyl Deprotection



Scheme 1c: Alcohol Deprotection

The *p*-toluoyl ester protecting groups were cleaved from the hydroxyl groups of a mixture of anomers of the glycoside **(47)** by treatment with 0.5M NaOMe at ambient temperature for 90 minutes. This gave compounds **(48)** in 80% yield after chromatography. These conditions were sufficiently mild to allow selectivity between the esters and the amide protecting group on the primary amine. The same conditions were then used to cleave the ester groups from samples of the individual anomers of glycoside **(47)**, giving isolated yields of 82% for the β isomer and 63% for the α isomer with no change to the stereochemistry at C1 as seen by ^{1}H-NMR integration.

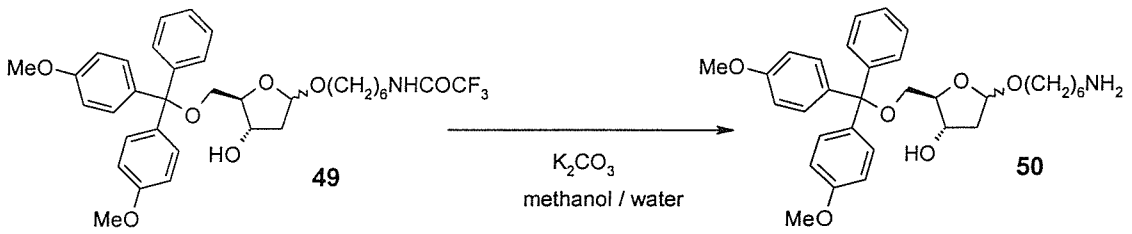
2.1.5 DMT Ether Formation



Scheme 1d: DMT Protection

The primary C-5 hydroxyl functions of diols **(48)** were then protected selectively with DMT by treating a mixture of anomers with 1.1 eq. DMT chloride in pyridine at ambient temperature for 2 hours. This gave an isolated yield of 80% after chromatography. The two anomers of compound **(49)** could easily be separated by wet flash chromatography at this stage and were isolated as amorphous solids. The ratio of anomers was seen by ^{1}H-NMR to be unaffected by this step which indicated that DMT protection was in no way selective for either anomer. The same conditions were used to protect the individual anomers of diol **(48)** and gave yields of 62% for the β anomer (with 9% of the bis-dimethoxytrityl compound also being recovered) and 77% for the α anomer. The discrepancy in yield between the two anomers here was due to mechanical losses because the reactions were carried out on a small scale.

2.1.6 Cleavage of the Trifluoroacetamide



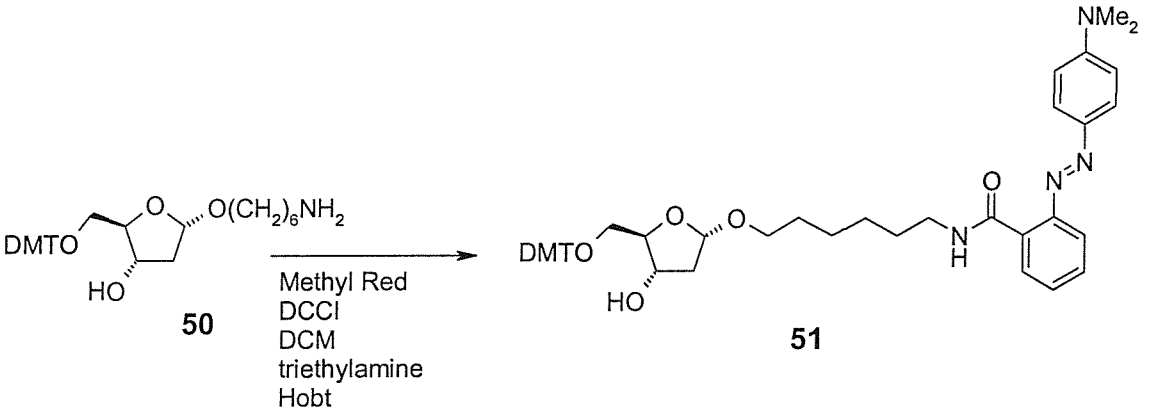
Scheme 1e: Amine Deprotection

The N-trifluoroacetyl protecting group was initially removed from the α-anomer of compound **(49)** by treating with 2.5 eq. hydrazine monohydrate in anhydrous THF / methanol (5:2), at ambient temperature. This reaction took 60 hours and did not proceed to completion. Chromatographic purification of a portion of the crude product showed that amine **(α-50)** had been formed in 63% yield. The reaction was repeated using 4 eq. of the hydrazine monohydrate in the same solvent mixture, and was heated at 50°C for 5 hours giving 68% yield after chromatography. The poor yields and the toxicity of hydrazine monohydrate

prompted the screening of other conditions for this step. The alternative conditions of stirring with 2 eq. K_2CO_3 in MeOH / H_2O (8.5:1.5) at 60°C for 45 minutes, gave a 91% yield after chromatography.

The methanolic deprotection was carried out on a small scale in 66% yield using the β anomer of the amide **(49)**. The basic reaction conditions did not effect the stereochemistry at the anomeric position of either anomer, as seen by NMR.

2.1.7 Coupling to Methyl Red



Scheme 2a: Coupling to the Quencher

Compound **(α-50)** was treated with 1.1 eq. methyl red, 1.5 eq. DCCI, 1.5 eq. 1-Hobt and 2 eq. Et_3N in DCM at ambient temperature for 14 hours and gave dye conjugate **(α-51)** in 85% yield after chromatography. This methyl red conjugate was isolated as an amorphous, red solid.

2.1.8 Phosphitylation

The C-3 hydroxyl function of dye conjugate **(α-51)** was phosphitylated by treating with 1.2 eq. 2-cyanoethyl N,N-diisopropylchlorophosphoramidite and DIPEA / anhydrous THF (1:6) at ambient temperature for 30 minutes. This gave the

methyl red phosphoramidite monomer **(α-52)** in 74% yield after work up and further purification by two cycles of precipitation from n-hexane at -78°C.

Compound **(α-52)** was tested in solid phase oligonucleotide synthesis and gave coupling efficiencies of 96.8% and 96.4% respectively for additions of this monomer in the DNA test oligonucleotide TMTMTT {where M denotes the methyl red monomer **(α-52)**}.

Overall this synthesis of compound **(α-52)** gave a 10.2% yield over 9 steps. The protecting group strategy was a success. The asymmetric glycosidation gave a surprising retention of configuration and was therefore of no use as a route to the β-series, however compound **(α-52)** was seen to be a useful building block for molecular beacons (Section 2.5).

2.2 Palladium Catalysed Synthesis of Alkynamino Nucleoside analogues

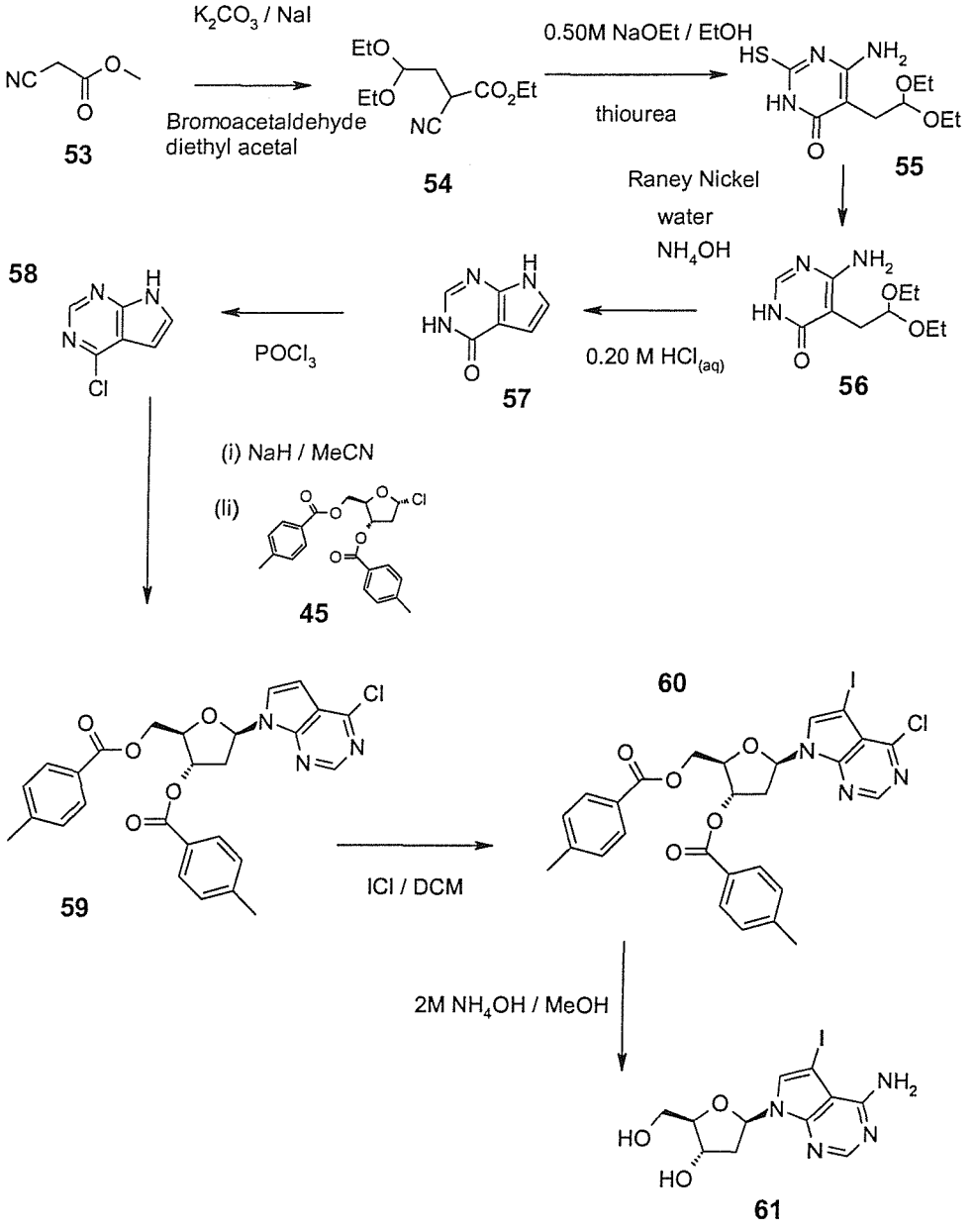
2.2.0 Overview

It would be good to design molecular beacons that give near total quenching of fluorescence when in the stem and loop conformation (*i.e.* the beacon is totally 'dark' when 'switched off'). If this can be combined with a high quantum yield for fluorescence in the probe-target complex then there will be a strong fluorescent signal on hybridisation. The 7 positions of purines and 7-deaza purine nucleosides and the C-5 position of pyrimidines have been used in the literature as sites to direct substituents into the major groove of double-helical DNA.[63-66] The major groove may be good site for quenching within molecular beacon stem sections as the physical constraint of the dye and quencher within the major groove may increase collisional quenching. The spatial aspect of quencher / fluorophore association may well be governed by competing forces between the dye / quencher and the solvent (water) or the DNA itself. Either way a good starting point for investigation is the synthesis of monomers that can physically direct the dye and quencher together. Pyrimidine monomers with C-5 attachment of fluorescein are commercially available. 7-Functionalised purine analogues can be accessed *via* the 7-deaza-iodo compounds.

Iodo-nucleosides and analogues are used as common intermediates towards more complex molecules. Alkynes can easily be attached to iodo-modified nucleosides under Sonagashira palladium coupling conditions. The formation of alkynyl derivatives of 5-iodo-2'-deoxycytidine, 5-iodouridine, 5-iodo-2'-deoxyuridine, 5-iodo-2',3'-dideoxycytidine, 5-iodo-2',3'-dideoxyuridine and 7-iodo-2',3'-dideoxy-7-deazapurine nucleosides has been reported this way.[67-70] This chemistry occurs under mild conditions and has been shown to be very versatile.

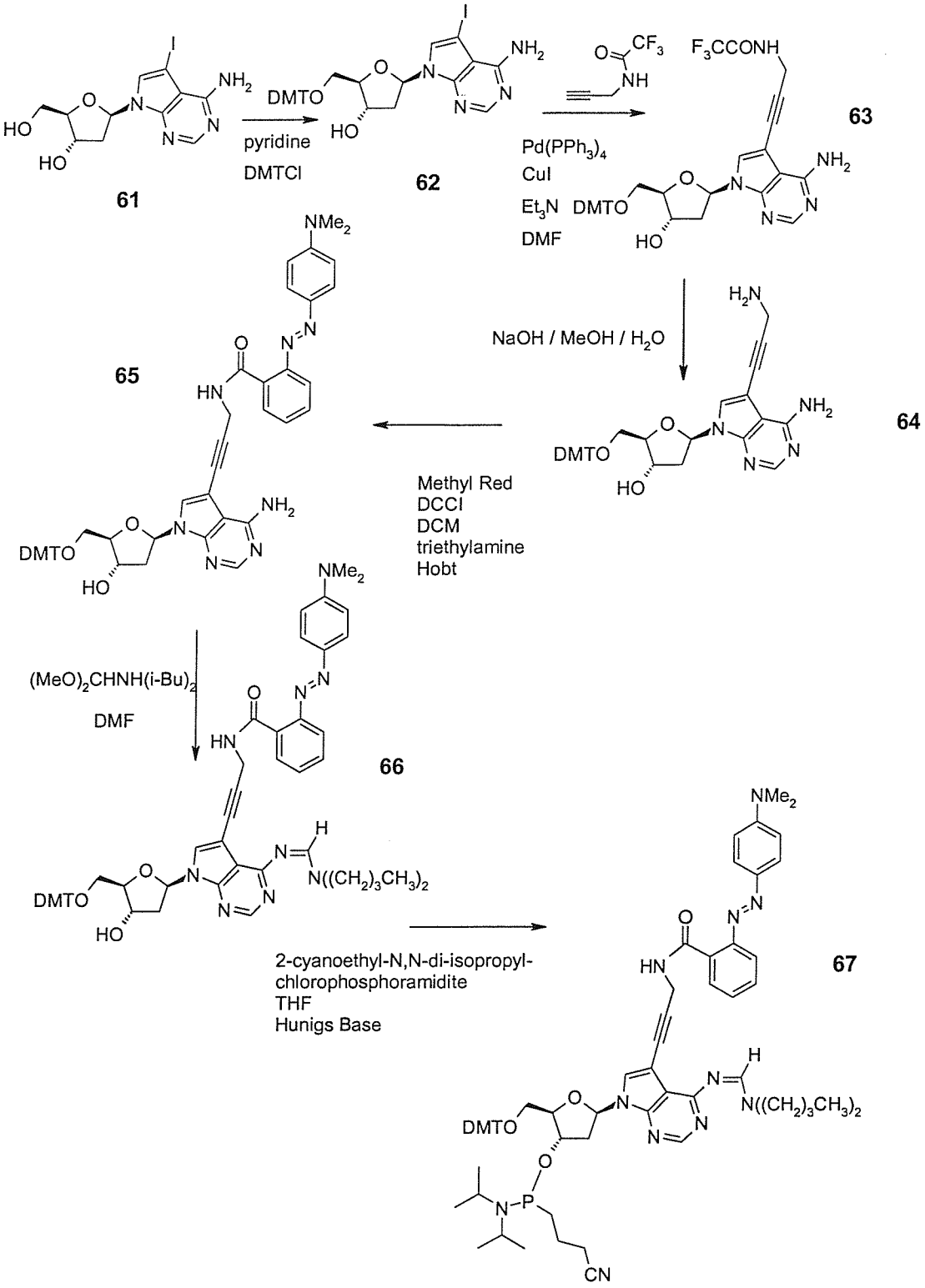
Pyrrolo[2,3-d]pyrimidines have been used in the literature to prepare 7-iodo-7-deaza analogues of purine nucleosides.[71-73]

Compounds **(54)** through to **(58)** (Scheme 3) were synthesised following the literature procedure of Davoll.[71] Compound **(59)** was prepared using the procedure of Kazimierkzuk *et al.*[72] Compounds **(60)** and **(61)** were synthesised following the literature procedure of Froehler *et al.*[73]



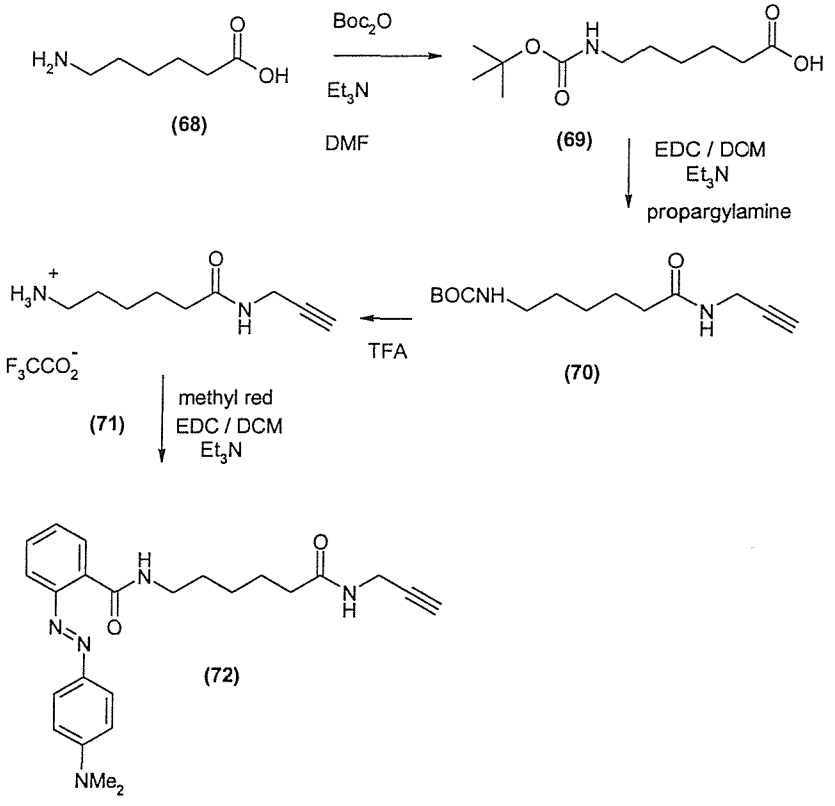
Scheme 3: Towards 7-Iodo-2'-deoxy-7-deazaadenosine **(61)**

Compound **(61)** was derivatised in further reactions that attach propargylamine and then couple to methyl red *via* an amide bond (Scheme 4) leading to 4-(di-n-butylamino-methyleneamino)-7-(O-(5-bis-(4-methoxyphenyl)-phenylmethyl)-O-(3-(2-cyanoethyl-N,N-diisopropyl)-phosphoramidite)-2-deoxy-β-D-ribosyl)-5-(3-N-(4-dimethylamino-phenylazo-benzamido)-prop-2-ynyl)-pyrrolo[2,3-d]pyrimidine **(67)** in 14 steps with a yield of 0.9%. The practical impact of the low overall yield was reduced because the low-yielding steps were in the first half of the synthesis. These early reactions were scaled-up accordingly to provide enough material for the second half of the synthesis. Many of the yields for the later reactions were effectively quantitative and material was then mainly lost during purification. Chemical purity (by NMR, TLC and LCMS) was generally maintained at each stage by chromatography or crystallisation.

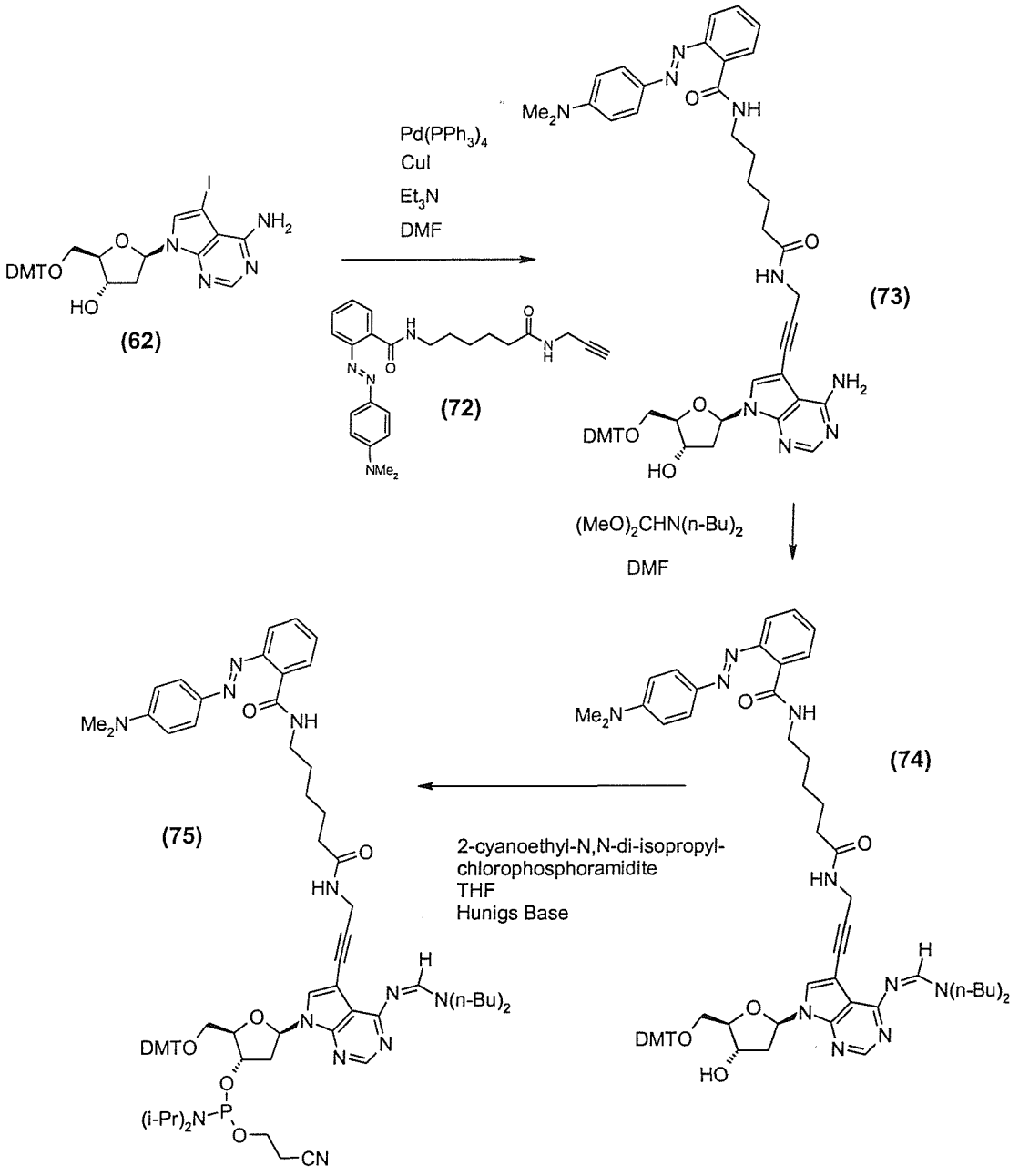


Scheme 4: Construction of Phosphoramidite Monomer **(67)**

Compound **(61)** was also derivatised with tethered methyl red fragment **(72)** and taken on to give monomer **(75)** (Schemes 5 and 6). Overall 4-(di-n-butylamino-methyleneamino)-7-[(O-5-bis-(4-methoxyphenyl)-phenylmethyl)-2-deoxy-β-D-ribosyl-O-3-(2-cyanoethyl-N,N-di-isopropyl-phosphoramidite)]-5-(6-(2-(dimethylamino-phenylazo)-benzoyl)-prop-2-ynyl-hexanamido)-pyrrolo[2,3-d]pyrimidine **(75)** was synthesised in 0.73% yield over 12 steps. This convergent tactic was much simpler than a stepwise synthesis of the methyl red conjugate. The success of this strategy was due to the robustness of the Sonagashira palladium coupling step.

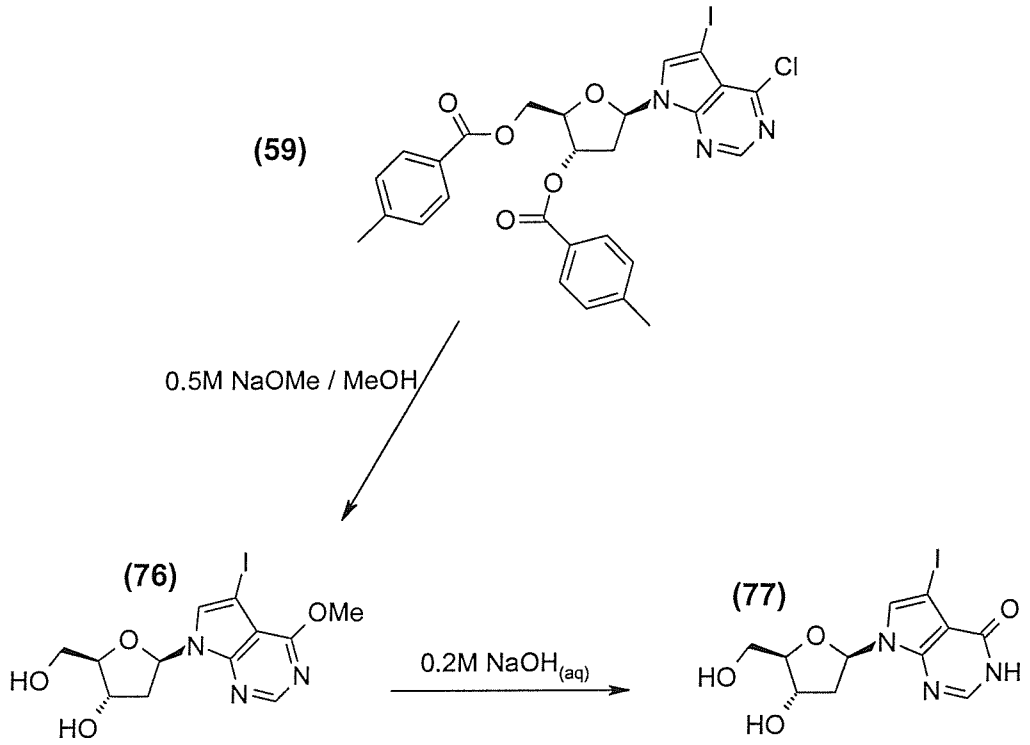


Scheme 5: Synthesis of the Tethered Methyl Red Fragment **(72)**



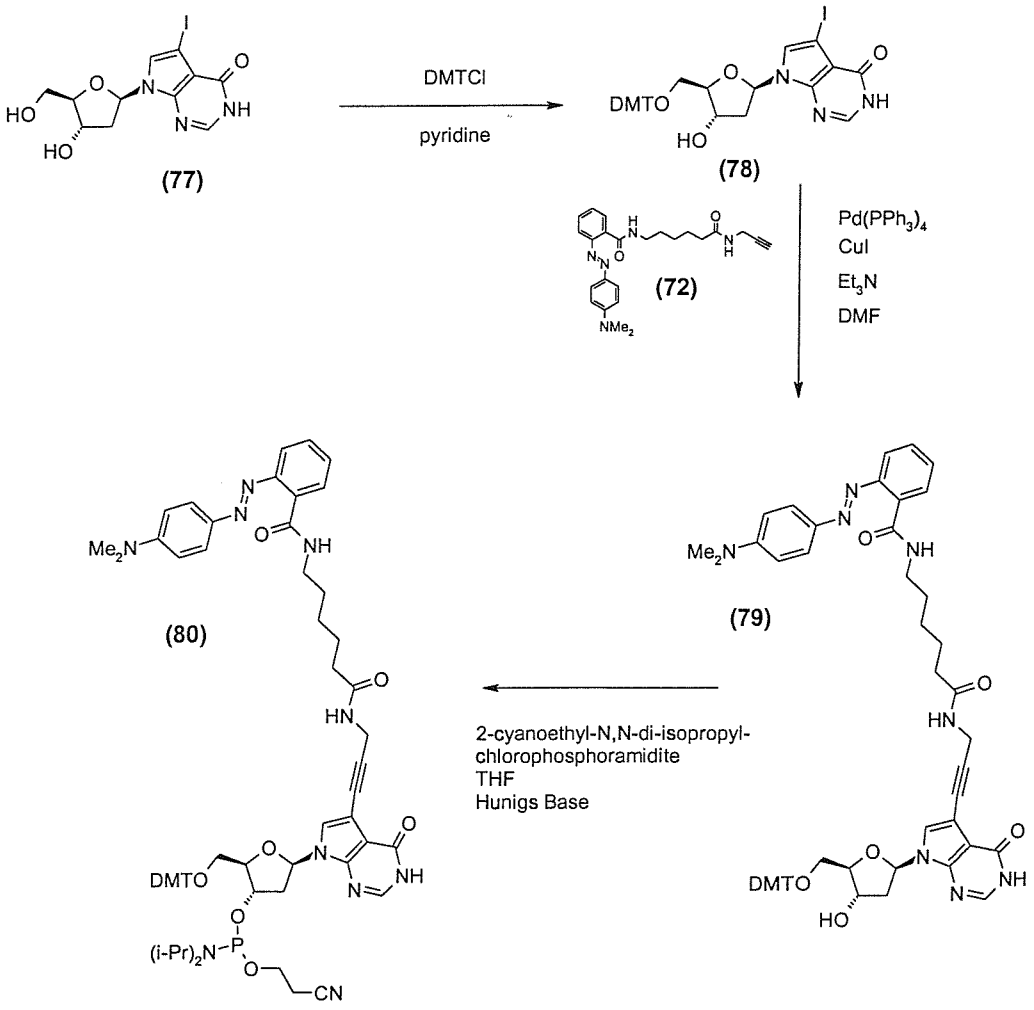
Scheme 6: Synthesis of the Methyl Red Adenosine Monomer **(75)**

Compound **(59)** was converted to compound **(77)** in 2 steps using the general methods of Seela *et al* to offer access to the 7-deaza inosine series (Scheme 7).[75-77]



Scheme 7: Formation of the Inosine Analogue **(77)**

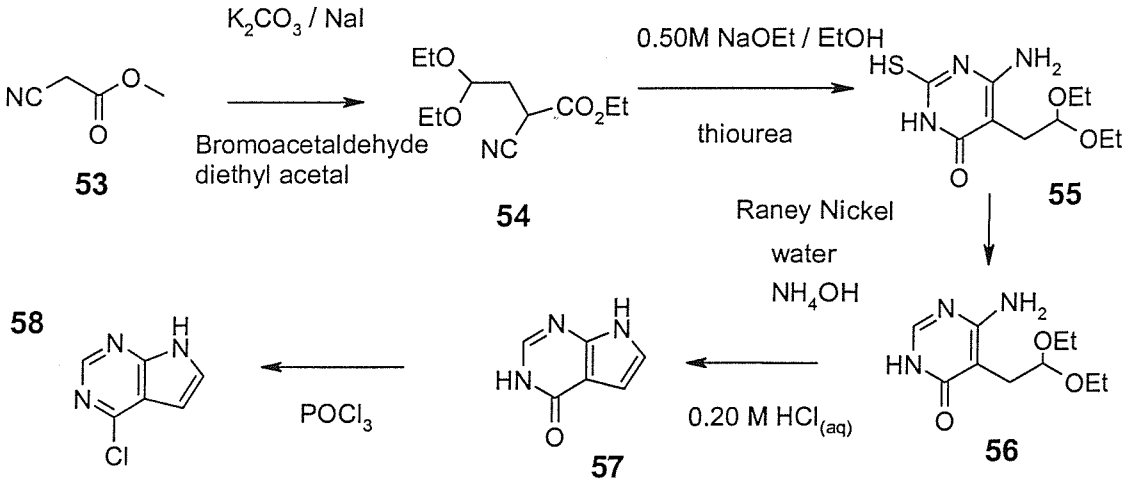
Compound **(77)** was DMT protected and coupled to tethered methyl fragment **(72)** by a Sonagashira palladium coupling reaction using general conditions taken from Froehler *et al* and then taken on to give phosphoramidite **(80)** (Scheme 8).[74] Overall 7-[O-(5-bis-(4-methoxyphenyl)-phenylmethyl)-2-deoxy-β-D-ribosyl-O-3-(2-cyanoethyl-N,N-diisopropyl-phosphoramidite)]-5-(6-(2-(dimethylamino-phenylazo)-benzoyl)-prop-2-ynyl-hexanamido)-pyrrolo[2,3-d]pyrimidin-5-one **(80)** was synthesised in 0.50% yield over 12 steps.



Scheme 8: Preparation of Monomer **(80)**

2.2.1 Enolate Displacement

Ethyl 2-cyano-4,4-diethoxybutanoate **(54)** was prepared with a low yield of 38% {lit. 46%}[71] using the procedure of Davoll.[71] This was 8% lower than the literature yield, perhaps due to losses on distillation. The low initial yield was tolerated as the first 5 steps in this synthesis were carried out on a litre scale.



Scheme 3a: Towards Chloro Compound **(58)**

2.2.2 Condensation with Thiourea

4-Amino-5-(2,2-diethoxyethyl)-6-hydroxy-2-pyrimidinethiol **(55)** was prepared from compound **(54)** by a condensation reaction with thiourea iin the presence of sodium ethoxide using the procedure of Davoll.[71] The literature work up and purification were changed to allow purification of the product without chromatography. The product was formed in the reaction as a sodium thiolate. This could be precipitated from the aqueous medium by a simple acidification with acetic acid. The crude precipitate was recovered in near quantitative yield but was purified by crystallisation from methanol with an isolated yield of 59% {lit. 82%},[71] which was lower than the literature yield because of the losses encountered on crystallisation. The simplicity of the purification and the quality of the crystalline product compensated for the drop in yield. The shortfall in material was accounted for by evaporation of the mother liquor and this material was held as a crude solid.

2.2.3 Reduction of the Thiol

The thiol group was cleaved from compound **(55)** using the procedure of Davoll to give 4-amino-5-(2,2-diethoxyethyl)-6-hydroxypyrimidine **(56)** by heating an aqueous solution of **(55)** with Raney nickel for 1 hour at reflux. The procedure for work up and purification was modified to improve the purity of the product, which was obtained by filtration, concentration and crystallisation from methanol. This gave compound **(56)** in 74% yield {lit. 75%}.[71]

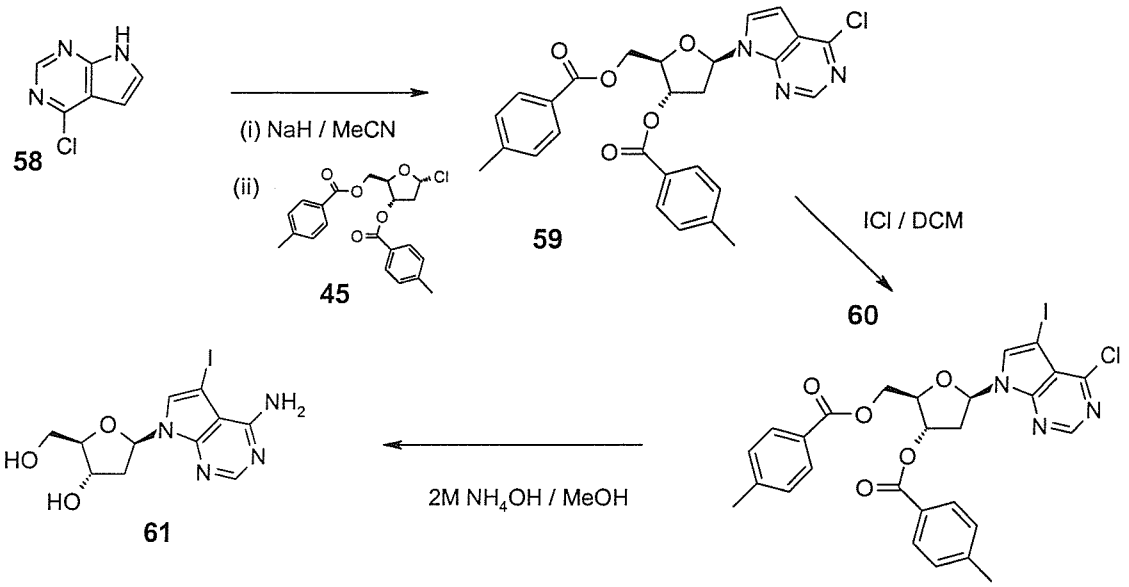
2.2.4 Ring Closure by Condensation

4-Hydroxy[2,3-d]pyrimidine **(57)** was readily prepared by shaking a suspension of **(56)** in dilute aqueous acid for 24 hours using the procedure of Davoll.[71] The product **(57)** was obtained in 91% yield by filtration {lit. 96%}.[71]

2.2.5 Chlorination of Keto Compound (57)

4-Chloropyrrolo[2,3-d]pyrimidine **(58)** was prepared in 44% yield {lit. 82%}[71] by heating a solution of compound **(59)** in $POCl_3$ at reflux for 45 minutes after the procedure of Davoll.[71] The product was obtained after extraction between DCM and water and evaporation of the organic extract. The shortfall in material was later found to be due to losses to the aqueous phase during the extraction. At the time this did not present a problem as sufficient material was taken through for the remainder of the synthesis.

2.2.6 Diastereoselective Glycosidation



Scheme 3b: Steps to Nucleoside Analogue **(61)**

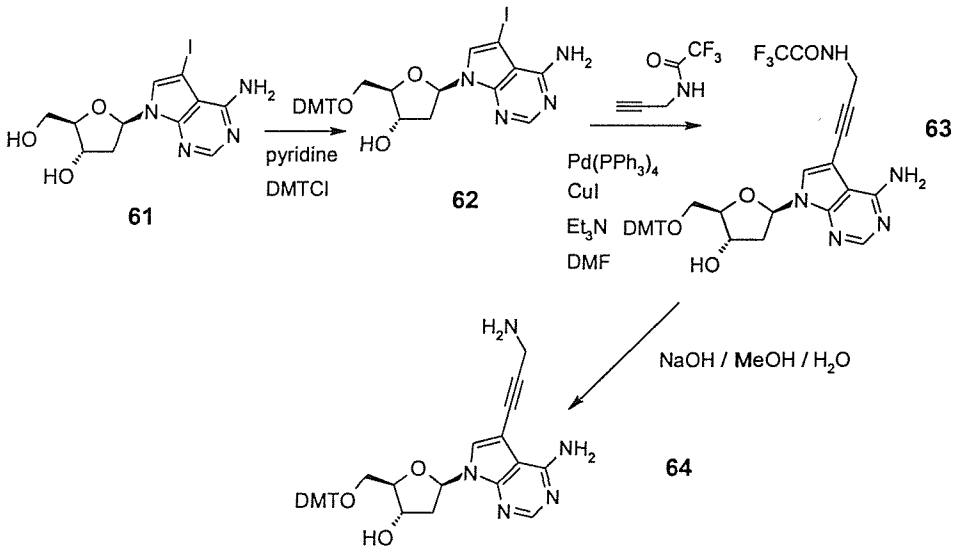
The aglycone **(58)** was combined with 1-chloro-2-deoxy-3,5-di-O-*p*-toluoyl-α-D-*erythro*-pentofuranoside **(45)** to give 4-chloro-7-(2-deoxy-3,5-di-O-*p*-toluoyl-β-D-*erythro*-pentofuranosyl)pyrrolo[2,3-d]pyrimidine **(59)** using the literature procedure of Kazimierkzuk *et al.*[71] The sodium salt of the aglycone was pre-formed by treating with sodium hydride in acetonitrile prior to reacting with the sugar chloride **(45)**. This procedure gave the nucleoside analogue **(59)** in 50% yield {lit. 71%}[72] after chromatography and crystallisation from ethyl acetate / hexane as the β-anomer. The shortfall in material over the literature reaction was accounted for on evaporation of the mother liquor. This material was held as a crude solid.

2.2.7 Iodination of Chloro Compound (59)

Compound **(59)** was iodinated by stirring overnight with iodine monochloride and sodium carbonate in DCM at ambient temperature using the literature procedure of Froehler *et al.*[73] The product **(60)** was obtained in 84% {lit. 88%}[73] yield by trituration of the organic concentrate.

2.2.8 Aminolysis of (60)

Compound **(60)** was simultaneously deprotected at the 3' and 5' hydroxyl positions of the sugar and aminated at the heterocycle using the literature procedure of Froehler *et al* to give compound **(61)** in 95% yield after crystallisation from methanol (lit. 60%).[73] The aminolysis required a 24 hour treatment with 2M NH_4OH / MeOH at 150°C in a Parr hydrothermal bomb. The increased yield over the literature route for this step was due to an improved work up because the product was purified by crystallisation rather than by chromatography.



Scheme 4a: Steps Towards Monomer **(67)**

2.2.9 DMT Protection of (61)

Compound **(61)** was protected at the primary hydroxyl position with a DMT ether group, under standard conditions by stirring for 90 minutes at ambient temperature with 1.2 eq. DMT-Cl in anhydrous pyridine to give compound **(62)** in 60% yield after chromatography. This was quite a poor yield for a DMT protection however no development work was done at this stage because the early stages of this synthesis had been carried out on a suitably large scale.

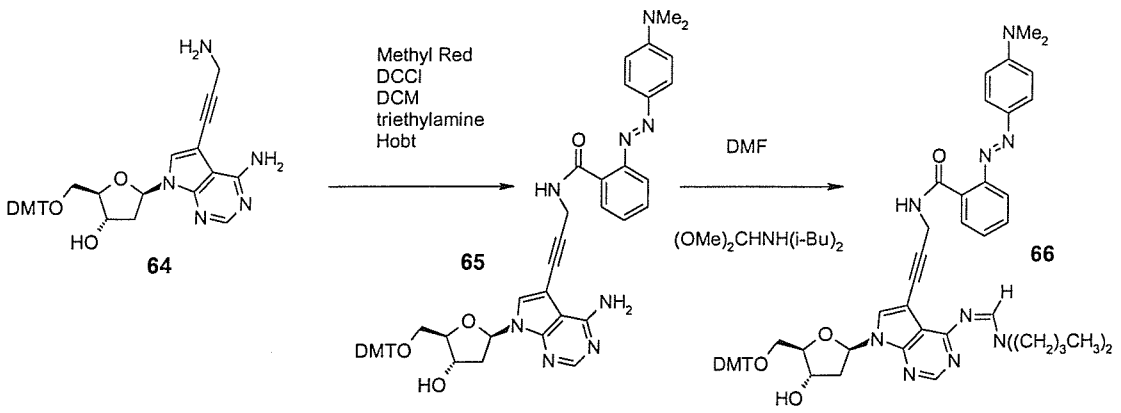
2.2.10 Palladium Coupling to Iodo Compound (62)

Compound **(62)** was converted to compound **(63)** in 97% yield under Sonagashira conditions by stirring at ambient temperature with $Pd(PPh_3)_4$ (0.18 eq.), CuI (0.36 eq.), N-prop-2-ynyl-trifluoroacetamide (5.4 eq.), triethylamine (18 eq.), in anhydrous DMF overnight using the general procedure of Froehler *et al.*[73] Compound **(63)** was purified by flash column chromatography followed by precipitation from hexane.

2.2.11 Nitrogen Deprotection of Compound (63)

The N-trifluoroacetyl protecting group of compound (**63**) was cleaved by treating with 2M methanolic methylamine at ambient temperature overnight, on a trial scale. The reaction did not give a clean profile by TLC hence a trial deprotection was attempted using instead 0.5M NaOH in H_2O / methanol (1:10). The TLC profile was much cleaner and flash column chromatography lead to a recovered yield of 74%. This reaction was repeated on a larger, preparative scale, leading to an acceptable recovered yield of compound **(64)** of 87% as a white solid.

2.2.12 Coupling to Methyl Red



Scheme 4b: Conjugation to the Quencher and Formamidine Protection

The preparation of the Hobt ester of methyl red was investigated using DCCI (1.3 eq.) and Hobt (1.2 eq.) in a range of solvents at ambient temperature. The solubility of both Hobt and methyl red in DCM was fairly low and solubility was found to be much better in pyridine, THF and DMF. Aqueous work-up of TLC samples (partitioning between ether / water) lead to rapid hydrolysis of the active ester, as seen by TLC. In addition the active ester was found to be sensitive to chromatography and TLC lead to hydrolysis but pre-treatment of the silica TLC plates with 10% triethylamine / DCM followed by oven drying minimised this. All these reactions were complete after 1 hour and each gave principally one component (presumed to be the methyl red Hobt ester). The reaction in DCM gave the cleanest TLC profile.

This active ester could not be purified by flash column chromatography on silica gel as it underwent hydrolysis back to methyl red. An attempt was therefore made to use the active ester without purification. The addition of compound **(64)** and excess triethylamine to a *ca.* tenfold excess of the active ester (prepared in DCM) led to a recovered yield of only 55% of the dye conjugate **(65)** after

overnight stirring at ambient temperature and purification by flash column chromatography.

For comparison the reaction was repeated on a similar (50 mg) scale using the simple procedure of combining compound **(64)**, methyl red (1.2 eq.), Hobt (1.5 eq), DCCI (1.5 eq.) and triethylamine (2.0 eq.) in DCM and stirring overnight at ambient temperature. This led to a recovered yield of compound **(65)** of 76% after chromatography. This reaction was repeated on a 1 g scale and gave compound **(65)** in 88% yield as an orange solid.

2.2.13 Formamidine Protection of the Exocyclic Amino Group

The exocyclic amino group of compound **(65)** was protected with a di-n-butyl formamidine protecting group in order to be compatible with solid phase synthesis. This was effected by treating with an excess of di-n-butylformamidedimethylacetal in DMF at ambient temperature with stirring for 48 hours using a general procedure taken from Froehler and Matteucci.[74] This led to an isolated yield of 76% for compound **(66)** after chromatography.

2.2.14 Phosphitylation

The secondary hydroxyl function of compound **(66)** was phosphitylated by stirring with 1.2 eq. 2-cyanoethyl-N,N-di-isopropyl-chlorophosphoramidite in DIPEA / anhydrous THF (1:10) at ambient temperature for 75 minutes to give the phosphoramidite target compound **(67)** in quantitative yield after work up.

2.2.15 Testing Phosphoramidite (67) in Solid-Phase Synthesis

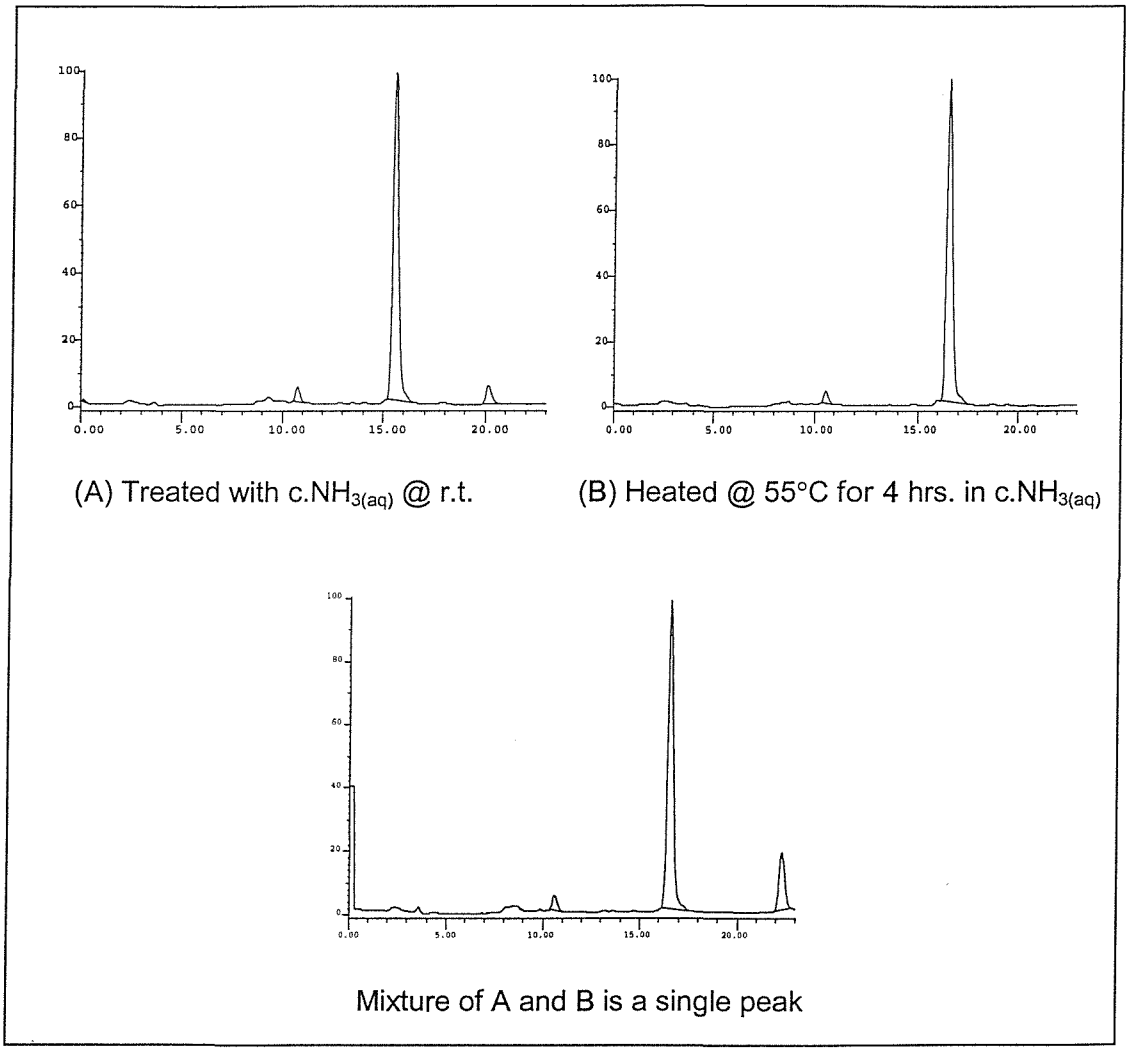
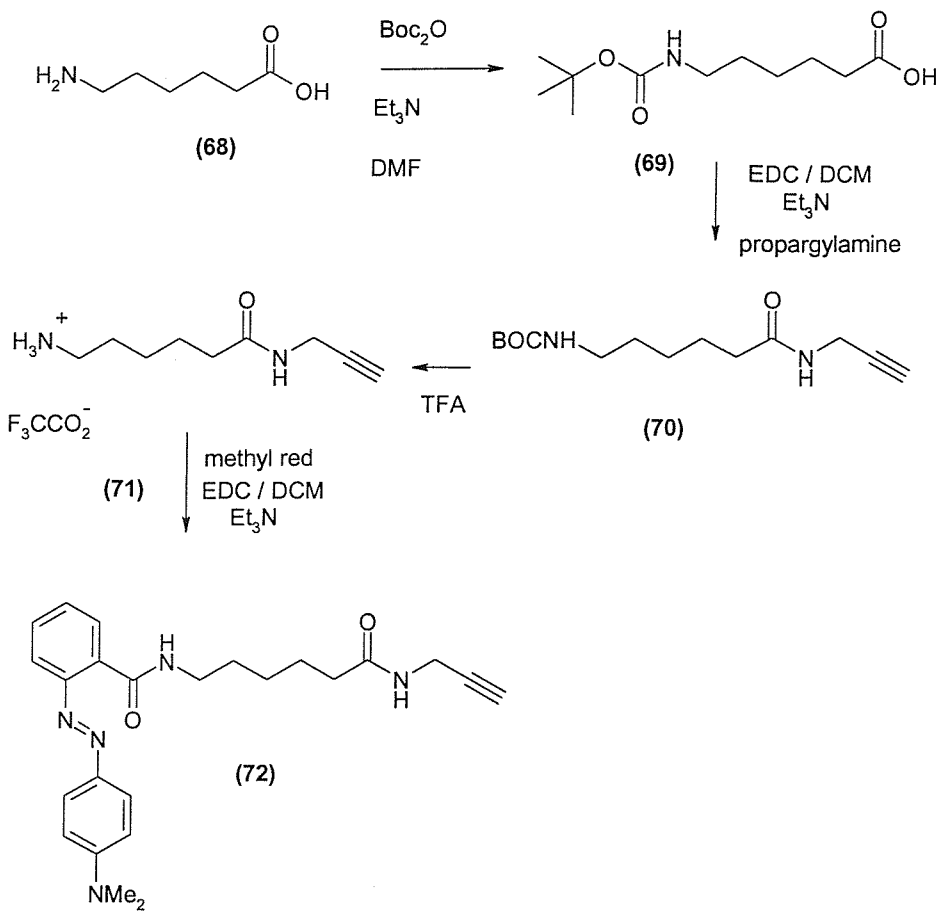


Figure 2.2-1: Reverse Phase HPLC Traces of Test Oligodeoxynucleotide TXTTT (Where 'x' Denotes Propargyl Methyl Red Monomer **(67)**) Comparing Post-synthetic Treatment with $c.NH_{3(aq)}$ @ Ambient Temperature *Versus* @ 55°C

Monomer **(67)** was incorporated into two identical test oligodeoxynucleotides with sequence TXTTT where 'X' denotes monomer addition using compound **(67)**. Online DMT+ analysis gave a coupling efficiency of 97.1% for inclusion of this monomer in both cases. The stability of the methyl red residue was then tested in standard DNA deprotection conditions (c.$NH_{3(aq)}$ @ 55°C). One of the pentamers was treated for 4 hrs. with c.$NH_{3(aq)}$ @ 55°C and the other was left overnight in c.$NH_{3(aq)}$ @ ambient temperature. The two samples were then analysed and compared by reverse phase HPLC. The HPLC traces (Figure 2.2-1) show that there was no change in retention time caused by heating during deprotection. This suggests that the deoxynucleotide residue derived from compound **(67)** is stable under standard DNA deprotection conditions.

2.3 Synthesis of a Tethered Methyl Red Fragment



Scheme 5a: Synthesis of the Tethered Methyl Red Fragment **(72)**

2.3.0 Overview

The final two monomers used the convergent tactic of incorporating the methyl red dye *via* palladium coupling reactions between tethered dye fragment **(72)** and the appropriate iodo compounds. 6-Aminohexanoic acid **(68)** was chosen as a suitable 7-atom spacer. The quencher was attached to one end of the alkynyl spacer *via* an amide bond. Overall the tethered methyl red compound **(72)** was synthesised in 10% yield over 4 stages.

2.3.1 Boc Protection of 6-Aminohexanoic Acid

6-Aminohexanoic acid **(68)** was Boc protected at the nitrogen by treating with stoichiometric quantities of Boc anhydride and triethylamine in anhydrous DMF at ambient temperature to give compound **(69)** in 64% yield after chromatography. This reaction was not optimised as this was the first reaction in the synthesis.

2.3.2 EDC Coupling to Propargylamine

The acid terminus of compound **(69)** was coupled to propargylamine *via* an amide bond. This was established by treating with EDC in anhydrous DCM at ambient temperature in the presence of triethylamine. The conjugate **(70)** was isolated in 76% yield as a white, crystalline solid after chromatography and subsequent crystallisation from ethyl acetate.

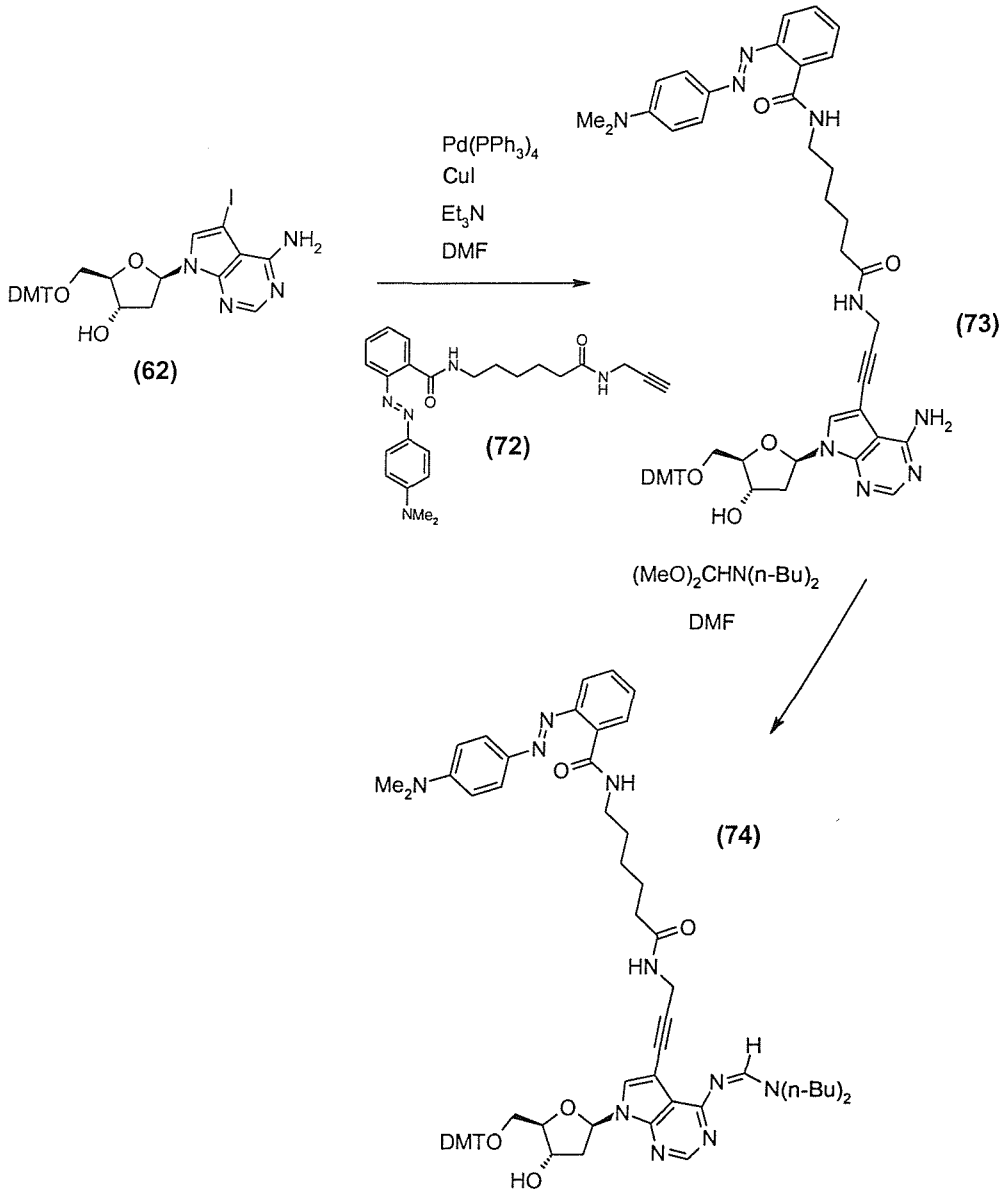
2.3.3 Cleavage of Boc

The Boc protection group was cleaved from compound **(70)** by treating with TFA at 0°C for 20 minutes. The product **(71)** was isolated in 64% yield as the TFA salt which was a white, crystalline solid. Purification was by chromatography followed by crystallisation from ethyl acetate.

2.3.4 Coupling to Methyl Red

Compound **(71)** was coupled to methyl red by stirring with EDC and triethylamine in anhydrous DCM at ambient temperature leading to compound **(72)** an orange

solid, in 32% yield after chromatography. This step was low yielding but was tolerated because the synthesis of this fragment was relatively short. Further development work would include a more thorough assessment of solvent, temperature, coupling reagent and possible additives for this step.



Scheme 6a: Convergent Steps Towards Methyl Red Adenosine Monomer **(75)**

2.3.5 Palladium Coupling between Iodo Compound (62) and Alkynyl Compound (72)

Compound **(62)** was coupled to fragment **(72)** to give compound **(73)** in 73% yield by stirring at ambient temperature with $Pd(PPh_3)_4$ (0.10 eq.), CuI (0.20 eq.), compound **(72)** (1.2 eq.), triethylamine (10 eq.), in anhydrous DMF for 16 hours. Compound **(73)** was purified by flash column chromatography.

2.3.6 Formamidine Protection of Compound (73)

The exocyclic amino function of compound **(73)** was protected with a di-n-butyl formamidine protecting group using the conditions of Froehler and Matteucci to give compound **(74)** in 75% yield after chromatography as an orange solid.[74]

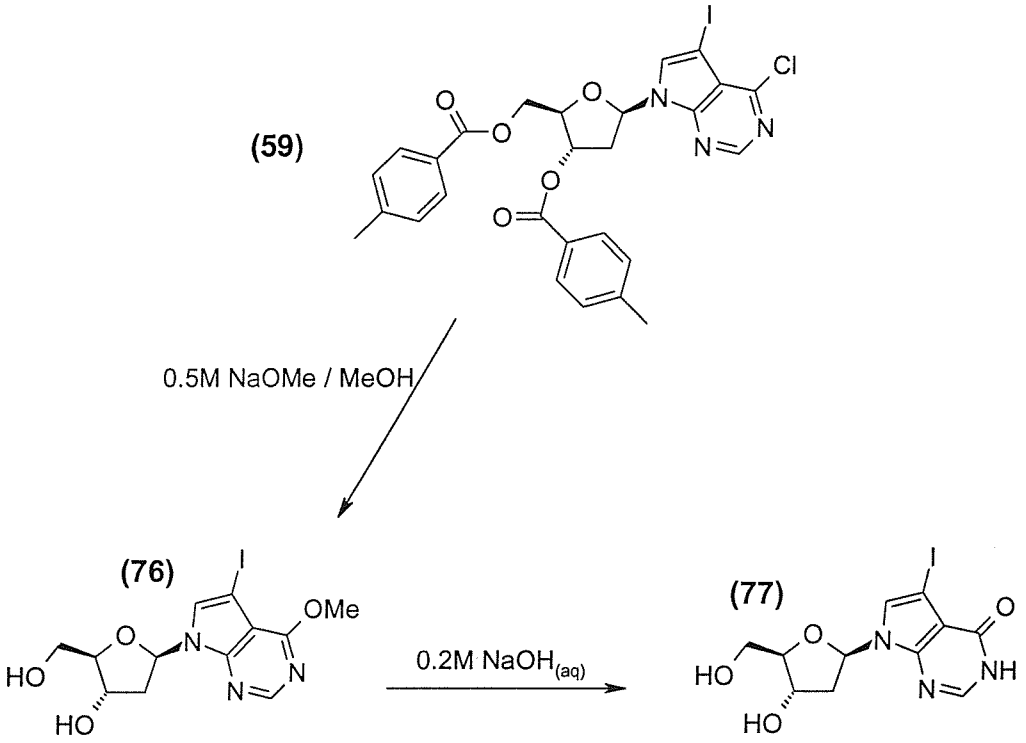
2.3.7 Phosphitylation of Compound (74)

Compound **(74)** was then phosphitylated under standard conditions: treatment with 1.1 eq. of 2-cyanoethyl-N,N di-isopropyl chlorophosphoramidite and 3 eq. of Hunigs' base in anhydrous THF at ambient temperature to give phosphoramidite monomer **(75)** in 84% yield after chromatography as a red solid.

2.4 Expanding the Chemistry to a Close Analogue of Inosine: the 'Universal Base'

2.4.0 Overview

Inosine analogue **(77)** was prepared in two steps from compound **(59)** using general conditions taken from Seela *et al.*[75-77]



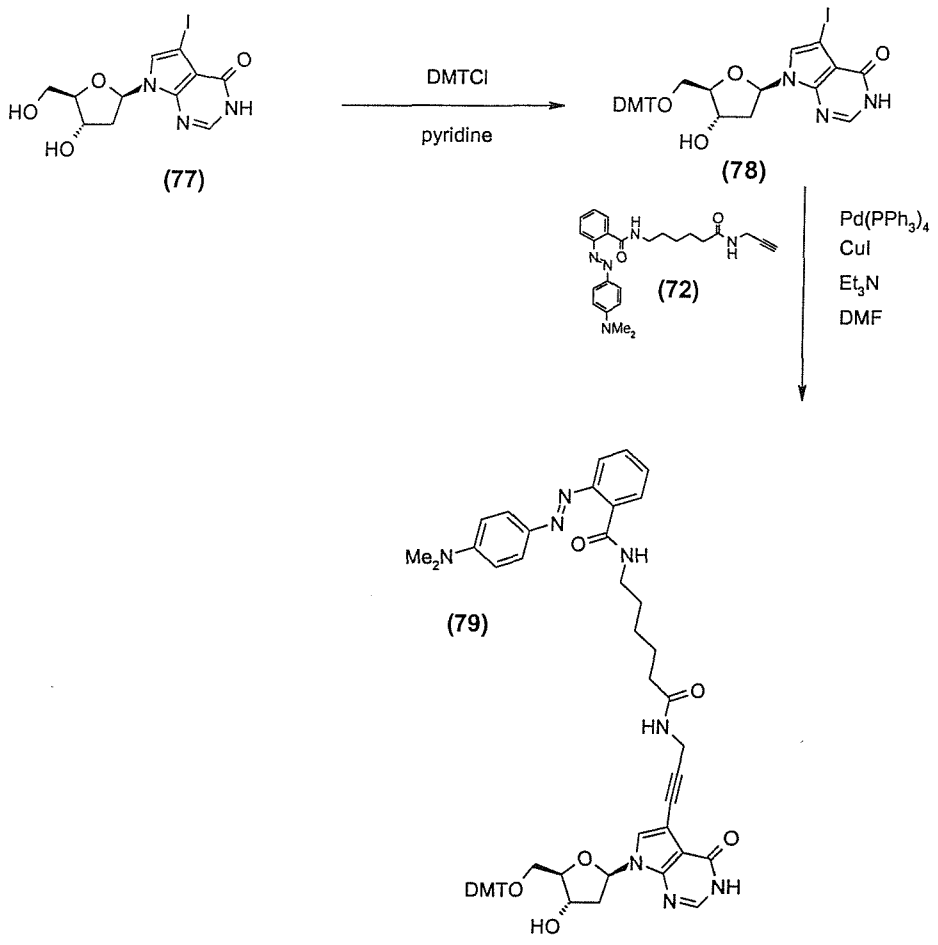
Scheme 7a: Formation of the Inosine Analogue **(77)**

2.4.1 Deprotection and Chloride Displacement with Sodium Methoxide

The ester protecting groups were cleaved from compound **(59)** and the chloro group was displaced with a methoxy group in one step by treating with 0.50M NaOMe / methanol at reflux for 1 hour. This afforded compound **(76)** as a white solid in 89% yield after chromatography.

2.4.2 Hydrolysis of the Methoxy Group

The methoxy group of compound **(76)** was hydrolysed to give the keto tautomer of the corresponding hydroxyl compound by treating with 0.2 M $NaOH_{(aq)}$ at reflux for four hours. This lead to compound **(77)** as a white solid in 74% yield after chromatography.



Scheme 8a: Convergent Steps Towards Monomer **(80)**

2.4.3 DMT Protection

The primary hydroxyl function of compound **(77)** was protected with a DMT ether group by treating with 1.10 eq. of DMT chloride in anhydrous pyridine at ambient

temperature for 3 hrs. Compound **(78)** was recovered in 91% yield as a white solid after crystallisation from EtOAc / Et_2O.

2.4.4 Palladium Coupling between Iodo Compound (78) and Alkynyl Compound (72)

Compound **(78)** was coupled to fragment **(72)** to give compound **(79)** in 48% yield by stirring at ambient temperature with $Pd(PPh_3)_4$ (0.10 eq.), CuI (0.20 eq.), compound **(72)** (1.2 eq.), triethylamine (10 eq.), in anhydrous DMF for 16 hours. Compound **(79)** was purified by flash column chromatography.

2.4.5 Phosphitylation of Compound (79)

Compound **(79)** was phosphitylated by treatment with 1.1 eq. of 2-cyanoethyl-N,N di-isopropyl chlorophosphoramidite and 3 eq of Hunigs' base in anhydrous THF at ambient temperature for 1 hour to give phosphoramidite monomer **(80)** in 62% yield after chromatography as a red solid.

2.5 Molecular Beacon Experiments

2.5.0 Overview

A number of molecular beacon experiments were carried out to demonstrate fluorogenic behaviour using monomers **(α-52)** and **(67)**.

2.5.1 Fluorescence Melting Studies

Monomer **(67)** was incorporated into scorpion PCR primers with the following sequences (the stem sequences are italicised).

Probe 1: The fluorophore and quencher are attached to complementary bases within the stem section of the beacon.

5'_*CCC**Fam**GCGCGG*AACATTTAGAAAAAACTTGGATC*CCGCGC**X**GGG*(HEG)TTCTT GATCACTCCACTGTTC_3'

Fam = fluoresceinyl caproyl deoxyU **(81)** (see Figure 2.5-1)
X = methyl red monomer **(67)**

Probe 2: The fluorophore and quencher are attached to complementary bases at the end of the stem section of the beacon.

5'_(**Fam**)*CCCGCGCGG*AACATTTAGAAAAAACTTGGATC*CCGCGCGGG***X**(HEG)TTT CTTGATCACTCCACTGTTC_3'

Fam = fluoresceinyl caproyl deoxyU **(81)** (see Figure 2.5-1)
X = methyl red monomer **(67)**

Probe 3:

5'_(**Fam**)CCCGCGCGGAACATTTAGAAAAAACTTGGATCCCGCGCGGG**Y**(HEG)TTT CTTGATCACTCCACTGTTC_3'

Fam = fluoresceinyl caproyl deoxyU **(81)** (see Figure 2.5-1)
Y = monomer **(β-52)**

Probe 3 is similar to probe 2 as it has a quencher and fluorophore at the end of the stem section. However these do not constitute a base pair because the quencher was attached using the deoxyribose aminohexyl glycoside monomer **(β-52)**. Therefore there is one less base pair in the stem section for this beacon.

All three probes had the same target sequence:

Complementary Sequence: 5'_GGGATCCAAGTTTTTTCTAAATGTTCC _3'

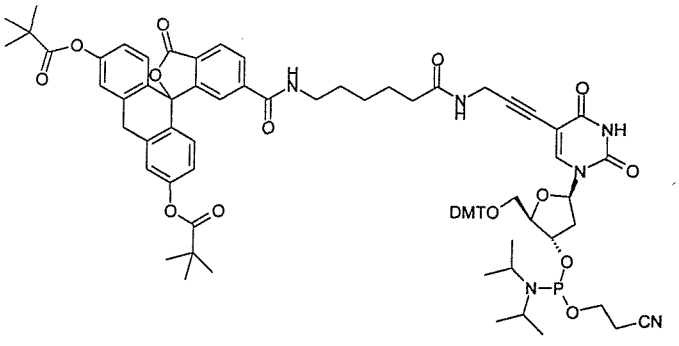
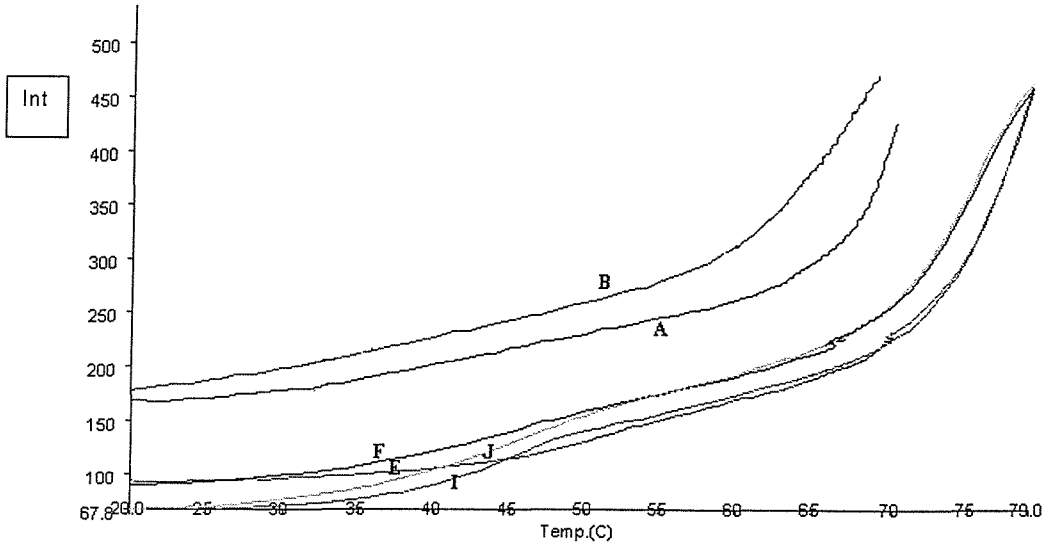


Figure 2.5-1: Fluoresceinyl caproyl deoxyU **(81)**

Fluorescence melting curves were generated for each of the probes both in isolation and in the presence of 10 eq. of their complementary target sequence. The three probes had the following concentrations; [Probe 1] = 88 nM, [Probe 2] = 80 nM, [Probe 3] = 69 nM.

2.5.1.1 Variable Temperature Fluorimetry for the Beacons in Isolation



A = Probe 1 heating B = Probe 1 cooling
E = Probe 2 heating F = Probe 2 cooling
I = Probe 3 heating J = Probe 3 cooling

Figure 2.5-2: Fluorescence Melt Data for the Probes in Isolation

In the absence of the target sequence the probes show low fluorescence intensities at room temperature due to the internal quenching of the fluorophore caused by the stem duplex. Probe 1 can be seen to show greater fluorescence (less efficient quenching) at room temperature than probes 2 or 3. This strongly suggests that stem duplex is destabilised by appending the fluorophore / quencher to a base-pair. It is known that the use of long alkyl linkers can reduce this type of destabilisation (Section 1.4.6).

As the temperature is raised there is a steady rise in the fluorescence intensities of all three oligomers. This corresponds to heat energy shifting the hybridisation equilibria towards the open (melted) stem conformers. This increase in fluorescence is gradual and roughly linear until 65-75°C and then becomes dramatic. The highest fluorescence is found at *ca.* 90°C (not shown) when the oligomers are entirely single-stranded.

2.5.1.2 Variable-Temperature Fluorimetry for the Beacons in the Presence of the Target Sequence

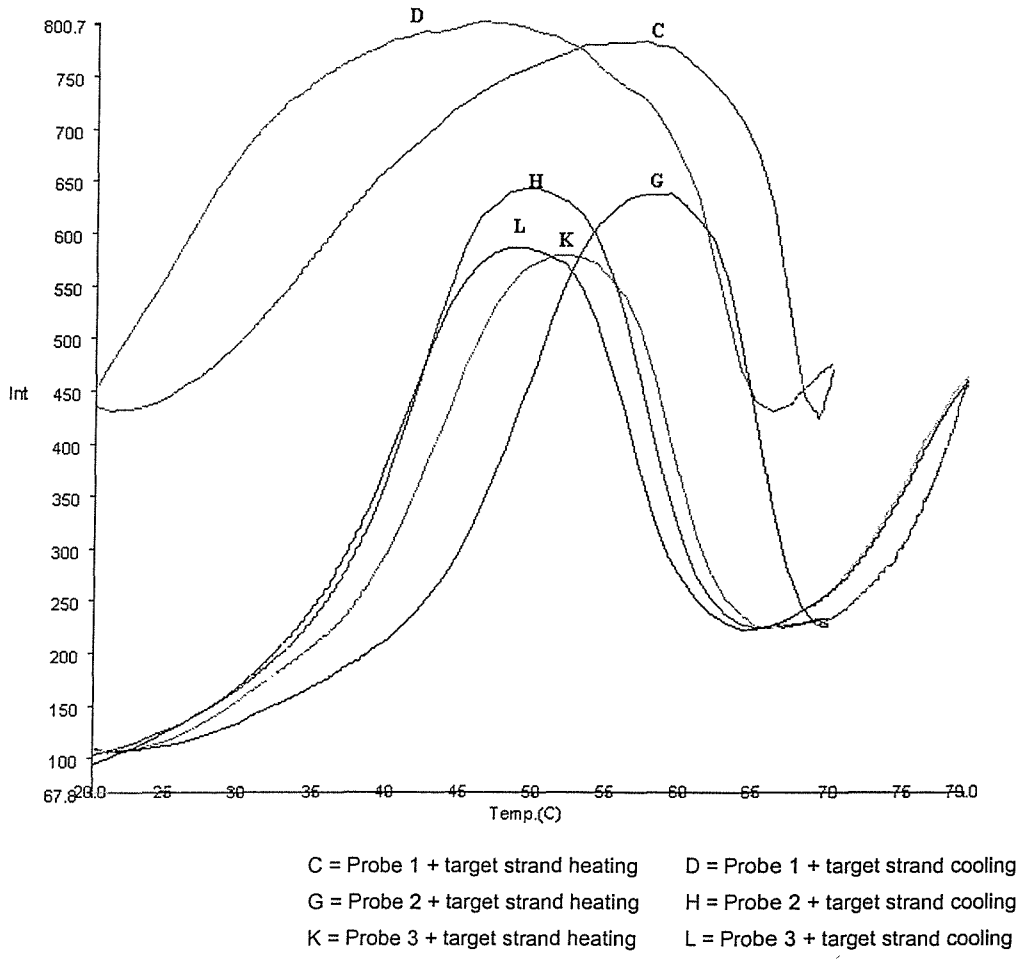


Figure 2.5-3: Fluorescence Melt Data for the Probes in the Presence of the Target Sequence

In the presence of the target sequence there is an increase in fluorescence intensity at low temperatures due to an equilibrium being established between the hairpin structure and the probe-target complex.

Thermal energy supplied by heating to *ca.* 50°C encourages the internal hybrid to melt and be replaced by the (longer) probe-target duplex. The associated

increase in fluorescent dye / quencher separation leads to a fluorescence maximum.

For probe 1 the room temperature fluorescence is very high. This must be a consequence of the reduced stability of the stem sequence. The lability of this internal hybrid also explains the relatively small increase in fluorescence on warming to the 70°C fluorescence peak. A broader curve profile for probe 1 also suggests a proliferation of accessible alternate secondary structures for the probe as would be encouraged by destabilisation of the stem section.

At higher temperatures (above *ca.* 70°C) the probe-target duplex melts and the curves become the same as those in the absence of the target strand leading to the second fluorescence maximum (not shown) @ *ca.* 90°C.

The conclusion from this experiment is that placing the methyl red dye in the major groove of the double-helical stem section probably destabilises the duplex. This is a reasonable observation considering the short length of the rigid propargyl linker holding the dye. If this is true then the incorporation of an aminocaproyl spacer unit between the propargyl amino group and the dye should resolve this problem.

2.5.2 Fluorescence Melting Studies Comparing Single *versus* Multiple Quenchers

The scorpion PCR primers shown in Figures 2.5-4 and 2.5-5 were prepared using either single or multiple incorporation of methyl red using monomer (**α-52**).

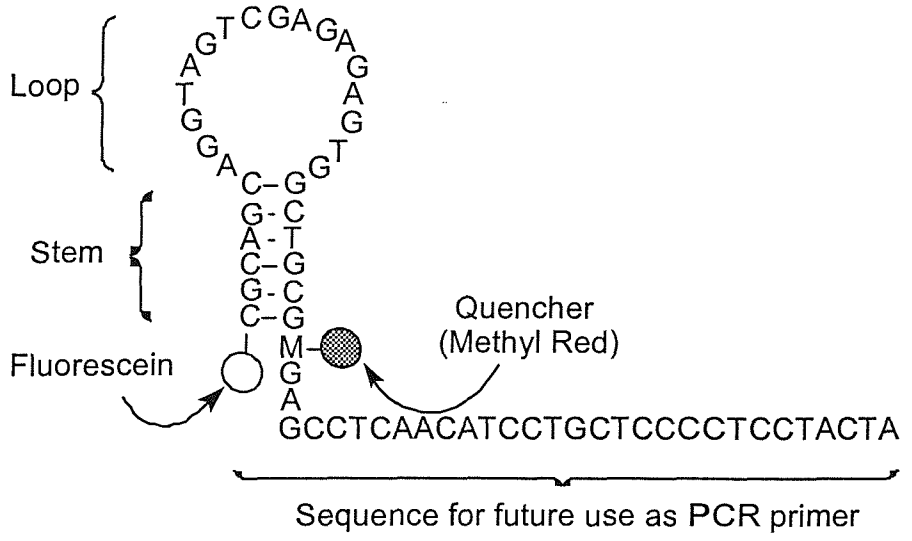


Figure 2.5-4: Beacon with a Single Quencher

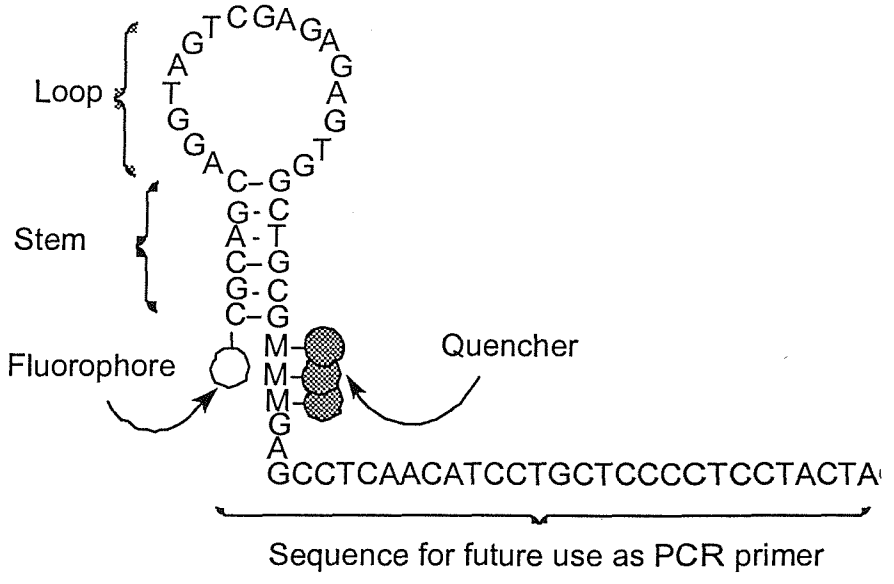


Figure 2.5-5: Beacon with Three Quenchers

In this experiment the objective was to measure and compare the increase in fluorescence for each beacon that occurs on target recognition. To get a clear picture of this behaviour it is necessary to separate at each temperature the behaviour of the free beacons in solution from that of the probe-target complexes. The fluorescence from free beacons is especially strong at high temperatures when the internal stem hybrids have all dissociated as seen in Figure 2.5-2. This can dominate the fluorescence profiles and hide other important trends.

Fluorescence melting studies were performed on solutions of the beacons both in isolation and in combination with their target DNA strand. At each temperature the fluorescence intensities for the beacons melting in isolation were subtracted from those for the beacons in the presence of their target sequences. The resulting fluorescence profile focuses on the increase in fluorescence intensity representing each probe binding the target sequence. These two curves are shown in Figure 2.5-6.

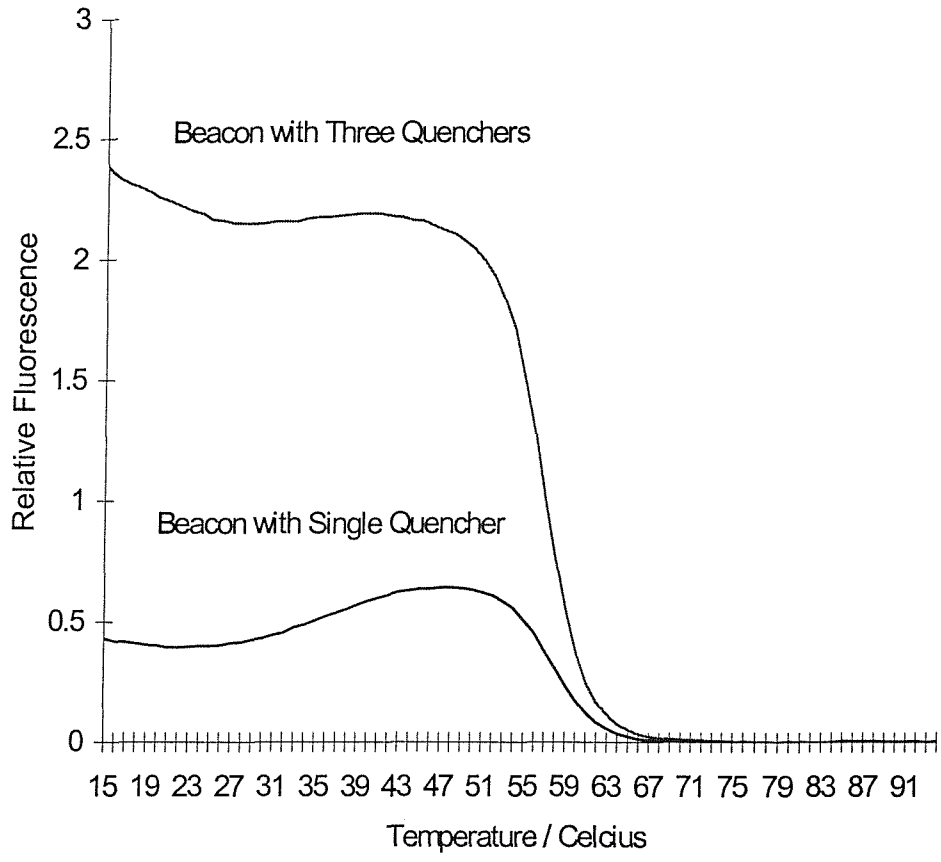


Figure 2.5-6: Fluorescence Melting Curves Comparing Single *versus* Multiple Quenchers

It can now be clearly seen that the beacon with three adjacent quenchers has the greater relative change in fluorescence intensity on binding to the target.

This can be assigned to additional quenching being made possible by an increased chance of Forster / collisional quenching between the fluorophore and at least one of the quenchers.

The conclusion from this experiment is that the degree of quenching in molecular beacon probe / target complexes can be increased by employing more than one quencher per beacon. In fact on a single molecule basis methyl red is quite capable of totally quenching all the fluorescence from fluorescein if it is close enough to the dye. This suggests that quenching in the hairpin conformation should also be improved by closely directing the dye / quencher pair together.

Section 3 Experimental

3.1.0 General Methods

3.1.1 Nuclear Magnetic Resonance Spectroscopy

^{13}C (75.42 MHz) and ^{1}H (300 MHz) spectra were recorded on a Bruker AM 300 Spectrometer using an appropriate deuterated solvent. All ^{13}C spectra were supported by DEPT 135. Selected spectra were supported by 2D C-H and H-H correlation experiments.

3.1.2 Drying of Solvents

Anhydrous THF was prepared by distillation from sodium wire / benzophenone.
Anhydrous acetonitrile, DCM, pyridine and triethylamine were prepared by distillation from CaH_2.
Anhydrous DMF was purchased from Aldrich.
Anhydrous methanol was prepared by distillation from a solution of the magnesium alkoxide formed *in situ* from magnesium turnings and catalytic iodine.

3.1.3 Thin Layer Chromatography

All compounds were shown to give a single spot by TLC. This was carried out using pre-coated aluminium backed plates (0.25mm, type 60 silica gel with fluorescent indicator).
Detection was by:-

1. u.v. light 254nm.
2. 5% *p*-anisaldehyde / 1% AcOH / 1% H_2SO_4 / EtOH
3. 5% H_2SO_4 / EtOH
4. fumes from c.$NH_{3(aq)}$
5. 5% phosphomolybdic acid / EtOH

3.1.4 Flash Column Chromatography

Flash column chromatography was carried out using silica gel 60, obtained from MERCK.

3.1.5 Oligonucleotide synthesis

All oligonucleotides were synthesised on an ABI 394 DNA synthesiser by automated solid-phase methods using β-cyanoethyl phosphoramidites. All monomers were added as phosphoramidites during oligonucleotide synthesis. Oligonucleotides were purified by reversed-phase HPLC on a C8 (octyl) column, eluting with a gradient of acetonitrile in ammonium acetate buffer.

3.1.6 Fluorescence Melting

All melting curves were carried out on a Roche LightCycler. Each reaction was made up from 1 µL of 10 X buffer (Advanced Biotechnologies Buffer IV: 200mM $(NH_4)_2SO_4$, 750mM Tris-HCL, 0.1% Tween), 1 µL of a 5 µM solution of Scorpion Primer / molecular beacon, 250 ng/µl of bovine serum albumin, 1.6 µL of a 25 mM solution of $MgCl_2$ and water to a final volume 20 µL. Only 10 µL of these mixtures was needed for each run. The cycling conditions were: initial denaturation for 5 minutes at 95°C, cooling to 30°C for 0 seconds, melting from

30°C to 95°C with a 0.2°C / second transition rate and cooling at 40°C for 30 seconds. Fluorescence was measured continuously during the melting phase.

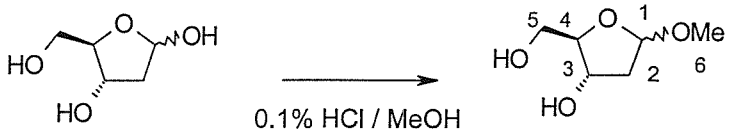
3.1.7 Mass Spectrometry

Mass Spectra were recorded on a Fisons VG platform instrument.

3.2.0 Synthetic Procedures

3.2.1

1-O-Methyl-2-deoxy-D-ribose (43) Hoffer *et al* [54], Kotera *et al* [58]



2-Deoxy-D-ribose (15.5 g, 116 mmol) was dissolved in anhydrous MeOH (170 mL) with stirring under argon then 0.7% (W:W) HCl / anhydrous MeOH {prepared by the cautious addition of acetyl chloride (0.5 mL) to anhydrous MeOH (30 mL) with stirring and external cooling with ice} was added and the reaction was stirred at ambient temperature for 2 hours. TLC analysis after that time showed that the reaction was complete. The reaction mixture was neutralised by the portionwise addition of $NaHCO_3$ (6.0 g), filtered and concentrated *in vacuo* to afford 1-O-methyl-2-deoxy-D-ribose **(43)** as a colourless oil (16.95 g, 99%) which was used without further purification. Analysis was concordant with the proposed structure and in agreement with the literature.

R_F 0.13, 0.22 (EtOAc, anisaldehyde)

Es+ve: 166 $[M + NH_4]^+$

Es-ve: 183 and 185 $[M + Cl]^-$

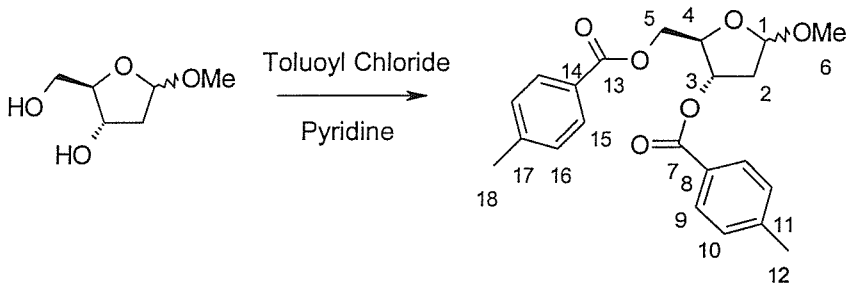
ν_{MAX} /cm^{-1} (KBr disc): 3200-3600 O-H str, 1036 and 1092 C-O str

δ^1H (300MHz, $CDCl_3$): 1.80-2.25 (m. 2H, $\underline{H}_2$), 2.55 and 2.70 (b.s., 1H, -O$\underline{H}$), 3.12-3.22 (m., 1H, $\underline{H}_4$), 3.23 and 3.26 (two s., 3H, $\underline{H}_6$), 3.35 and 3.52 (m., 2H, $\underline{H}_5$), 3.85-4.06 (m., 1H, $\underline{H}_3$), 4.05 (bs., -O$\underline{H}$), 4.48 and 5.00 (two m., 1H, $\underline{H}_1$)

δ^{13}C (75.42MHz, $CDCl_3$): 41.52 and 42.28 ($\underline{C}_2$), 55.07 and 55.51 ($\underline{C}_6$), 62.99 and 63,68 ($\underline{C}_5$), 71.98 and 72.68 ($\underline{C}_4$), 87.16 and 87.37 ($\underline{C}_3$), 105.54 and 105.62 ($\underline{C}_1$)

3.2.2

1-O-Methyl-2-deoxy-3,5-di-O-*p*-toluoyl-D-ribose (44) Hoffer *et al* [54], Kotera *et al* [58]



1-O-Methyl-2-deoxy-D-ribose **(43)** (16.9g, 114 mmol) was co-evaporated with anhydrous pyridine (3 x 15 mL) and dissolved in anhydrous pyridine (90 mL). The solution was stirred under a headspace of argon at 0°C with cooling over ice and *p*-toluoyl chloride (2.2 eq., 33.7 mL, 252 mmol) was added dropwise, with stirring over 30 minutes. The mixture was allowed to warm to ambient temperature then heated to 65°C and stirred at that temperature for 4 hours. Heating was then switched off and the reaction mixture was allowed to cool to ambient temperature overnight with stirring. At that time TLC analysis showed the reaction to be complete. The reaction mixture was partitioned between water (150 mL) and DCM (100 mL) and the aqueous phase was extracted with further DCM (2 x 100 mL). The combined organic extracts were washed with 2M $HCl_{(aq)}$ (2 x 100 mL) and then 6% $NaHCO_{3(aq)}$ (200 mL), dried over Na_2SO_4 and concentrated *in vacuo*. The resulting yellow oil was co-evaporated with toluene

(3 x 15 mL) to remove residual pyridine, then with diethyl ether (3 x 15 mL) and dried *in vacuo* to constant mass to afford methyl 1-O-methyl-2-deoxy-3,5-di-O-*p*-toluoyl-D-ribose **(44)**, a 1:1 mixture of anomers by ^{1}H-NMR, as a yellow oil (46.1 g, 105% crude with residual DCM by ^{1}H-NMR).

A sample of this crude oil (1.0 g) was purified for characterisation by flash silica column chromatography, eluting with DCM, affording 1-O-methyl-2-deoxy-3,5-di-O-*p*-toluoyl-D-ribose **(44)** (0.87 g) as a colourless oil. This was dried overnight *in vacuo* over P_2O_5. Analysis was concordant with the proposed structure and in agreement with the literature.

R_F = 0.60, 0.65 (2:1 hexane / EtOAc; u.v., anisaldehyde)

ES+ve: 402 $(M+NH_4)^+$, 407 $(M+Na)^+$, 423 $(M+K)^+$

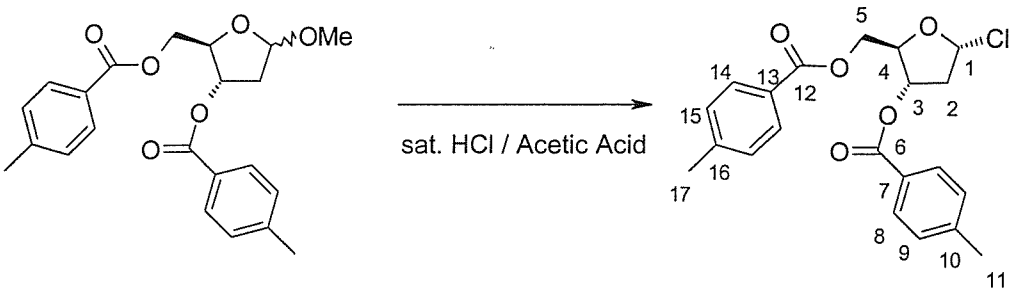
ν_{MAX} /cm^{-1} (KBr disc): 1710 C=O str, 1272 and 1107 C-O str, 1068 C-O str

δ^1H (300MHz, $CDCl_3$): 2.15-2.40 and 2.20-2.55 (2m., each 1H, $\underline{H}_2$), 2.29 and 2.30 (2s., each 3H, $\underline{H}_{12}$ and $\underline{H}_{18}$), 3.25 and 3.31 (2s., 3H, $\underline{H}_6$), 4.32-4.50 (m., 3H, $\underline{H}_4$ and $\underline{H}_5$), 5.05-5.16 (m., 1H, $\underline{H}_3$), 5.28 to 5.57(m., 1H, $\underline{H}_1$), 7.19 and 7.24 (2d. J=8Hz 4H, $\underline{H}_{10}$ and $\underline{H}_{16}$), 7.80-7.95 (2d. J=8Hz 4H, $\underline{H}_9$ and $\underline{H}_{15}$)

δ^{13}C (75.42MHz, $CDCl_3$): 21.84 ($\underline{C}_{12}$ and $\underline{C}_{18}$), 39.44 ($\underline{C}_2$), 55.26 and 55.36 ($\underline{C}_6$), 64.46 and 65.30 ($\underline{C}_5$), 74.76 and 75.56 ($\underline{C}_4$), 81.13 and 82.06 ($\underline{C}_3$), 105.22 and 105.78 ($\underline{C}_1$), 127.02, 127.17, 127.24 and 127.35 ($\underline{C}_{11}$ and $\underline{C}_{18}$), 129.27, 129.85 and 129.96 (aryl $\underline{C}$-H), 143.85, 143.96 144.10 and 144.19 ($\underline{C}_{14}$ and $\underline{C}_8$), 166.28, 166.45 and166.66 ($\underline{C}_{13}$ and $\underline{C}_7$).

3.2.3

1-α-Chloro-2-deoxy-3,5-di-O-*p*-toluoyl-D-ribose (45) Hoffer *et al* [54], Kotera *et al* [58]



Crude methyl 1-O-methyl-2-deoxy-3,5-di-O-*p*-toluoyl-D-ribose **(44)** (5.00 g, 12.4 mmol) was dissolved in glacial AcOH (6.0 mL) and stirred at ambient temperature. A pre-saturated solution of HCl in glacial AcOH (10.0 mL) {prepared by adding acetyl chloride (16.3 mL) to a mixture of glacial AcOH (81.0 mL) and water (4.00 mL), at 0°C to ambient temperature} was added in a steady stream with vigorous stirring. Sufficient acetyl chloride (3.00 mL) was added dropwise with additional trituration from a glass rod to cause a thick white precipitate. The mixture was then cooled on ice and shaken vigorously with ice-cold diethyl ether (10 mL). The white precipitate was obtained by filtration and washed by displacement with ice cold diethyl ether (2 x 10 mL). The precipitate was quickly transferred to a desiccator and dried *in vacuo* over KOH affording 1-α-chloro-2-deoxy-3,5-di-O-*p*-toluoyl-D-ribose **(45)** (2.92 g, 60% from deoxyribose, [lit.[58]; 58% from deoxyribose]), as a microcrystalline white solid. Analysis was concordant with the proposed structure and in agreement with the literature.

R_F = 0.4 (2:1 hexane / EtOAc; UV, anisaldehyde [sensitive to chromatography])

m.p. 98-105°C {lit. 107-109°C}[58]

ES+ve: 406 and 408 $(M+NH_4)^+$

ν_{MAX} /cm^{-1}(KBr disc): 1707 C=O str, 1276 and 1177 C-O str, 1096 C-O str

δ^1H (300MHz, $CDCl_3$): 2.20-2.35 and 2.62-2.94 (2m., each 1H, $\underline{H}_2$), 2.38 and 2.39 (2s., each 3H, $\underline{H}_{11}$ and $\underline{H}_{17}$), 4.38-4.82 (m., 3H, $\underline{H}_4$ and $\underline{H}_5$), 5.37-5.72 and 6.30-6.48 (m., 2H, $\underline{H}_3$ and $\underline{H}_1$), 7.19 and 7.24 (2d. J=8Hz 4H, $\underline{H}_9$ and $\underline{H}_{15}$), 7.89-8.95 (2d. J=8Hz 4H, $\underline{H}_{14}$ and $\underline{H}_8$)

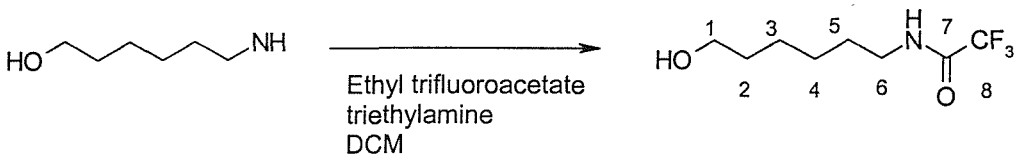
δ^{13}C (75.42MHz, $CDCl_3$): 21.81 ($\underline{C}_{11}$ and $\underline{C}_{17}$), 44.66 ($\underline{C}_2$), 63.63 ($\underline{C}_5$), 73.68 ($\underline{C}_4$), 84.82 ($\underline{C}_3$), 95.43 ($\underline{C}_1$), 126.93 (aryl C), 129.26, 129.34, 129.81 and 130.04 (aryl $\underline{C}$H), 144.18 and 144.41 (aryl $\underline{C}$),166.8 ($\underline{C}$=O of ester).

A sample of 1-α-chloro-2-deoxy-3,5-di-O-*p*-toluoyl-D-ribose **(42)** (4.00g, 10.3 mmol) was crystallised from anhydrous diethyl ether (250 mL) with cooling from reflux to 4°C. This afforded a pure sample of the title compound, as evinced by the melting point, (2.62g, 6.74 mol) in three crops of fine, white needles.

m.p. 104-107°C, 106-108°C and 105-109°C {lit.[58] 107-109°C}, ^{1}H-NMR, ^{13}C-NMR, MS, TLC and ir as above.

3.2.4

N-(6-Hydroxyhexyl)-trifluoroacetamide (46)



6-Aminohexanol (25.0 g, 214 mmol) was dissolved in anhydrous DCM (500 mL) and stirred with anhydrous Na_2SO_4 (15g) for five minutes at ambient temperature. The drying agent was removed by filtration and the filtrate was concentrated *in vacuo* to give an oil (24.09 g, 206 mmol). This was co-evaporated with anhydrous DCM (3 x 25 mL) and dissolved in anhydrous DCM (300 mL) under a headspace of argon. Anhydrous triethylamine (1.50 eq., 309

mmol, 43.0 mL) was added with stirring followed by the dropwise addition of ethyl trifluoroacetate (1.50 eq., 309 mmol, 36.8 mL). The reaction was then stirred under argon at ambient temperature for 3 hours. At that time TLC analysis showed that no substrate remained. The reaction mixture was washed by extraction with 10% citric acid (2 x 150 ml), dried over Na_2SO_4 and concentrated *in vacuo*. Residual volatile components were displaced by co-evaporation with DCM (3 x 15 mL) and the resulting off-white solid (41.78 g) was crystallised from EtOAc / hexane and dried *in vacuo* over P_2O_5 affording N-(6-hydroxyhexyl)-trifluoroacetamide **(46)** (38.5 g, 88%) as a white, microcrystalline solid. Analysis was concordant with the proposed structure.

R_F = 0.60 (1:9 hexane / EtOAc; molybdenum blue)

ES+ve: 231 $(M+NH_4)^+$ ES-ve: 248 and 250 $(M+Cl)^-$

m.p. 42-45°C

ν_{MAX} /cm^{-1}(KBr disc): 3307 OH str, 1700 C=O str, 1554 N-H def, 1181 and 1354 C-O str or OH def (coupled), ν_{MAX} /cm^{-1} (DCM solution cell): 3432 OH str, 1723 C+O str, 1540 N-H def, 1160 and 1350 OH def

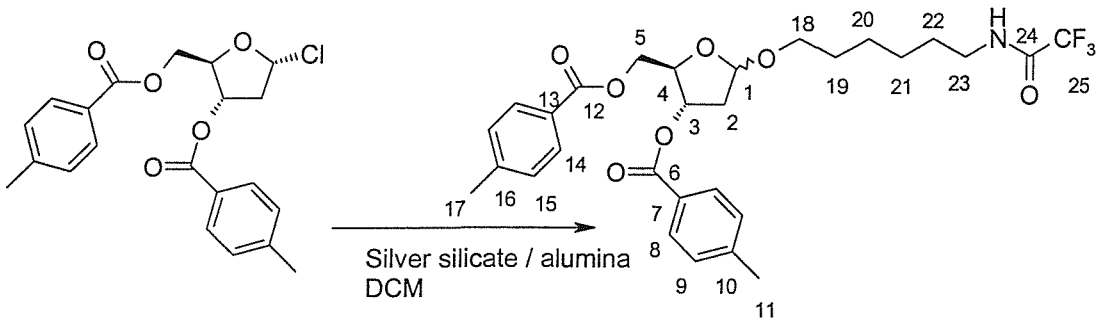
δ^1H (300MHz, $CDCl_3$): 1.23-1.42 (m., 4H, $\underline{H}_3$ and $\underline{H}_4$), 1.47-1.64 (m., 4H, $\underline{H}_2$ and $\underline{H}_5$), 2.30 (s., 1H, OH), 3.23-3.35 (m., 2H, $\underline{H}_6$), 3.56-3.65 (m., 2H, $C\underline{H}_1$, J=9.6Hz and 6.6Hz), 6.5 (b.s., N-$\underline{H}$ of amide)

δ^{13}C (75.42MHz, $CDCl_3$): 25.32, 26.42, 28.89, 32.44, 39.94, 62.65, 157.59 quartet (CF_3).

3.2.5

1'-O-(6-(2,2,2-Trifluoroacetyl)-aminohexyl)-3',5'-di-O-*p*-toluoyl-2'-deoxy-D-ribose (47)

Heterogeneous Process



N-(6-Hydroxyhexyl)-trifluoroacetamide

N-(6-Hydroxyhexyl)-trifluoroacetamide **(46)** (2.93 g, 13.7 mmol, 1.2eq.) and silver silicate / alumina (8.80 g) were charged to a reaction flask and the mixture was co-evaporated with anhydrous DCM (3 x 40 mL) to displace residual solvents and then suspended in anhydrous DCM (50 mL). Powdered 3Å molecular sieves (8.8 g) were added and the resulting mixture was stirred at ambient temperature for 2 hours under a headspace of nitrogen and then cooled to -15°C (ethylene glycol / dry ice slush-bath). 1-α-Chloro-2-deoxy-3,5-di-*p*-toluoyl-D-ribose **(46)** (4.45 g, 11.5 mmol, 1eq.) in anhydrous DCM (30 mL) was added dropwise, with stirring and the reaction was stirred at -15°C for 2 $^{1}/_{2}$ hours and than allowed to warm to ambient temperature and stirred for a further 2 hours. At that time TLC analysis showed the reaction was complete giving an excess of the lower-running α-anomer of the product glycoside. The reaction mixture was diluted with DCM (20 mL), filtered and the residue was washed by displacement with DCM (2 x 20 mL). The combined organic solutions were washed by extraction with sat. $NaHCO_{3(aq)}$ (100 mL), dried over Na_2SO_4 and concentrated *in vacuo* to give a

dark oil. Flash silica column chromatography, eluting with 20% EtOAc / hexane, allowed purification and partial separation of the product anomers.

Overall this afforded 1'-α-O-(6-(2,2,2-trifluoroacetyl)aminohexyl)-3',5'-di-O-*p*-toluoyl-2'-deoxy-D-ribose **(α-47)** (1.60g) as a colourless oil and a mixture of anomers of compound **(47)** (4.17g) as a colourless oil (overall yield 5.77g, 89%, 49% d.e. for α anomer). The d.e was calculated by ^{1}H-NMR integration. Analysis was concordant with the proposed structure. The major (alpha) isomer, which was a colourless oil, spontaneously crystallised after several months at ambient temperature. Single crystal x-ray diffraction analysis gave unambiguous assignment of the stereochemistry at C_1.

1'-α-O-(6-(2,2,2-Trifluoroacetyl)-aminohexyl)-3',5'-di-O-*p*-toluoyl-2'-deoxy-D-ribose **(α-47)**

R_F = 0.60 (10% EtOAc / DCM, u.v., anisaldehyde)

Es+ve: 583 $(M+NH_4)^+$, (588 $M+Na)^+$

ν_{MAX} /cm^{-1}(KBr disc): 2939 C-H str, 1717 C=O str, 1273 and 1178 C-O str

δ^1H (300MHz, $CDCl_3$): 1.15-1.40 (m., 4H, $\underline{H}_{20}$ and $\underline{H}_{21}$), 1.40-1.62 (m., 4H, $\underline{H}_{19}$ and $\underline{H}_{22}$), 2.05-2.17 and 2.37-2.50 (2m., each 1H, $\underline{H}_2$), 2.30 and 2.32 (2s., each 3H, $\underline{H}_{11}$ and $\underline{H}_{17}$), 3.34 (d.t., 2H, $\underline{H}_{23}$, J = 7.3Hz and 7.3Hz), 3.40 and 3.73 (2d.t, each 1H, $\underline{H}_{18}$, J=9.6Hz and 6.6Hz {rotomeric effect}), 4.42-4.48 (m., 2H, $\underline{H}_5$), 4.54-4.59 (m., 1H, $\underline{H}_4$), 5.21-5.25 (m., 1H, $\underline{H}_1$) 5.32-5.40 (m., 1H, $\underline{H}_3$), 6.35 (b.s., 1H, N$\underline{H}$), 7.15-7.35 (2d. J=8Hz 4H, $\underline{H}_9$ and $\underline{H}_{15}$), 7.85-8.06 (2d. J=8Hz 4H, $\underline{H}_8$ and $\underline{H}_{14}$)

$\delta^{13}C$ (75.42MHz, $CDCl_3$): 21.81 ($\underline{C}_{17 \text{ and } 11}$), 25.92 ($\underline{C}_{22}$), 26.55 ($\underline{C}_{21}$), 29.06 ($\underline{C}_{19}$), 29.66 ($\underline{C}_{20}$), 39.38 ($\underline{C}_{2}$), 40.035 ($\underline{C}_{23}$), 64.49 ($\underline{C}_{5}$), 67.38 ($\underline{C}_{18}$), 74.91 ($\underline{C}_{4}$), 81.16 ($\underline{C}_{3}$), 103.98 ($\underline{C}_{1}$), 127.19 ($\underline{C}_{10 \text{ and } 16}$), 129.24, 129.29, 129.81 and 129.91 (aryl $\underline{C}$-H), 144.04 and 144.20 ($\underline{C}_{7}$ and $\underline{C}_{13}$), 157-weak-($\underline{C}F_3$), 166.51, 166.60 ($\underline{C}$=O of esters).

A pure sample of the microcrystalline 1'-β-O-(6-(2,2,2-trifluoroacetyl)-aminohexyl)-3',5'-di-O-*p*-toluoyl-2'-deoxy-D-ribose **(β-47)** was obtained during chromatography by spontaneous crystallisation.

1'-β-O-(6-(2,2,2-Trifluoroacetyl)-aminohexyl)-3',5'-di-O-*p*-toluoyl-2'-deoxy-D-ribose **(β-47)**

m.p. 92-92.5°C

R_F = 0.70 (10% EtOAc / DCM, uv, anisaldehyde)

Es+ve: 583 $(M+NH_4)^+$, (588 $M+Na)^+$

ν_{MAX} /cm^{-1}(KBr disc): 2943 C-H str, 1703 C=O str, 1269 and 1173 C-O str

δ^1H (300MHz, $CDCl_3$): 1.25-1.42 (m., 4H, $\underline{H}_{20}$ and $\underline{H}_{21}$), 1.44-1.67 (m., 4H, $\underline{H}_{19}$ and $\underline{H}_{22}$), 2.30-2.45 and 2.50-2.63 (2m., each 1H, $\underline{H}_{2}$), 2.48 and 2.49 (2s., each 3H, $\underline{H}_{11}$ and $\underline{H}_{17}$), 3.28-3.43 (m., 2H, $\underline{H}_{23}$), 3.35 and 3.72 (2d.t, each 1H, $\underline{H}_{18}$, J=9.6Hz and 6.6Hz {rotomeric effect}), 4.42-4.65 (m., 2H, $\underline{H}_{5}$), 4.57-4.61 (m., 1H, $\underline{H}_{4}$), 5.34-5.39 (m., 1H, $\underline{H}_{1}$) 5.59-5.64 (m., 1H, $\underline{H}_{3}$), 6.75 (b.s., 1H, N$\underline{H}$), 7.15-7.35 (2d. J=8Hz 4H, $\underline{H}_{9}$ and $\underline{H}_{15}$), 7.85-8.06 (2d. J=8Hz 4H, $\underline{H}_{8}$ and $\underline{H}_{14}$)

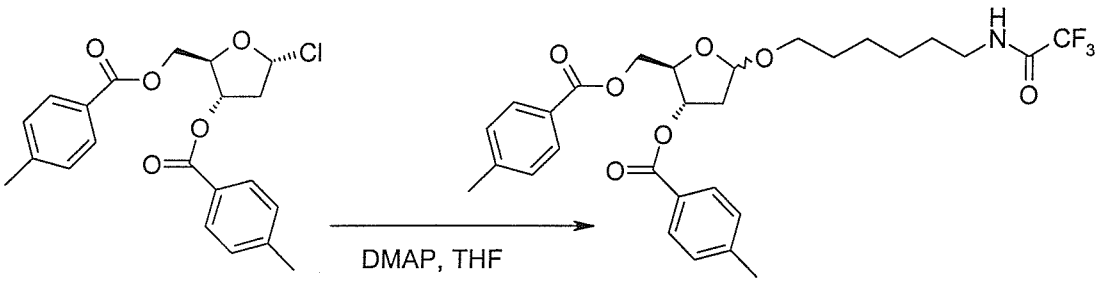
$\delta^{13}C$ (75.42MHz, $CDCl_3$): 21.85 ($\underline{C}_{17}$ and $\underline{C}_{11}$), 25.84 ($\underline{C}_{22}$), 26.38 ($\underline{C}_{21}$), 28.93 ($\underline{C}_{19}$), 29.39 ($\underline{C}_{20}$), 39.45 ($\underline{C}_{2}$), 40.03 ($\underline{C}_{23}$), 65.60 ($\underline{C}_{5}$), 67.91 ($\underline{C}_{18}$), 75.84 ($\underline{C}_{5}$), 81.89 ($\underline{C}_{3}$), 104.84 ($\underline{C}_{1}$), 127.01 and 127.16 ($\underline{C}_{10 \text{ and } 16}$), 129.29, 129.86 and

129.92 (aryl C-H), 144.06, 144.24 (C_7 and C_{13}), 157.40 (CF_3), 166.31, 166.68 (C=O of esters).

3.2.6

1'-O-(6-(2,2,2-Trifluoroacetyl)-aminohexyl)-3',5'-di-O-*p*-toluoyl-2'-deoxy-D-ribose (47)

Homogeneous Process



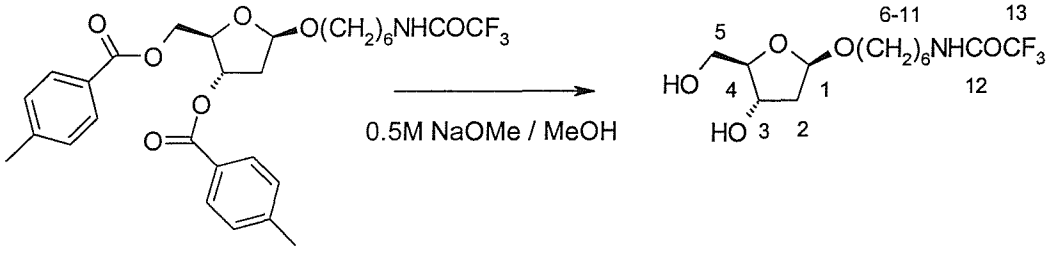
N-(6-Hydroxyhexyl)-trifluoroacetamide

N-(6-Hydroxyhexyl)-trifluoroacetamide **(46)** (10.5 g, 1.2 eq, 49.0 mmol)) was co-evaporated with anhydrous THF (3 x 15 mL) and dissolved in anhydrous THF (40 mL) under a headspace of nitrogen. 4-DMAP (1.0 g, 8.20 mmol, 0.20 eq) was added with stirring followed by 1-α-chloro-2-deoxy-3,5-di-*p*-toluoyl-D-ribose **(45)** (15.9 g, 40.9 mmol, 1.00 eq) and the reaction was stirred at ambient temperature overnight after which time TLC analysis indicated that the reaction was complete. The mixture was concentrated *in vacuo* to give an oil and residual solvents were displaced by co-evaporation with DCM (3 x 20 mL). ^{1}H-NMR of the resulting oil showed a 1:1 mixture of anomers in the product glycoside. The close running anomers were partially separated by flash silica column chromatography, eluting with 20% EtOAc / hexane giving the high-running anomer **(β-47)** (2.74 g) as a white solid, the lower anomer **(α-47)** (2.69 g) as a colourless oil and mixed anomers **(47)** (10.5 g) as an oil to afford overall a 1:1 mixture of anomers (by ^{1}H-NMR) of 1'-O-(6-(2,2,2-trifluoroacetyl)-aminohexyl)-3',5'-di-O-*p*-toluoyl-2'-deoxy-

D-ribose **(47)** (15.9 g, 69%). Analysis was concordant with the proposed structure (analytical details were as above).

3.2.7

1'-β-O-(6-(2,2,2-Trifluoroacetyl)-aminohexyl)-2'-deoxy-D-ribose (β-48)



1'-β-O-(6-(2,2,2-Trifluoroacetyl)-aminohexyl)-3',5'-di-O-*p*-toluoyl-2'-deoxy-D-ribose **(β-47)** (2.31 g, 4.1 mmol) was co-evaporated with anhydrous MeOH (3 x 15 mL) and dissolved in 0.5M NaOMe / MeOH (13 mL) with stirring under a headspace of nitrogen. The resulting solution was stirred at ambient temperature for 90 minutes. At this time TLC analysis showed absence of the diester substrate. The reaction mixture was pre-adsorbed directly onto silica gel (1.0g) by concentration *in vacuo* and purified by flash silica column chromatography eluting with 10% MeOH / DCM. This afforded 1'-β-O-(6-(2,2,2-trifluoroacetyl)-aminohexyl)-2'-deoxy-D-ribose **(β-48)** (1.11 g, 82%) as a colourless oil. Analysis was concordant with the proposed structure.

R_F = 0.4 (EtOAc, anisaldehyde)

Es+ve: 330 $(M+H)^+$, 347 $(M+NH_4)^+$, 352 $(M+Na)^+$, 368 (M+K)

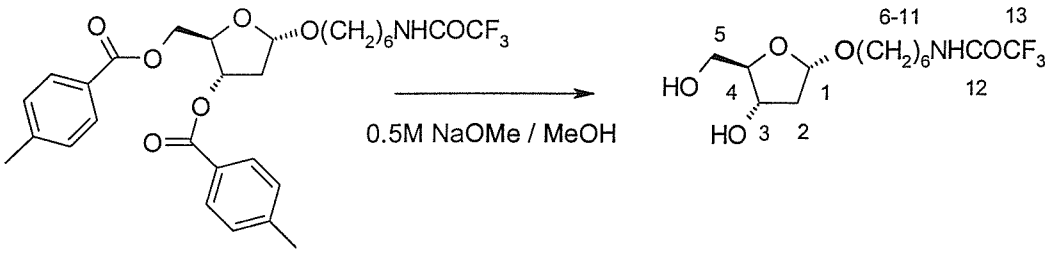
ν_{MAX} /cm^{-1}(KBr Disc): 1710 and 1560 C=O str, 1038 C-O str

δ^1H (300MHz, $CDCl_3$): 1.20-1.42 (m., 4H, $\underline{H}_8$ and $\underline{H}_9$), 1.42-1.63 (m., 4H, $\underline{H}_7$ and $\underline{H}_{10}$), 2.03-2.28 (m., 2H, $\underline{H}_2$), 2.88 (b.s., 1H, -O$\underline{H}$), 3.05-3.16 (m., 1H, $\underline{H}_4$), 3.23-3.39 (m., 2H, $\underline{H}_{11}$), 3.30-3.42 and 3.64-3.71 (2m., each 1H, $\underline{H}_6$ {rotomeric effect}), 3.51-3.70 (m., 2H, $\underline{H}_5$), 3.94-4.01 (m., 1H, $\underline{H}_3$), 4.45 (b.s., 1H, -O$\underline{H}$), 5.13-5.20 (m, 1H, $\underline{H}_1$), 6.95 (b.s., 1H, N$\underline{H}$)

δ^{13}C (75.42MHz, $CDCl_3$): 25.56 ($\underline{C}_9$), 26.16 ($\underline{C}_{10}$), 28.69 ($\underline{C}_7$), 29.29 ($\underline{C}_8$), 39.71 ($\underline{C}_2$), 42.53 ($\underline{C}_{11}$), 63.44 ($\underline{C}_5$), 68.27 ($\underline{C}_6$), 72.03 ($\underline{C}_4$), 87.42 ($\underline{C}_3$), 104.49 ($\underline{C}_1$), 157.4 ($\underline{C}_{13}$)

3.2.8

1'-α-O-(6-(2,2,2-Trifluoroacetyl)-aminohexyl)-2'-deoxy-D-ribose (α-48)



The same reaction conditions were applied to 1'-α-O-(6-(2,2,2-trifluoroacetyl)aminohexyl)-3',5'-di-O-*p*-toluoyl-2'-deoxy-D-ribose **(α-47)** (0.60 g, 1.15 mmol) to afford 1'-β-O-(6-(2,2,2-trifluoroacetyl)-aminohexyl)-2'-deoxy-D-ribose **(α-48)** (220 mg, 63%) as a colourless oil. Analysis was concordant with the proposed structure.

R_F = 0.4 (EtOAc, anisaldehyde)

Es+ve: 347 $(M+NH_4)^+$, 352 $(M+Na)^+$, 368 $(M+K)^+$

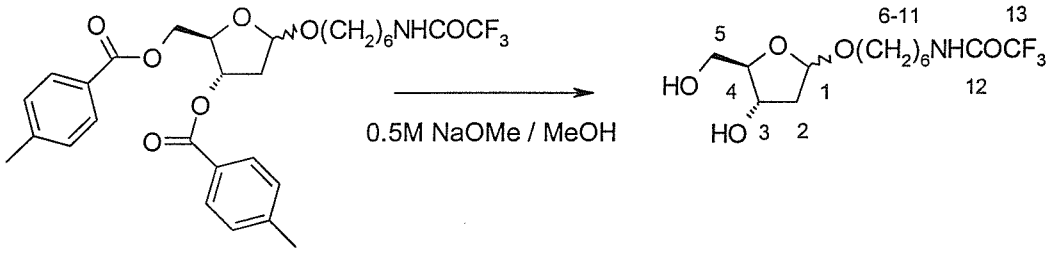
ν_{MAX} /cm^{-1}(KBr Disc): 1709 and 1559 C=O str, 1082 C-O str,

δ^1H (300MHz, $CDCl_3$): 1.23-1.39 (m., 4H, $\underline{H}_8$ and $\underline{H}_9$), 1.41-1.62 (m., 4H, $\underline{H}_7$ and $\underline{H}_{10}$), 1.86-1.98 and 2.01-2.12 2 (m., each 1H, $\underline{H}_2$), 2.49 (b.s., 1H, C^5-O$\underline{H}$), 3.10-3.19 (m., 1H, $\underline{H}_4$), 3.20-3.32 (m., 2H, $\underline{H}_{11}$), 3.25-3.39 and 3.60-3.72 (2m., each 1H, 1H $\underline{H}_6$ {rotomeric effect}), 3.51-3.68 (m., 2H, $\underline{H}_5$), 4.00-4.13 (m., 1H, $\underline{H}_3$), 4.05 (b.s., 1H, C^3-O$\underline{H}$), 5.10-5.14 (m., 1H, $\underline{H}_1$), 6.95 (b.s., 1H, N$\underline{H}$)

δ^{13}C (75.42MHz, $CDCl_3$): 25.83 ($\underline{C}_9$), 26.38 ($\underline{C}_{10}$), 28.90 ($\underline{C}_7$), 29.43 ($\underline{C}_8$), 39.94 ($\underline{C}_2$), 41.47 ($\underline{C}_{11}$), 63.14 ($\underline{C}_5$), 67.44 ($\underline{C}_6$), 72.95 ($\underline{C}_4$), 87.40 ($\underline{C}_3$), 104.37 ($\underline{C}_1$), 157.47 ($\underline{C}F_3$).

3.2.9

1'-O-(6-(2,2,2-Trifluoroacetyl)-aminohexyl)-2'-deoxy-D-ribose (48)

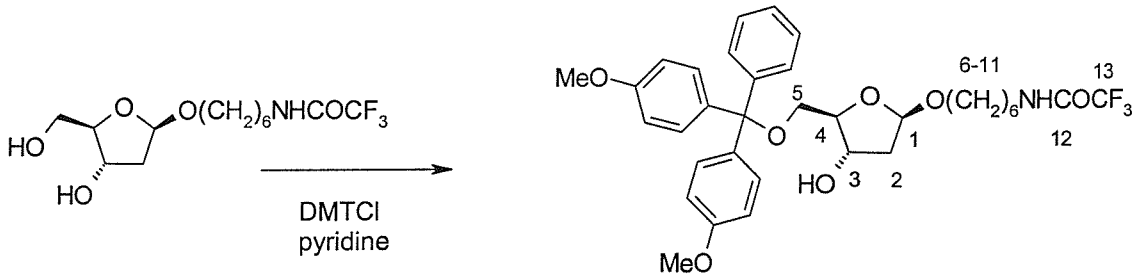


The same reaction conditions were applied to a (1:1) mixture of anomers of 1'-α-O-(6-(2,2,2-trifluoroacetyl)-aminohexyl)-3',5'-di-O-*p*-toluoyl-2'-deoxy-D-ribose **(47)**

(21.2 g, 37.5 mmol) to afford 1'-O-(6-(2,2,2-trifluoroacetyl)-aminohexyl)-2'-deoxy-D-ribose **(48)** (9.89 g, 80%) as a colourless oil. Analysis was concordant with the proposed structure and the ratio of anomers was seen (by ^{1}H-NMR) to be unaffected by these conditions.

3.2.10

1'-β-O-(6-(2,2,2-Trifluoroacetyl)-aminohexyl)-5'-O-(5-bis-(4-methoxyphenyl)-phenylmethyl)-2'-deoxy-D-ribose (β-49)



1'-β-O-(6-(2,2,2-Trifluoroacetyl)-aminohexyl)-2'-deoxy-D-ribose **(β-48)** (1.02 g, 3.10 mmol) was co-evaporated with anhydrous pyridine (3 x 10 mL) and dissolved in anhydrous pyridine (12 mL). DMT-Cl (1.1 eq., 1.18 g) was added in one portion and the resulting solution was stirred at ambient temperature for 3 hours. At that time TLC analysis showed an absence of starting material. The reaction mixture was diluted with DCM (30 mL), washed by extraction with sat. $NaHCO_{3(aq)}$ (20 mL) and the organic phase was dried over Na_2SO_4 (2.0g) and concentrated *in vacuo* to give an orange oil. This was co-evaporated with toluene (5 x 30 mL) to displace residual pyridine and purified by flash silica column chromatography, pre-equilibrating the silica using 1% Et_3N / EtOAc, and eluting with 40% EtOAc / hexane. This afforded 1'-β-O-(6-(2,2,2-trifluoroacetyl)-aminohexyl)-5'-O-(5-bis-(4-methoxyphenyl)-phenylmethyl)-2'-deoxy-D-ribose **(β-49)** (1.22 g, 62%) as a white, waxy solid. Analysis was concordant with the proposed structure.

m.p. 58-62°C

R_F=0.27 (EtOAc / hexane 1:1, uv., HCl fumes, anisaldehyde)

ES+ve: 649 $(M+NH_4)^+$, 654 $(M+Na)^+$, 670 $(M+K)^+$

HRMS: 631.2783 (calc. 631.2756)

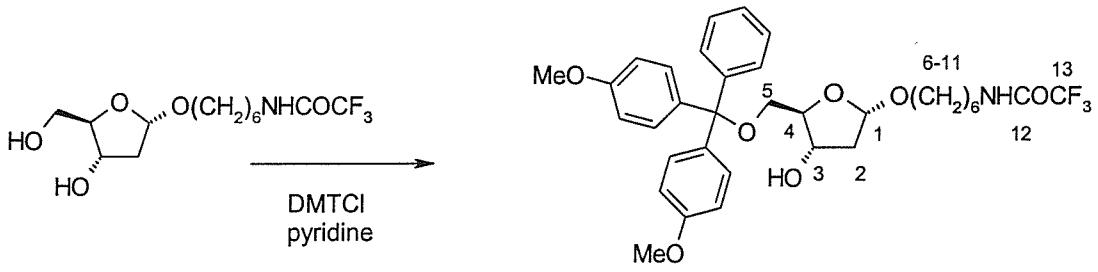
ν_{MAX} /cm^{-1}(KBr Disc): 1720 C=O str, 1608 C=O str, 1175 (OH def)

δ^1H (300MHz, $CDCl_3$): 1.31-1.25(m., $\underline{H}_8$ and $\underline{H}_9$), 1.35-1.49 (m., 4H, $\underline{H}_7$ and $\underline{H}_{10}$), 1.91-2.20 (m., 2H, $\underline{H}_2$), 1.95 (s., 1H, O$\underline{H}$), 2.95-3.23 (m., 2H, $\underline{H}_5$), 3.14-3.23 (m., 2H, $\underline{H}_{11}$), 3.13-3.23 and 3.50-3.60 (2m., each 1H, $\underline{H}_6$ {rotomeric effect}), 3.68 (s., 6H, -OC$\underline{H}_3$), 3.86-3.95 (m., 1H, $\underline{H}_4$), 4.30-4.42 (m., 1H, $\underline{H}_3$), 5.05-5.10 (m., 1H, $\underline{H}_1$), 6.40 (b.s., 1H, N$\underline{H}$), 6.71-6.82 (d., 4H, aromatic C-$\underline{H}$ *ortho* to -OMe), 7.10-7.48 (m., 9H, aromatic C-$\underline{H}$)

δ^{13}C (75.42MHz, $CDCl_3$): 25.81 ($\underline{C}_9$), 26.51 ($\underline{C}_{10}$), 28.89 ($\underline{C}_7$), 29.49 ($\underline{C}_8$), 40.01 ($\underline{C}_2$), 41.32 ($\underline{C}_{11}$), 55.39 ($H_3\underline{C}O$), 65.21 ($\underline{C}_5$), 67.68 ($\underline{C}_6$), 73.54 ($\underline{C}_4$), 84.74 ($\underline{C}_3$), 86.27 ($\underline{C}Ar_3$), 104.03 ($\underline{C}_1$), 113.27, 126.94, 127.99, 128.24, 130.23 (aryl $\underline{C}$H), 136.21, 136.28, 145.05 (aryl $\underline{C}_{quat}$), 158.57 ($\underline{C}F_3$),

3.2.11

1'-α-O-(6-(2,2,2-Trifluoroacetyl)-aminohexyl)-5'-O-(5-bis-(4-methoxyphenyi)-phenyimethyl)-2'-deoxy-D-ribose (α-49)



The same reaction conditions were applied 1'-α-O-(6-(2,2,2-trifluoroacetyl)-aminohexyl)-2'-deoxy-D-ribose **(α-48)** (190 mg, 0.395 mmol) to afford 1'-α-O-(6-(2,2,2-trifluoroacetyl)-aminohexyl)-5'-O-(5-bis-(4-methoxyphenyl)-phenylmethyl)-

2'-deoxy-D-ribose **(α-49)** (280mg, 77%) as an amorphous white solid. Analysis was concordant with the proposed structure.

m.p. 65-68°C

R_F = 0.44 (EtOAc / hexane 1:1, uv., HCl fumes, anisaldehyde)

ES+ve: 649 $(M+NH_4)^+$, 654 $(M+Na)^+$, 670 $(M+K)^+$

HRMS: 631.2754 (calc. 631.2756)

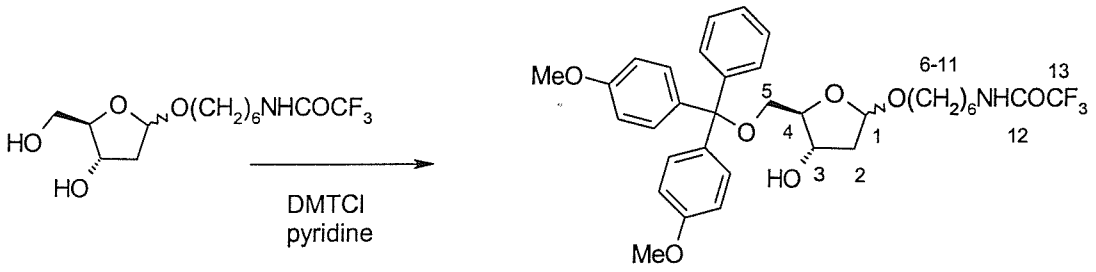
ν_{MAX} /cm^{-1}(KBr Disc): 1712 C=O str, 1608 C=O str, 1176 OH def

δ^1H (300MHz, $CDCl_3$): 1.24-1.35 (m., 4H, $\underline{H}_8$ and $\underline{H}_9$), 1.45-1.60 (m., 4H, $\underline{H}_7$ and $\underline{H}_{10}$), 1.60 (b.s., 1H, $\underline{OH}$), 1.91-2.20 (m., 2H, $\underline{H}_2$), 1.95 (s., 1H, O$\underline{H}$), 2.92-3.10 (m., 2H, $\underline{H}_5$), 3.23-3.21 (m., 2H, $\underline{H}_{11}$), 2.90-3.10 and 3.20-3.40 (2m., each 1H, $\underline{H}_6$ {rotomeric effect}), 3.70 (s., 6H, -OC$\underline{H}_3$), 4.08-4.19 (2m., each 1H, $\underline{H}_4$ and $\underline{H}_3$), 5.18-5.20 (m., 1H, $\underline{H}_1$), 6.42 (b.s., 1H, N$\underline{H}$), 6.71-6.82 (d., 4H, aromatic C-$\underline{H}$ *ortho* to -OMe), 7.10-7.48 (m., 9H, aromatic C-$\underline{H}$)

δ^{13}C (75.42MHz, $CDCl_3$): 25.89 ($\underline{C}_9$), 26.43 ($\underline{C}_{10}$), 29.02 ($\underline{C}_7$), 29.52 ($\underline{C}_8$), 39.94 ($\underline{C}_2$), 41.02 ($\underline{C}_{11}$), 55.36 ($H_3\underline{C}$-O), 64.16 ($\underline{C}_5$), 67.29 ($\underline{C}_6$), 73.71 ($\underline{C}_4$), 86.23 ($\underline{C}_3$), 87.01 ($\underline{C}Ar_3$), 104.49 ($\underline{C}_1$), 113.25, 126.93, 127.97, 128.28, 130.20 (aryl $\underline{C}$H), 136.04, 136.14, 144.93 (aryl $\underline{C}_{quat}$), 158.60 ($\underline{C}F_3$)

3.2.12

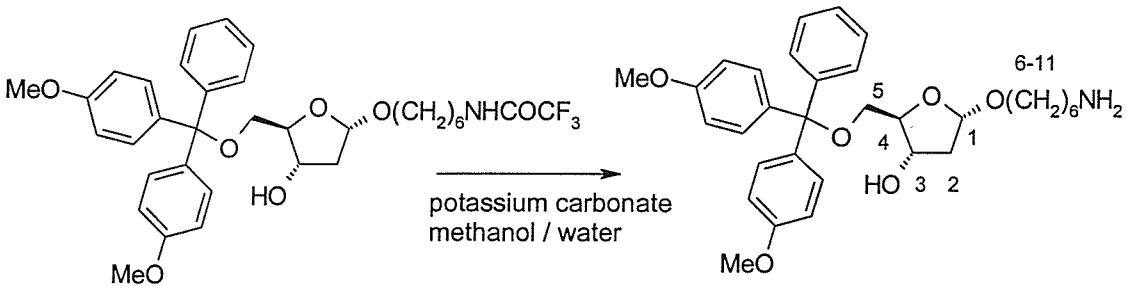
1'-O-(6-(2,2,2-Trifluoroacetyl)-aminohexyl)-5'-O-(5-bis-(4-methoxyphenyl)-phenylmethyl)-2'-deoxy-D-ribose (49)



The same reaction conditions were applied a (1:1) mixture of anomers of 1'-O-(6-(2,2,2-trifluoroacetyl)-aminohexyl)-2'-deoxy-D-ribose **(48)** (9.67g, 29.4 mmol) to afford 1'-α-O-(6-(2,2,2-trifluoroacetyl)-aminohexyl)-5'-O-(5-bis-(4-methoxyphenyl)-phenylmethyl)-2'-deoxy-D-ribose **(49)** (14.5g, 80%) as an amorphous white solid. Analysis was concordant with the proposed structure (details as above).

3.2.13

1'-α-O-(6-Aminohexyl)-5'-O-(5-bis-(4-methoxyphenyl)-phenylmethyl)-2'-deoxy-D-ribose (α-50)



1'-α-O-(6-(2,2,2-Trifluoroacetyl)-aminohexyl)-5'-O-(5-bis-(4-methoxyphenyl)-phenylmethyl)-2'-deoxy-D-ribose **(α-49)** (0.69 g, 1.09 mmol) was dissolved in MeOH (7.0 mL) and deionised water (1.2 mL) was added dropwise with stirring, to give a homogeneous solution. K_2CO_3 (2.0 eq., 2.18 mmol, 0.30g) was added and the resulting mixture was heated to 60°C and stirred at that temperature for 45 minutes. After that time TLC analysis showed that all of the substrate had

reacted. The reaction mixture was allowed to cool to ambient temperature, diluted with DCM (30 mL) and washed by extraction with sat. $KCl_{(aq)}$ (30 mL). The aqueous phase was extracted with DCM (2 x 30 mL) and the combined organic extracts were dried over Na_2SO_4 (1.0 g), filtered and concentrated *in vacuo* to give a colourless oil (640 mg). This was purified by flash column chromatography on silica gel, eluting with DCM / MeOH / Et_3N (94:5:1) to afford 1'-α-O-(6-aminohexyl)-5'-O-(5-bis-(4-methoxyphenyl)-phenylmethyl)-2'-deoxy-D-ribose **(α-50)** (0.53g, 0.99mmol, 91%) as an amorphous white solid. Analysis was concordant with the proposed structure.

m.p. 126-135°C decomp.

R_F=0.36 (DCM / MeOH / Et_3N (8.75:1:0.25), u.v., 5% H_2SO_4 / EtOH, anisaldehyde)

ES+ve: 536 $(M+H)^+$, 558 $(M+Na)^+$

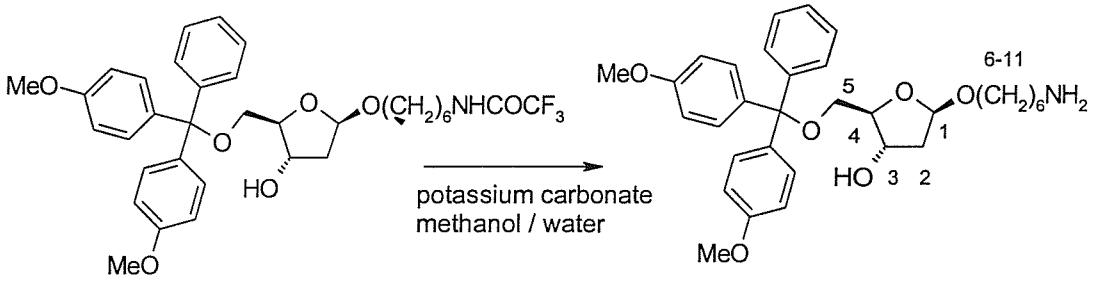
HRMS: 558.2832 $(M+Na)^+$ (calc. 558.2856)

δ^1H (300MHz, $CDCl_3$): 1.19-1.37 (m., 4H, $\underline{H}_8$ and $\underline{H}_9$), 1.30-1.47 (m., 2H, $\underline{H}_{10}$), 1.43-1.60 (m., 2H, $\underline{H}_7$), 1.87-2.00 and 1.85-2.20 (2m., each 2H, $\underline{H}_2$), 2.28 (b.s, 3H, O$\underline{H}$ and N$\underline{H}_2$), 2.58-2.64 (m., 2H, $\underline{H}_{11}$), 3.00-3.13 (m., 2H, $\underline{H}_5$), 3.28-3.39 and 3.60-3.72 (2m., each 1H, $\underline{H}_6$), 3.68 (s., 6H, -OC$\underline{H}_3$), 4.08-4.17 (m., 1H, $\underline{H}_4$), 4.08-4.17 (m., 1H, $\underline{H}_3$), 5.12-5.17 (m., 1H, $\underline{H}_1$), 6.71-6.82 (d., 4H, aromatic C-$\underline{H}$ *ortho* to -OMe), 7.10-7.48 (m., 9H, aromatic C-$\underline{H}$)

$\delta^{13}C$ (75.42MHz, $CDCl_3$): 26.22 ($\underline{C}_9$), 26.74 ($\underline{C}_{10}$), 29.76 ($\underline{C}_7$), 33.40 ($\underline{C}_8$), 41.11 ($\underline{C}_2$), 42.06 ($\underline{C}_{11}$), 55.36 ($H_3\underline{C}$-O), 64.21 ($\underline{C}_5$), 67.61 ($\underline{C}_6$), 73.57 ($\underline{C}_4$), 86.18 ($\underline{C}Ar_3$), 86.87 ($\underline{C}_3$), 104.48 ($\underline{C}_1$), 113.24, 126.90, 127.96, 128.30, 130.21 (aryl $\underline{C}$H), 136.08, 136.17, 144.98 (aryl $\underline{C}_{quat}$)

3.2.14

1'-β-O-(6-Aminohexyl)-5'-O-(5-bis-(4-methoxyphenyl)-phenylmethyl)-2'-deoxy-D-ribose (β-50)



The same reaction conditions were applied to 1'-β-O-(6-(2,2,2-Trifluoroacetyl)-aminohexyl)-5'-O-(5-bis-(4-methoxyphenyl)-phenylmethyl)-2'-deoxy-D-ribose **(β-49)** (1.57g, 2.49 mmol) to afford 1'-β-O-(6-aminohexyl)-5'-O-(5-bis-(4-methoxyphenyl)-phenylmethyl)-2'-deoxy-D-ribose **(β-50)** (0.88g, 66%) as an amorphous white solid. Analysis was concordant with the proposed structure.

m.p. 138-160°C decomp.

R_F=0.43 (DCM / MeOH / Et_3N (8.75:1:0.25), u.v., 5% H_2SO_4 / EtOH, anisaldehyde

ES+ve: 536 $(M+H)^+$

HRMS: $(M+Na)^+$ 558.2837 (calc. 558.2832)

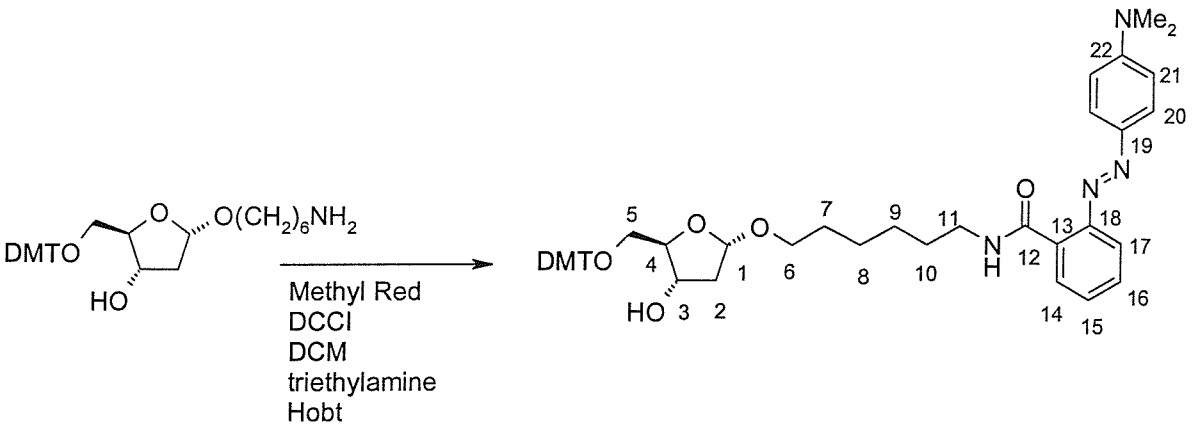
δ^1H (300MHz, $CDCl_3$): 1.13-1.20 (m., 4H, $\underline{H}_8$ and $\underline{H}_9$), 1.20-1.29 (m., 2H, $\underline{H}_{10}$), 1.29-1.38 (m., 2H, $\underline{H}_7$), 1.82-2.00 and 2.03-2.10 (2m., each 2H, $\underline{H}_2$), 1.88 (b.s, 3H, O$\underline{H}$ and N$\underline{H}_2$), 2.47-2.58 (m., 2H, $\underline{H}_{11}$), 3.03-3.20 (m., 2H, $\underline{H}_5$), 3.14-3.25 and 3.45-3.54 (2m, each 1H, $\underline{H}_6$ {rotomeric effect}), 3.68 (s., 6H, -OC$\underline{H}_3$), 3.85-3.92

(m., 1H, $\underline{H}_4$), 4.28-4.56 (m., 1H, $\underline{H}_3$), 5.08-5.17 (m., 1H, $\underline{H}_1$), 6.71-6.82 (d., 4H, aromatic C-$\underline{H}$ *ortho* to -OMe), 7.10-7.48 (m., 9H, aromatic C-$\underline{H}$)

δ^{13}C (75.42MHz, $CDCl_3$): 26.10 ($\underline{C}_9$), 26.78 ($\underline{C}_{10}$), 29.67 ($\underline{C}_7$), 33.59 ($\underline{C}_8$), 41.36 ($\underline{C}_2$), 42.14 ($\underline{C}_{11}$), 55.35 ($H_3\underline{C}$-O), 65.32 ($\underline{C}_5$), 67.85 ($\underline{C}_6$), 73.10 ($\underline{C}_4$), 84.86 ($\underline{C}_3$), 86.24 ($\underline{C}Ar_3$), 103.99 ($\underline{C}_1$), 113.23, 126.91, 127.95, 128.34, 130.21 (aryl $\underline{C}$H), 136.26, 136.26, 145.04 (aryl $\underline{C}_{quat}$)

3.2.15

1'-α-O-[(2-(Dimethylamino-phenylazo)-benzoyl)-6-aminohexyl]-5'-O-(5-bis-(4-methoxyphenyi)-phenylmethyl)-2'-deoxy-D-ribose (α-51)



1'α-O-(6-Aminohexyl)-5'-O-(5-bis-(4-methoxyphenyl)-phenylmethyl)-2'-deoxy-D-ribose **(α-50)** (2.12 g, 4.0 mmol) was co-evaporated with anhydrous DCM (2 x 15 mL), dissolved in anhydrous DCM (18 mL) and stirred under a headspace of argon at ambient temperature. Anhydrous Et_3N (2 eq., 0.81g, 1.1 mL) was added, followed by 2-(4-dimethylamino-phenylazo)-benzoic acid (methyl red) (1.1 eq, 4.4 mmol, 1.20 g) and 1-Hobt (1.5 eq., 6.0 mmol, 0.81 g). A solution of DCCI (1.5 eq., 6.0 mmol, 1.24 g) in anhydrous DCM (2 mL) was then added dropwise and the reaction was stirred at ambient temperature overnight. At that time TLC analysis showed the reaction to be complete. The reaction mixture was filtered

and the filtrate was diluted with DCM (60 ml) and washed by extraction with sat. $NaHCO_{3(aq)}$ (50 mL), then 2M $NaOH_{(aq)}$ (2 x 50 mL). The organic solution was dried over $MgSO_4$, filtered and concentrated *in vacuo*. The residual oil (5.32g) was purified by flash column chromatography on silica gel (pre-equilibrated with 1% Et_3N in hexane / EtOAc (2:1), eluting with hexane / EtOAc (2:1 to 1:1) to afford 1'-α-O-[(2-(dimethylamino-phenylazo)-benzoyl)-6-aminohexyl]-5'-O-(5-bis-(4-methoxyphenyl)-phenylmethyl)-2'-deoxy-D-ribose **(α-51)** (2.98 g, 3.79 mmol, 88%) as a red solid (dried *in vacuo* over P_2O_5 to constant mass). Analysis was concordant with the proposed structure.

m.p. 64-66°C

$\varepsilon_{max} = 20.05 \times 10^3\ mol^{-1}\ dm^3\ cm^{-1}$ @ 432.5nm / MeOH

R_F=0.25 (hexane / EtOAc (1:1), coloured (orange), uv., 5% H_2SO_4 / EtOH, anisaldehyde)

ES+ve: 809 $(M+Na)^+$

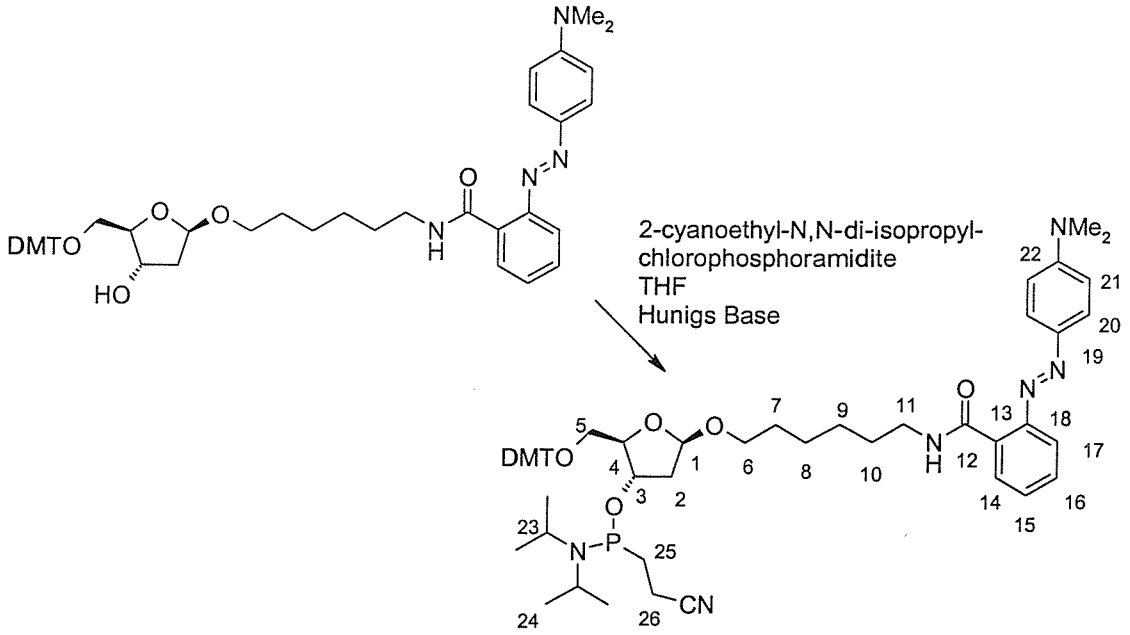
HRMS: 786.398 (calc. 786.399)

δ^1H (300MHz, $CDCl_3$): 1.20-1.48 (m., 4H, $\underline{H}_8$ and $\underline{H}_9$), 1.38-1.59 and 1.50-1.62 (2m., each 2H, $\underline{H}_{10}$ and $\underline{H}_7$), 1.78 (b.s., 1H, O$\underline{H}$), 1.90-2.00 and 2.05-2.18 (2m., each 1H, $\underline{H}_2$), 2.91-3.10 (m., 2H, $\underline{H}_5$), 3.03 (s., 6H, -N($C\underline{H}_3$)$_2$) 3.21-3.36 and 3.54-3.69 (2m., each 1H, $\underline{H}_6$ {rotomeric effect}), 3.41-3.53 (m., 2H, $\underline{H}_{11}$), 3.67 (s., 6H, -O$C\underline{H}_3$), 4.00-4.19 (2m., each 1H, $\underline{H}_3$ and $\underline{H}_4$), 5.10-5.17 (m., 1H, $\underline{H}_1$), 6.75 (d. J = 8Hz, 2H, $\underline{H}_{21}$), 6.65-6.80 (m., 4H, aromatic C-$\underline{H}$ *ortho* to -OMe), 7.10-7.30 (m., 9H, trityl aromatic C-$\underline{H}$), 7.42-7.50 and 7.60-7,70 (2m., each 1H $\underline{H}_{15}$ and $\underline{H}_{16}$), 7.70 (d. J = 8 Hz, 2H, $\underline{H}_{20}$), 8.39-8.46 (m., 1H, $\underline{H}_{17}$), 8.95-9.05 (m., 1H, $\underline{H}_{14}$)

$\delta^{13}C$ (75.42MHz, $CDCl_3$): 26.15 ($\underline{C}_9$), 27.16 ($\underline{C}_{10}$), 29.72 ($\underline{C}_7$), 29.84 ($\underline{C}_8$), 40.12 ($\underline{C}_2$), 40.45 (N($\underline{C}H_3)_2$), 41.07 ($\underline{C}_{11}$), 55.37 ($H_3\underline{C}$-O), 64.20 ($\underline{C}_6$), 67.55 ($\underline{C}_5$), 73.66 ($\underline{C}_4$), 86.20 ($\underline{C}_3$), 86.92 (-$\underline{C}(Ar)_3$), 104.45 ($\underline{C}_1$), 111.73 ($\underline{C}_{21}$), 113.26 (aryl $\underline{C}$ *ortho* to -OMe), 116.08 ($\underline{C}_{20}$), 125.86, 126.92, 127.97, 128.30, 129.69, 130.21, 131.54, 131.70 (aryl $\underline{C}$-H), 136.07, 136.17 and 144.97 (aryl $\underline{C}_{quat}$), 143.58 ($\underline{C}_{22}$), 150.65 ($\underline{C}_{19}$), 153.26 ($\underline{C}_{18}$), 158.60 ($\underline{C}_{12}$), 166.28 ($\underline{C}_{13}$)

3.2.16

1'-α-O-[(2-(Dimethylamino-phenylazo)-benzoyl)-6-aminohexyl]-5'-O-(5-bis-(4-methoxyphenyl)-phenylmethyl)-2'-deoxy-D-ribose-3'-(2-cyanoethyl-N,N-diisopropyl-phosphoramidite) (α-52)



1'-α-O-[(2-(Dimethylamino-phenylazo)-benzoyl)-6-aminohexyl]-5'-O-(5-bis-(4-methoxyphenyl)-phenylmethyl)-2'-deoxy-D-ribose **(α-51)** (1.00 g, 1.27 mmol) was co-evaporated with anhydrous THF (3 x 5 mL) and dissolved in anhydrous THF (6.0 mL). The resulting solution was stirred at ambient temperature under a headspace of argon and N,N-diisopropylamine (4.5 eq., 5.8 mmol, 0.75 g, 1.0 mL) was added as a co-solvent. This was followed by the dropwise addition of 2-cyanoethyl-N,N-diisopropyl-chlorophosphoramidite (1.17 eq., 1.48 mmol, 0.35 g,

0.35 mL) and the resulting solution was stirred at ambient temperature for 1 hour, after which time TLC analysis showed all the starting material to have reacted. The reaction mixture was diluted with degassed DCM (100 mL) {degassed with a stream of argon at ambient temperature} and washed by extraction with sat. $KCl_{(aq)}$ (100 mL). The organic extract was dried over Na_2SO_4, (5 g) with transfer *via* cannula and filtered by cannula transfer through a glass wool plug to remove the drying agent. The resulting solution was concentrated *in vacuo* to give a red oil. This was dissolved in anhydrous DCM (5.0 mL) and precipitated by dropwise addition to anhydrous hexane (500 mL) at –78°C (dry ice / acetone bath) with vigorous magnetic stirring. The suspension of the product was transferred by cannula through a glass wool plug and the precipitate was then washed from the glass wool with anhydrous DCM (100 mL). This organic solution was concentrated *in vacuo* to give a red oil. This was dissolved in anhydrous acetonitrile (6.0 mL) and passed through a Gelman Acrodisc filter (0.45 μm) to remove particulate matter. The filtrate was concentrated *in vacuo* and residual solvents were displaced by co-evaporation with anhydrous DCM (3 x 15 mL). The residual oil (1.14g) was dried *in vacuo* over P_2O_5 for 8 hours and precipitated again from hexane (as above). The second precipitation afforded 1'-α-O-[(2-(dimethylamino-phenylazo)-benzoyl)-6-aminohexyl]-5'-O-(5-bis-(4-methoxyphenyl)-phenylmethyl)-2'-deoxy-D-ribose-3'-(2-cyanoethyl-N,N-diisopropyl-phosphoramidite) **(α-52)** (0.93g, 0.94mmol, 74%), an air-sensitive red solid (dried *in vacuo* over P_2O_5 for 8 hours). Analysis was concordant with the proposed structure.

R_F=0.57, 0.65 (Hexane / EtOAc (1:1), coloured (orange), uv., 5% H_2SO_4 / EtOH, anisaldehyde)

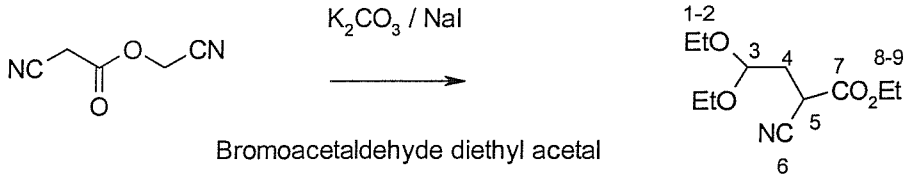
ES+ve: 1009 $(M+H)^+$

δ^1H (300MHz, $CDCl_3$): 0.95-1.18 (2m., 12H. $\underline{H}_{24}$), 1.20-1.48 (m., 4H, $\underline{H}_8$ and $\underline{H}_9$), 1.38-1.59 and 1.50-1.62 (2m., each 2H, $\underline{H}_{10}$ and $\underline{H}_7$), 1.40-1.55 (m., 2H, $\underline{H}_{26}$),

2.00-2.11 (m., 2H, $\underline{H}_2$), 3.00 (s., 6H, -N($C\underline{H}_3$)$_2$), 3.05-3.20 (m., 2H, $\underline{H}_5$), 3.35-3.64 (m., 6H, $\underline{H}_6$, $\underline{H}_{11}$ and $\underline{H}_{25}$), 3.67 (s., 6H, -O$C\underline{H}_3$), 4.00-4.12 (m., 1H, $\underline{H}_4$), 4.25-4.50 (m., 1H, $\underline{H}_3$), 5.05-5.12 (m., 1H, $\underline{H}_1$), 6.75 (d. J = 8Hz, 2H, $\underline{H}_{21}$), 6.65-6.80 (m., 4H, aromatic C-$\underline{H}$ *ortho* to -OMe), 7.10-7.40 (m., 9H, trityl aromatic C-$\underline{H}$), 7.42-7.50 and 7.60-7,70 (2m., each 1H $\underline{H}_{15}$ and $\underline{H}_{16}$), 7.70 (d. J = 8 Hz, 2H, $\underline{H}_{20}$), 8.20-8.28 (m., 1H, $\underline{H}_{17}$), 8.92-9.01 (m., 1H, $\underline{H}_{14}$)

3.2.17

Ethyl 2-cyano-4,4-diethoxybutanoate (54) Davoll [71]



Bromoacetaldehyde diethyl acetal (81.81 g, 0.415 mol), ethyl cyanoacetate (5.70 eq., 2.35 mol, 266 g), anhydrous K_2CO_3 (dried @ 1mmHg / 160°C for 12 hrs.) (1 eq., 0.415 mol, 57.3 g) and sodium iodide (0.05 eq., 20.8 mmol, 3.11 g) were heated to 150°C (oil bath), 115°C (internal) over 2 hours, stirred at that temperature for 6 hours and allowed to cool overnight to ambient temperature. The reaction mixture was diluted with de-ionised water (600 mL) (sufficient to dissolve the inorganic solids) and extracted with Et_2O (4 x 200 mL). The combined organic extracts were dried over Na_2SO_4 (50 g) and concentrated *in vacuo* giving a coloured (dark brown) oil. This was purified by distillation under reduced pressure through a 15 cm Fenske column yielding ethyl 2-cyano-4,4-diethoxybutanoate **(54)** (36.23 g, 0.158 mol, 38%, {lit.; 46%)) as a colourless oil (b.p. 110-120°C @ 1mmHg: Lit.; 111-115°C @ 1.3 mmHg).[71] Analysis was concordant with the proposed structure and in agreement with the literature.

R_F 0.35 (DCM, phosphomolybdic acid, iodine sublimation)

ES+: 130.8 $(M+Na)^+$

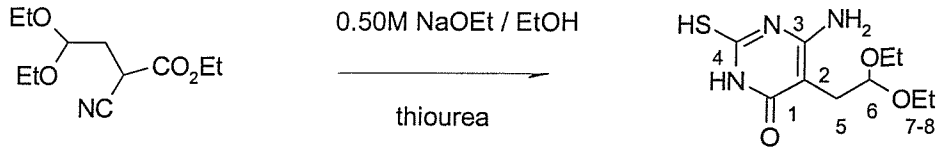
ν_{MAX} /cm^{-1} (Neat oil):2252 CN str., 1752 CO str.

δ^1H (300MHz, $CDCl_3$): 1.17 and 1.19 (2t., each 3H, J = 7.35 Hz diastereotopic $\underline{H}_1$), 1.23 (t., 3H, 7.36 Hz, $\underline{H}_9$), 2.13-2.31 (m., 2 H, H_4), 3.45-3.57 (m., 1H, $\underline{H}_5$), 3.45-3.75 (m., 4H, $\underline{H}_2$), 4 17 (q., 2H, J = 7.35 Hz, $\underline{H}_8$), 4.65 (t., 1H, J = 5.88 Hz $\underline{H}_3$)

$\delta^{13}C$ (75.42MHz, $CDCl_3$): 13.86, 15.08, 15.11 ($\underline{C}_1$ and $\underline{C}_9$), 33.46 ($\underline{C}_5$), 33.50 ($\underline{C}_4$), 62.71, 62.87, 62.95 ($\underline{C}_2$ and $\underline{C}_8$), 99.88 ($\underline{C}_3$), 116.31 ($\underline{C}_6$), 165.82 ($\underline{C}_7$)

3.2.18

4-Amino-5-(2,2-diethoxyethyl)-6-hydroxy-2-pyrimidinethiol (55) Davoll[71]



Ethyl 2-cyano-4,4-diethoxybutanoate **(55)** (36.08 g, 157 mmol) was dissolved in 0.50M NaOEt / EtOH (100 mL) and stirred magnetically at ambient temperature. A suspension of thiourea (1.0 eq., 157 mmol, 12.0 g) in 0.5M NaOEt / EtOH (215 mL) was added portionwise by decantation to the stirred reaction mixture. The reaction was heated at reflux for 4 hours after which time TLC analysis indicated that the reaction was complete. The reaction mixture was allowed to cool to ambient temperature and concentrated *in vacuo* to give a coloured solid. The solid was dissolved in water (50 mL) (pH12) and glacial acetic acid (10 mL) was added dropwise until pH6.5. This resulted in a white precipitate that was obtained by filtration and washed by displacement with i-PrOH (50 ml) and then

Et_2O (50 ml) and crystallised from MeOH (250 ml). The crystals were recovered by suction, washed with i-PrOH (50 mL) and then Et_2O (2 x 20 mL), air dried by suction for 2 hours and then dried *in vacuo* over P_2O_5 for 1 hour. This afforded 4-amino-5-(2,2-diethoxyethyl)-6-hydroxy-2-pyrimidinethiol **(55)** (23.94g, 92.4 mmol 59%, {lit.; 82%}) as a white, crystalline solid.[71] Analysis was concordant with the proposed structure and in agreement with the literature.

R_F = 0.45 (10% MeOH / DCM, u.v., phosphomolybdic acid)

ES+: 260 $(M+H)^+$, 282 $(M+Na)^+$

ν_{MAX} /cm^{-1} (Nujol Mull): 1640 CO str.

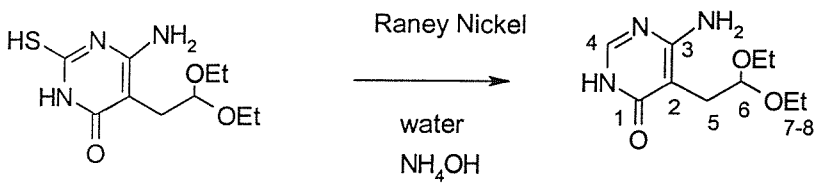
m.p. 143-156°C decomp.

δ^1H (300MHz, DMSO): 1.08 (t., 6H, J = 7.35 Hz, $\underline{H}_8$), 2.43 (d., 2 H, J=5Hz), $\underline{H}_5$), 3.25-3.41 and 3.52-3.65 (2m., each 2H, $\underline{H}_7$), 4 50 (t., 1H, J=5Hz, $\underline{H}_6$), 6.1 (s., 2H) NH_2, 11.45 and 11.64 (2s., each1H) SH and NH

δ^{13}C (75.42MHz, DMSO): 15.39 ($\underline{C}_8$), 27.96 ($\underline{C}_5$), 61.76 ($\underline{C}_7$), 85.74 (C_2), 101.77 ($\underline{C}_6$), 151.95 ($\underline{C}_3$), 161.85 ($\underline{C}_4$), 172.88 ($\underline{C}_1$).

3.2.19

4-Amino-5-(2,2-diethoxyethyl)-6-hydroxypyrimidine (56) Davoll[71]



Preparation of Raney Nickel

NaOH (69 g) was stirred in de-ionised water (265 mL) and was allowed to exotherm to 70°C. Al/Ni alloy (1:1) (53.1g) was cautiously added over 30 minutes at such a rate as to maintain a temperature between 70°C and 95°C. The mixture was then allowed to cool with stirring for 30 minutes. and transferred into a 250 mL measuring cylinder. The mixture was then rinsed with a steady stream of de-ionised water that was directed to the base of the measuring cylinder using a glass tube. The flow rate was adjusted to bring the catalyst to within a few centimetres of the measuring cylinder lip. The rinsing was continued until the pH of the washings was neutral (universal indicator paper), requiring 10 litres of de-ionised water in total. The catalyst was allowed to settle and the majority of the water was removed by decantation. The active Raney nickel was used directly as an aqueous slurry.

Desulfurisation

4-Amino-5-(2,2-diethoxyethyl)-6-hydroxy-2-pyrimidinethiol **(55)** (17.70 g 68.3 mmol) was dissolved in de-ionised water (750 mL) and c.$NH_{3(aq)}$ (53.1 mL) was added followed by Raney nickel (from 53.1g of alloy). The mixture was heated at reflux for 1 hour and hot-filtered through a sintered glass funnel. The Raney nickel residue was washed by displacement with MeOH (4 x 50 mL) and the combined filtrate and washings were concentrated *in vacuo*. The amorphous residue was crystallised from MeOH (150 mL) affording 4-amino-5-(2,2-diethoxyethyl)-6-hydroxypyrimidine **(56)** (11.53 g, 50.7 mmol, 74%, {lit. 75%}[71]) as a white, crystalline solid. Analysis was concordant with the proposed structure and in agreement with the literature.

R_F = 0.42 (15% MeOH / DCM, u.v., phosphomolybdic acid)

ES+: 228 $(M+H)^+$, 455 $(2M+H)^+$

ν_{MAX} /cm^{-1} (KBr disc): 1633 CO str.

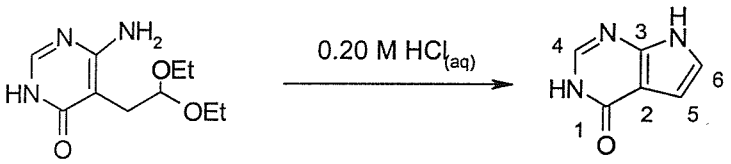
m.p. 177-180°C {lit. 185-186°C}.[71]

δ^1H (300MHz, DMSO): 1.04 (t., 6H, J=7.35 Hz, $\underline{H}_8$), 2.52 (d., 2 H, J=5 Hz, $\underline{H}_5$, 3.33-3.48 and 3.52-3.67 (2m., each 2H, $\underline{H}_7$), 4 55 (t., 1H, J=5 Hz, $\underline{H}_6$), 6.1 (s., 2H) NH_2, 7.7 (s., 1H, $\underline{H}_4$)

δ^{13}C (75.42MHz, DMSO): 15.38 ($\underline{C}_8$), 28.82 ($\underline{C}_5$), 61.46 ($\underline{C}_7$), 93.24 (C_2), 101.93 ($\underline{C}_6$), 147.08 (C_4), 161.54 (C_3), 161.86 (C_1).

3.2.20

4-Hydroxypyrrolo[2,3-d]pyrimidine (57) Davoll[71]



4-Amino-5-(2,2-diethoxyethyl)-6-hydroxypyrimidine **(56)** (6.27 g, 27.6 mmol) was suspended in 0.2M $HCl_{(aq)}$ (1.5 eq., 205 mL) and agitated at ambient temperature for 24 hours using a mechanical flask shaker. The mixture was filtered to give a solid that was rinsed by displacement with de-ionised water (2 x 20 mL) then Et_2O (2 x 20 mL) and dried overnight *in vacuo* over P_2O_5 affording 4-hydroxypyrrolo[2,3-d]pyrimidine **(57)** (3.40 g, 25.2 mmol, 91% {lit.; 96%) as a uniform white solid.[71] Analysis was concordant with the proposed structure and in agreement with the literature.

R_F = 0.22 (10% MeOH / DCM, u.v., phosphomolybdic acid)

ES+: 136 $(M+H)^+$, 153 $(M+NH_4)^+$

ν_{MAX} /cm^{-1} (Nujol Mull): 1662 CO str

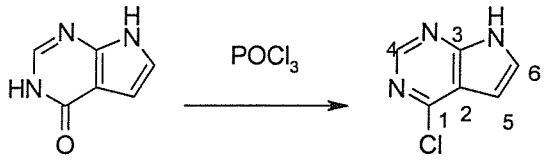
m.p. 335-342°C decomp.. {Lit.; 335-340°C decomp.}.[71]

δ^1H (300MHz, DMSO): 6.49 (d., 1H, J=3 Hz, $\underline{H}_5$), 7.08 (d., 1H, J=3 Hz, $\underline{H}_6$), 7.87 (s., 1H, $\underline{H}_4$), 11.79 and 11.80 (2s., each 1H) two N$\underline{H}$

δ^{13}C (75.42MHz, DMSO): 102.05 ($\underline{C}_5$), 107.71 ($\underline{C}_2$), 120.44 ($\underline{C}_6$), 143.30 ($\underline{C}_4$), 148.14 ($\underline{C}_1$), 158.57 ($\underline{C}_3$)

3.2.21

4-Chloropyrrolo[2,3-d]pyrimidine (58) Davoll [71]



4-Hydroxypyrrolo[2,3-d]pyrimidine **(57)** (8.48 g, 62.8 mmol) was suspended in $POCl_3$ (85 mL) and heated at reflux for 40 minutes. The reaction mixture was allowed to cool to ambient temperature and concentrated *in vacuo* to give an oil. This was quenched by pouring onto ice (250 mL) and stirring for 1 hour while the ice was allowed to melt. Sat. $KCl_{(aq)}$ (100 mL) was added, the mixture was extracted with DCM (10 x 100 mL) and the combined organic extracts were dried over Na_2SO_4 (20 g) and concentrated *in vacuo*.to give a residual solid, which was

dried overnight *in vacuo* over P_2O_5 affording 4-chloropyrrolo[2,3-d]pyrimidine **(58)** (4.20 g, 27.5 mmol, 44%, {lit.; 79%} [71]) as a white solid. Analysis was concordant with the proposed structure and in agreement with the literature.

R_F = 0.56 (10% MeOH / DCM, u.v., phosphomolybdic acid)

ES+: 153 and 155 $(M+H)^+$

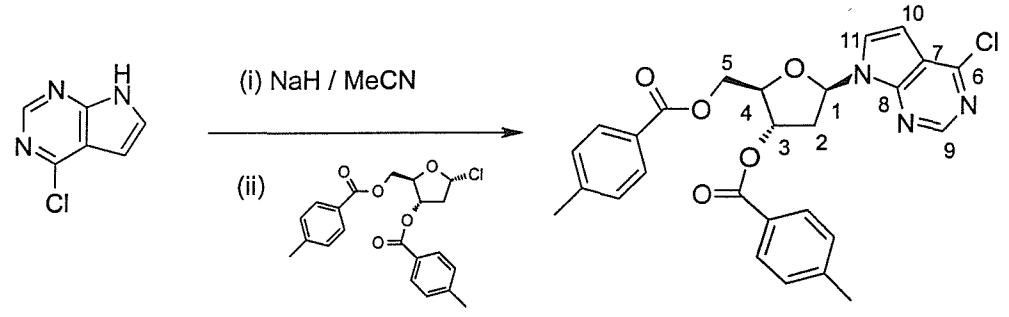
m.p. 189-190°C {lit.; 189-190°C} [71]

δ^1H (300MHz, DMSO): 6.59 (d., 1H, J=3Hz, $\underline{H}_5$), 7.67 (d., 1H, J=3Hz, $\underline{H}_6$), 8.60 (s., 1H, $\underline{H}_4$), 12.59 (b.s, 1H, N$\underline{H}$)

δ^{13}C (75.42MHz, DMSO): 98.84 ($\underline{C}_5$), 116.60 ($\underline{C}_2$), 128.39 ($\underline{C}_6$), 150.33 ($\underline{C}_4$), 150.51 ($\underline{C}_{1.}$), 151.82 ($\underline{C}_3$)

3.2.22

4-Chloro-7-(1-β- 2-deoxy-3,5-di-O-*p*-toluoyl-D-ribosyl)-pyrrolo[2,3-d]pyrimidine (59) Kazimierkzuk *et al* [72]



Sodium hydride 60 % dispersion in mineral oil (55 mmol, 2.19 g) was rinsed with anhydrous cyclohexane (2 x 5 mL) and dried under a stream of dry $N_{2(g)}$ for 1 hour. Anhydrous MeCN (180 mL) was added followed by 4-chloropyrrolo[2,3-

d]pyrimidine **(58)** (4.20 g, 27.4 mmol) and the mixture was stirred at ambient temperature under N_2 for 45 minutes. 1-α-Chloro-2-deoxy-3,5-di-O-*p*-toluoyl-D-ribose **(45)** (16.0 g, 41 mmol) was then added portionwise, with stirring over five minutes and the mixture was heated at 50°C for 3 hours. TLC analysis at that time showed that all of the 4-chloropyrrolo[2,3-d]pyrimidine **(58)** had by then reacted. The reaction mixture was allowed to cool to ambient temperature, partitioned between DCM (250 ml) and brine (150 ml) and the organic phase was dried over Na_2SO_4 (5.0g) and concentrated *in vacuo*. The residual solid was purified by flash column chromatography on silica gel, eluting with 20% EtOAc in hexane. Further purification was then possible by crystallisation from EtOAc / hexane.

This afforded 4-chloro-7-(1-β-2-deoxy-3,5-di-O-*p*-toluoyl-β-D-ribosyl)-pyrrolo[2,3-d]pyrimidine **(59)** (6.84 g, 13.5 mmol, 50%, {lit. 71%}[72]) as a white, crystalline solid. Analysis was concordant with the proposed structure and in agreement with the literature. Further material was retained in an impure form after evaporation of the mother liquor.

R_F = 0.20 (20% EtOAc / hexane, u.v. (purple under short wave), anisaldehyde)

ES+: 506, 508 $(M+H)^+$

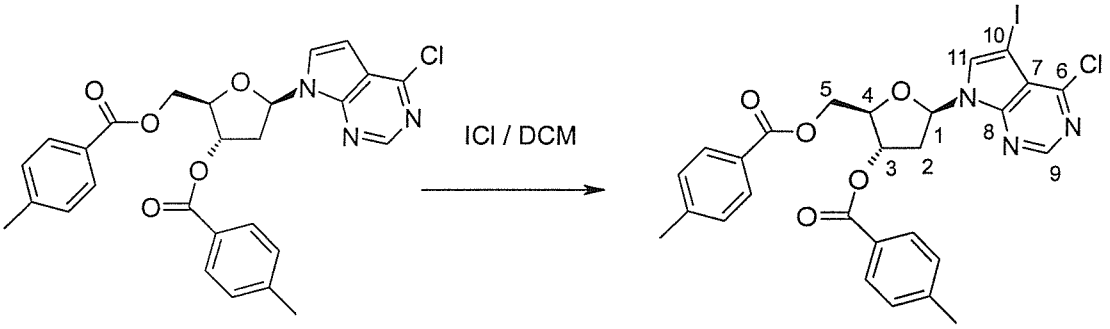
m.p. 114-115°C, {lit.; 118°C}[72]

δ^1H (300MHz, $CDCl_3$): 2.28 and 2.31 (2s., each 3H, Ar-C$\underline{H}_3$), 2.65-2.90 (m., 2H, $\underline{H}_2$), 4.39-4.70 (m., 3H, $\underline{H}_4$ and $\underline{H}_5$), 5.69-5.74 (m., 1H, $\underline{H}_3$), 6.53 (d., 1H, J=3Hz, $\underline{H}_{10}$), 6.70-6.80 (m., 1H, $\underline{H}_1$), 7.12-7.26 (2m., each 2H, aryl C-$\underline{H}$ *meta* to ester), 7.34 (d., 1H J=3Hz, $\underline{H}_{11}$), 7.80-7.95 (2m., each 2H, aryl C-$\underline{H}$ *ortho* to ester), 8.56 (s., 1H, $\underline{H}_9$)

$\delta^{13}C$ (75.42MHz, $CDCl_3$): 21.87 and 21.97 ($\underline{C}H_3$), 38.29 (C_2), 64.32 ($\underline{C}_5$), 75.25 ($\underline{C}_4$), 82.64 ($\underline{C}_3$), 84.59 ($\underline{C}_{10}$), 101.23 ($\underline{C}_1$), 118.50 ($\underline{C}_7$), 126.13 ($\underline{C}_{11}$), 126.56 and 126.89 (aryl $\underline{C}$-Me), 129.29, 129.45, 129.79 and 129.99 (aryl $\underline{C}$H), 144.38 and 144.69 ($\underline{C}$-1 of phenyl rings), 151.07 (C_9), 151.26 (C_6), 152.50 (C_8), 166.19 and 166.33 carbonyl

3.2.23

5-Chloro-7-(2-deoxy-3,5-di-O-*p*-toluoyl-β-D-ribosyl)-5-iodopyrrolo[2,3-d]pyrimidine (60) Froehler *et al* [73]



4-Chloro-7-(1-β-2-deoxy-3,5-di-O-*p*-toluoyl-β-D-ribosyl)-pyrrolo[2,3-d]pyrimidine **(59)** (10.39 g, 20.5 mmol) and sodium carbonate (4.00 eq, 82.2 mmol, 8.71 g) were charged to a reaction flask and stirred under a headspace of nitrogen. A solution of iodine monochloride (2.00 eq, 6.66 g, 41.1 mmol) in anhydrous DCM (75.0 mL) was charged and the resulting solution was stirred at ambient temperature for 18 hours. At that time TLC analysis showed complete conversion of the substrate to the desired product. The reaction was quenched with the addition of 0.1M $Na_2S_2O_{4(aq)}$ (300 mL) and the organic phase was separated and then washed by extraction with sat. $NaHCO_{3(aq)}$ (50.0 mL), dried over sodium sulfate (10 g) and concentrated *in vacuo* to give an amorphous white solid. This was triturated with ethyl acetate (30.0 mL) and recovered by filtration, washing by displacement with diethyl ether (10 mL). This afforded the title compound as a white solid (9.74 g). The combined filtrate and washings were then concentrated

sufficiently by evaporation to allow crystallisation of further product (1.19 g), which was of similar purity by TLC and ^{1}H-NMR, giving overall 4-chloro-7-(2-deoxy-3,5-di-O-*p*-toluoyl-β-D-ribosyl)-5-iodopyrrolo[2,3-d]pyrimidine **(60)** (10.93 g, 17.3 mmol, 84%, {lit.; 88%}[73]) as an amorphous white solid.

m.p. 158-159°C

R_F = 0.50 (30% EtOAc / hexane.) disclosed with; u.v., anisaldehyde

ES+: 632, 634 $(M+H)^+$

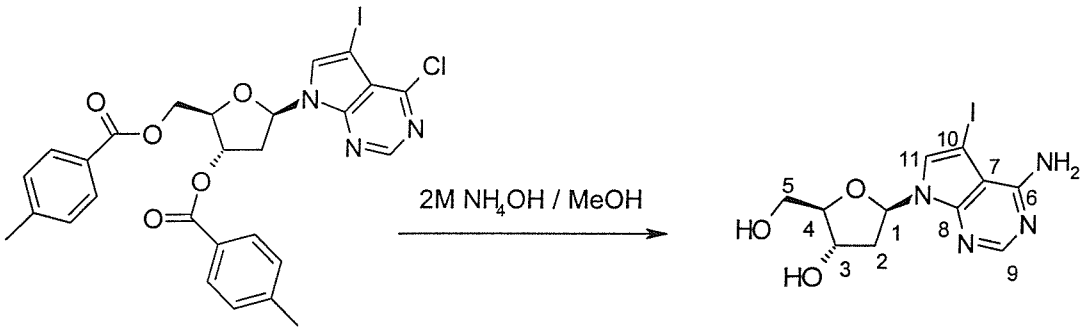
ν_{MAX} /cm^{-1} (Nujol Mull): 1740 CO str

δ^1H (300MHz, DMSO): 2.35 and 2.39 (2s., each 3H, Ar-C$\underline{H}_3$), 2.70-2.82 and 3.02-3.18 (2m., each 1H, $\underline{H}_2$), 4.50-4.72 (m., 3H, $\underline{H}_4$ and $\underline{H}_5$), 5.72-5.79 (m., 1H, $\underline{H}_3$), 6.70-6.80 (m., 1H, $\underline{H}_1$), 7.29-7.87 (2d., each 2H, aryl C-$\underline{H}$ *meta* to ester), 7.80-7.98 (2d., each 2H, aryl C-$\underline{H}$ *ortho* to ester), 8.14 (s., 1H, $\underline{H}_{11}$), 8.56 (s., 1H, $\underline{H}_9$)

δ^{13}C (75.42MHz, DMSO): 21.87 ($\underline{C}H_3$), 36.31 (C_2), 54.23 ($\underline{C}_{10}$), 64.08 ($\underline{C}_5$), 74.82 ($\underline{C}_4$), 81.67 ($\underline{C}_3$), 83.75 ($\underline{C}_1$), 116.85 ($\underline{C}_7$), 126.57 (aryl $\underline{C}$-Me), 129.33, 129.38, 129.41 and 129.53 (aryl $\underline{C}$H), 133.37 ($\underline{C}_{11}$), 143.91 and 144.13 ($\underline{C}$-1 of phenyl rings), 150.83 (C_9), 151.65 (C_6), 151.37 (C_8), 165.30 and 165.54 carbonyl

3.2.24

4-Amino-7-(2-deoxy-β-D-ribosyl)-5-iodopyrrolo[2,3-d]pyrimidine (61) Froehler *et al* [73]



4-Chloro-7-(2-deoxy-3,5-di-O-*p*-toluoyl-β-D-ribosyl)-5-iodopyrrolo[2,3-d]pyrimidine **(60)** (4.91 g, 7.76 mmol) was suspended in 2M NH_4OH / MeOH (75 mL) and heated at 150°C for 24 hours in a Parr hydrothermal bomb. The reaction vessel was allowed to cool to ambient temperature over five hours and the reaction mixture was removed and partitioned between water (200 mL) and diethyl ether (2 x 70 mL). The aqueous phase was concentrated *in vacuo* and the residual solid was crystallised from methanol. This afforded 4-amino-7-(2-deoxy-β-D-ribosyl)-5-iodopyrrolo[2,3-d]pyrimidine **(61)** (2.78g, 7.39 mmol, 95%, {lit.; 60%}[73]) as a white, crystalline solid. Analysis was concordant with the proposed structure and in agreement with the literature.

m.p. 163-172°C decomp.

R_F = 0.47 (2% Et_3N / 10% MeOH in EtOAc, u.v., anisaldehyde)

ν_{MAX} /cm^{-1} (DCM solution cell): 1257 C-N str

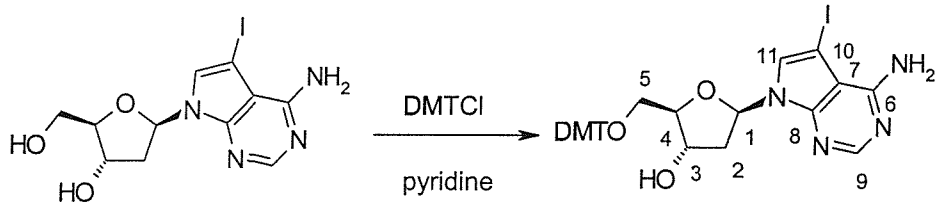
ES+: 377 $(M+H)^+$

δ^1H (300MHz, DMSO): 2.09-2.22 and 2.35-2.50 (m., 2H, $\underline{H}_2$), 3.45-3.60 (m., 2H, $\underline{H}_5$), 3.70-3.73 (m., 1H, $\underline{H}_4$), 4.33-4.38 (m., 1H, $\underline{H}_3$), 5.05 (t., 1H, C_5-O$\underline{H}$), 5.25 (d., 1 H, C_3-O$\underline{H}$), 6.45-6.52 (m., 1 H, $\underline{H}_1$), 6.65 (b.s., 2H, N$\underline{H}_2$), 7.65 (s., 1H, $\underline{H}_{11}$), 8.15 (s., 1H, $\underline{H}_9$)

δ^{13}C (75.42MHz, DMSO): 39.85 (C_2), 51.95 ($\underline{C}_{10}$), 61.95 ($\underline{C}_5$), 71.02 ($\underline{C}_3$), 83.01 ($\underline{C}_1$), 87.49 ($\underline{C}_4$), 103.20 ($\underline{C}_7$), 126.83 ($\underline{C}_{11}$), 149.78 ($\underline{C}_6$), 152.03 (C_9), 157.20 ($\underline{C}_8$)

3.2.25

4-Amino-7-[(O-5-bis-(4-methoxyphenyl)-phenylmethyl)-2-deoxy-β-D-ribosyl]-5-iodopyrrolo[2,3-d]pyrimidine (62)



4-Amino-7-(2-deoxy-β-D-ribosyl)-5-iodopyrrolo[2,3-d]pyrimidine **(61)** (100 mg, 0.266 mmol) was dissolved in anhydrous pyridine (0.5 mL) and stirred under a headspace of anhydrous N_2. A solution of DMT-Cl (1.10 eq., 0.292 mmol, 99 mg) in anhydrous pyridine (0.5 mL) was then added dropwise, and the reaction was stirred at ambient temperature for 90 minutes. TLC analysis at that time showed that all of the substrate had reacted. The reaction mixture was partitioned between DCM (5.0 mL) and 6% $NaHCO_{3(aq)}$ (5.0 mL, the organic phase was dried over Na_2SO_4 (0.50 g) and concentrated *in vacuo*. The residual solid was co-evaporated with toluene several times to displace residual pyridine and then purified by flash column chromatography on silica gel, eluting with 2% Et_3N / 7% MeOH / Et_2O. This afforded 4-amino-7-[(O-5-bis-(4-methoxyphenyl)-phenylmethyl)-2-deoxy-β-D-ribosyl]-5-iodopyrrolo[2,3-d]pyrimidine **(62)** (109mg,

0.161 mmol, 60%) as a white solid. Analysis was concordant with the proposed structure

R_F = 0.27 (2% Et_3N / 7% MeOH in Et_2O, u.v., anisaldehyde, 15% H_2SO_4 / EtOH)

ES+: 679 $(M+H)^+$, 701 $(M+Na)^+$, 717 $(M+K)^+$

ν_{MAX} /cm^{-1} (DCM solution cell): 1258 C-N str., 1179 CO str

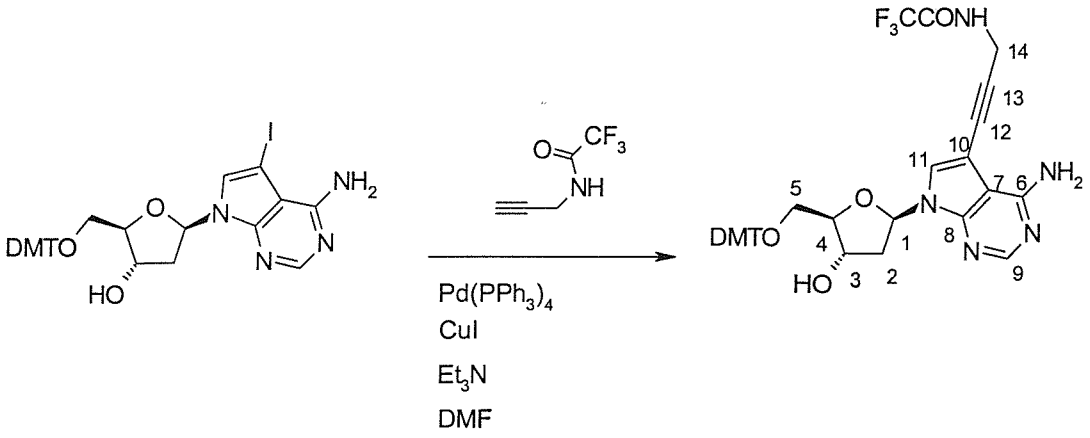
m.p. 102-108°C decomp.

δ^1H (300MHz, $CDCl_3$): 2.30-2.54 (m., 2H, $\underline{H}_2$), 3.24-3.40 (m., 2H, $\underline{H}_5$), 3.74 (s., 6H, $OC\underline{H}_3$), 4.05-4.12 (m., 1H, $\underline{H}_4$), 4.50-4.60 (m., 1H, $\underline{H}_3$), 5.70 (b.s., 2H, $N\underline{H}_2$), 6.55-6.65 (m., 1H, $\underline{H}_1$), 6.69-6.80 and 7.10-7.35 (2m., 13H, DMT C$\underline{H}$)_7.38 (s., 1H, $\underline{H}_{11}$), 8.15 (s., 1H, $\underline{H}_9$)

$\delta^{13}C$ (75.42MHz, DMSO): 41.16 (C_2), 50.84 ($\underline{C}_{10}$), 55.42 ($H_3\underline{C}$-O), 64.07 ($\underline{C}_5$), 72.74 ($\underline{C}_3$), 83.51 ($\underline{C}_1$), 85.73 ($\underline{C}_4$), 86.86 (-$\underline{C}(Ar_3)$, 104 ($\underline{C}_7$), 113.42 (aryl $\underline{C}$H), 126.52 ($\underline{C}_{11}$), 127.14, 128.15, 128.32, 130.20 and 130.27 (aryl $\underline{C}$H), 135.75, 135.90, 144.63 (aryl $\underline{C}_{quat}$), 150.21 ($\underline{C}_6$), 152.29 (C_9), 158.72 ($\underline{C}_8$)

3.2.26

4-Amino-7-[(O-5-bis-(4-methoxyphenyl)-phenylmethyl)-2-deoxy-β-D-ribosyl]-5- (prop-2-ynyl-trifluoroacetamido)-pyrrolo[2,3-d]pyrimidine (63)



4-Amino-7-[(O-5-bis-(4-methoxyphenyl)-phenylmethyl)-2-deoxy-β-D-ribosyl]-5-iodopyrrolo[2,3-d]pyrimidine **(62)** (1.00 g, 1.47 mmol), $Pd(PPh_4)_3$ (0.18 eq., 0.266 mmol, 307 mg) and CuI (0.36 eq., 0.532 mmol 101 mg) were charged to a reaction flask equipped with a stirrer bar and a vented septum and dried for 3 hours *in vacuo* over P_2O_5. The vaccum was broken with anhydrous nitrogen and anhydrous DMF (10.0 mL) was added with stirring under nitrogen, followed by Et_3N (18 eq., 26.6 mmol, 3.70 mL) and N-prop-2-ynyl-trifluoroacetamide (5.4 eq., 7.98 mmol, 1.20 g). The resulting mixture was stirred at ambient temperature under nitrogen and TLC analysis indicated that the reaction was complete after 36 hours. The reaction mixture was partitioned between Et_2O (150 mL) and water (50 mL) and the organic phase was dried over Na_2SO_4 (5.0 g) and concentrated *in vacuo*. The residue was purified by flash column chromatography on silica gel, eluting with 2% Et_3N / 4% MeOH / DCM to give a tan solid which was dissolved in DCM (2 mL) and precipitated by dropwise addition to stirred n-hexane (100 ml) at ambient temperature. The precipitate was recovered by filtration and air-dried by suction for 4 hours to afford 4-amino-7-[(O-5-bis-(4-methoxyphenyl)-phenylmethyl)-2-deoxy-β-D-ribosyl]-5-(prop-2-ynyl-trifluoroacetamido)-pyrrolo[2,3-d]pyrimidine **(63)** (1.00 g, 1.43 mmol, 97%) as an amorphous, tan solid. Analysis was concordant with the proposed structure.

R_F = 0.29 (2% Et_3N / 5% MeOH in DCM, u.v., anisaldehyde,15% H_2SO_4 / EtOH)

ES+: 702 $(M+H)^+$, 724 $(M+Na)^+$, 740 $(M+K)^+$

HRMS: $(M+H)^+$ 702.2682 (calc. 702.2534)

ν_{MAX} /cm^{-1} (DCM solution cell): 1726 C=O str

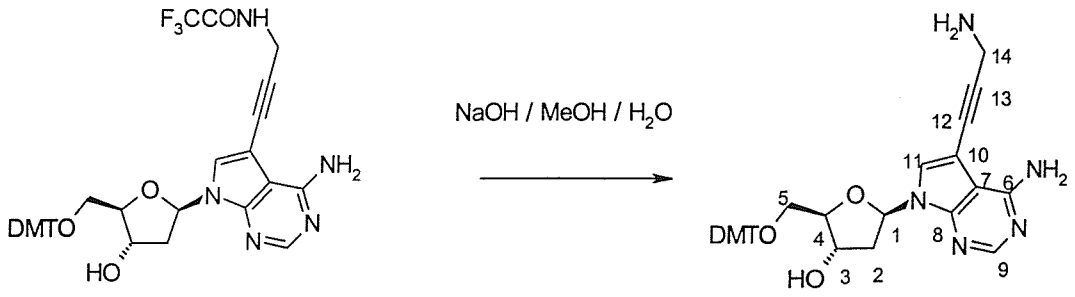
m.p. 97-105°C decomp.

δ^1H (300MHz, $CDCl_3$): 2.30-2.50 (m., 2H, $\underline{H}_2$), 3.39-3.43 (m., 2H, $\underline{H}_5$), 3.70 (s., 6H, $OC\underline{H}_3$), 4.98-4.10 (m., 1H, $\underline{H}_4$), 3.23-3.27 (m., 2H, $\underline{H}_{14}$), (4.48-4.60 (m., 1H, $\underline{H}_3$), 5.65 (b.s., 2H, $N\underline{H}_2$), 6.50-6.70 (m., 1H, $\underline{H}_1$), 6.69-6.80 and 7.10-7.35 (2m., 13H, DMT C$\underline{H}$) 7.38 (s., 1H, $\underline{H}_{11}$), 8.15 (s., 1H, $\underline{H}_9$)

$\delta^{13}C$ (75.42MHz, $CDCl_3$): 30.84 (C_{14}), 41.17 (C_2), 55.48 ($H_3\underline{C}$-O), 63.60 ($\underline{C}_5$), 72.02 ($\underline{C}_3$), 78.12 ($\underline{C}_{13}$), 83.53 ($\underline{C}_{12}$), 85.26 (-$\underline{C}(Ar)_3$), 85.65 ($\underline{C}_1$), 86.79 ($\underline{C}_4$), 94.87 (C_{10}), 104 ($\underline{C}_7$), 113.48 (aryl $\underline{C}$H), 126.72 ($\underline{C}_{11}$), 127.15, 128.11, 128.46 and 130.23 (aryl $\underline{C}$H), 136.12, 136.19, 144.31 (aryl $\underline{C}_{quat}$), 149.60 ($\underline{C}_6$), 152.99 (C_9), 158.56 ($\underline{C}_8$)

3.2.27

4-Amino-7-[(O-5-bis-(4-methoxyphenyl)-phenylmethyl)-2-deoxy-β-D-ribosyl]-5-(prop-2-ynyl-amino)-pyrrolo[2,3-d]pyrimidine (64)



4-Amino-7-[(O-5-bis-(4-methoxyphenyl)-phenylmethyl)-2-deoxy-β-D-ribosyl]-5-(prop-2-ynyl-trifluoroacetamido)-pyrrolo[2,3-d]pyrimidine **(63)** (1.21 g, 1.76 mmol) was dissolved in MeOH (10.0 mL) and a solution of NaOH (3.00 eq., 5.28 mmol, 211 mg) in H_2O (1.00 mL) was added dropwise, with stirring. The resulting homogeneous solution was stirred overnight at ambient temperature after which time TLC analysis indicated that all of the substrate had reacted. The reaction mixture was partitioned between DCM (80 mL) and brine (100 mL) and the organic phase was dried over Na_2SO_4 (0.30 g) and concentrated *in vacuo*. The residual solid was purified by flash column chromatography on silica gel eluting with 0.2% Et_3N / 8% MeOH / DCM. This gave 4-amino-7-[(O-5-bis-(4-methoxyphenyl)-phenylmethyl)-2-deoxy-β-D-ribosyl]-5-(prop-2-ynyl-amino)-[2,3-d]pyrimidine **(64)** (923 mg, 1.53 mmol, 87%) as a white solid after drying overnight *in vacuo* over P_2O_5. Analysis was concordant with the proposed structure.

R_F = 0.29 (4% $NH_{3(aq)}$ / 10% MeOH in DCM, u.v., anisaldehyde)

ES+: no mass peak could be found using electrospray techniques

m.p. 40-42 °C decomp.

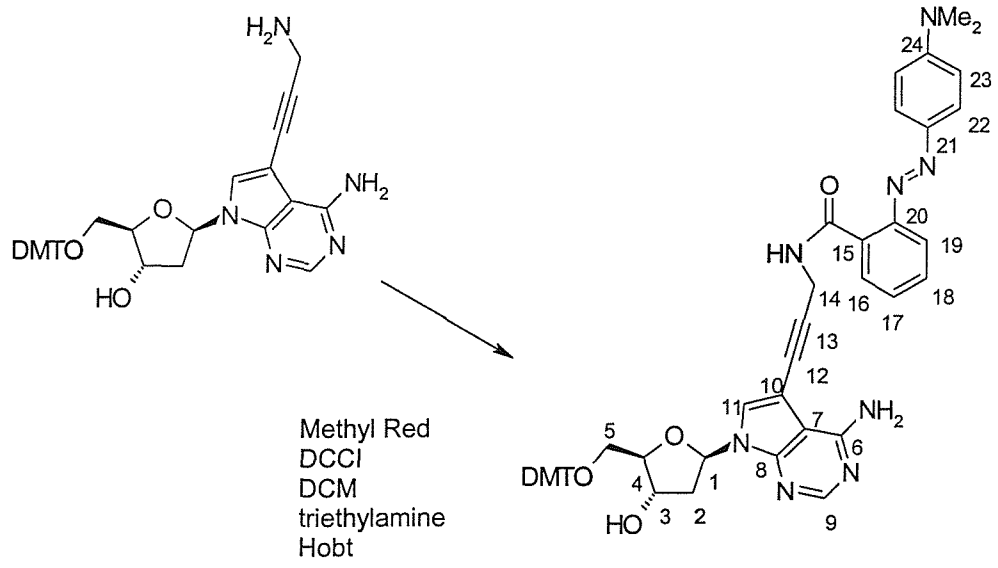
δ^1H (300MHz, $CDCl_3$): 2.35-2.52 (m., 2H, $\underline{H}_2$), 3.18-3.28 (m., 2H, $\underline{H}_5$), 3.56 (s., 2H, $\underline{H}_{14}$), 3.70 (s., 6H, $OC\underline{H}_3$), 3.98-4.05 (m., 1H, $\underline{H}_4$), (4.42-4.52 (m., 1H, $\underline{H}_3$), 5.60 (b.s., 2 H, $N\underline{H}_2$), 6.50-6.70 (m., 1H, $\underline{H}_1$), 6.69-6.80 and 7.10-7.35 (2m., 13H, DMT C$\underline{H}$) 7.38 (s., 1H, $\underline{H}_{11}$), 8.15 (s., 1H, $\underline{H}_9$)

δ^{13}C (75.42MHz, DMSO): 39.75 (C_2), 55.46 ($H_3\underline{C}$-O), 60.20 (C_{14}), 64.63 ($\underline{C}_5$), 71.05 ($\underline{C}_3$), 75.40 ($\underline{C}_{13}$), 83.16 ($\underline{C}_1$), 85.85 ($\underline{C}_4$), 85.91 (-$\underline{C}(Ar)_3$), 88.87 ($\underline{C}_{12}$), 95.05 (C_{10}), 103.03 ($\underline{C}_7$), 113.58 (aryl $\underline{C}$H), 126.09 ($\underline{C}_{11}$), 127.07, 128.13, 128.24 and

130.17 (aryl CH), 135.92, 136.06, 145.42 (aryl C_{quat}), 149.89 (C_6), 153.33 (C_9), 158.50 (C_8)

3.2.28

4-Amino-7-(O-(5-bis-(4-methoxyphenyl)-phenylmethyl)-2-deoxy-β-D-ribosyl)-5-[(2-(dimethylamino-phenylazo)-benzoyl)-prop-2-ynyl]-pyrrolo[2,3-d]pyrimidine (65)



4-Amino-7-[(O-5-bis-(4-methoxyphenyl)-phenylmethyl)-2-deoxy-β-D-ribosyl]-5-(prop-2-ynyl-amino)-pyrrolo[2,3-d]pyrimidine **(64)** (963 mg, 1.59 mmol), 2-(4-dimethylamino-phenylazo)-benzoic acid (methyl red) (1.2 eq., 515 mg, 1.91 mmol), Hobt (1.5 eq., 2.39 mmol, 322 mg) and DCCI (1.5 eq., 2.39 mmol, 492 mg) were charged to a reaction flask equipped with a vented septum and a stirrer bar and the flask contents were dried *in vacuo* over P_2O_5 for 2 hours and opened to nitrogen. Anhydrous DCM (12.0 mL) was added with stirring under nitrogen followed by anhydrous Et_3N (2.0 eq., 3.18 mmol, 0.443 mL) and the resulting mixture was stirred overnight at ambient temperature under a headspace of nitrogen. TLC analysis after this time showed complete reaction through to the desired product. The reaction mixture was diluted with DCM (80

mL) and washed by extraction with brine (80 mL). The organic phase was dried over Na_2SO_4 (0.3 g) and concentrated *in vacuo* to give a coloured oil which was purified by flash column chromatography on silica gel, eluting with 4% $NH_{3(aq)}$ / 4% MeOH / DCM. The resulting coloured solid showed trace impurities by TLC and was further purified through a short silica gel column (pre-equilibrated with 2% Et_3N / EtOAc), eluting with EtOAc. This afforded 4-amino-7-(O-(5-bis-(4-methoxyphenyl)-phenylmethyl)-2-deoxy-β-D-ribosyl)-5-[(2-(dimethylamino-phenylazo)-benzoyl)-prop-2-ynyl]-pyrrolo[2,3-d]pyrimidine **(65)** (1.02 g, 88%) as a coloured (orange) solid after drying for 48 hours *in vacuo* over P_2O_5. Analysis was concordant with the proposed structure.

R_F = 0.61 (4% $NH_{3(aq)}$ / 10% MeOH in DCM, u.v., anisaldehyde, 5% H_2SO_4 / EtOH) (pink), NH_3 fumes (orange))

ν_{MAX} /cm^{-1} (DCM solution cell): 1601 C=O str

ES+; 857 $(M+H)^+$, 879 $(M+Na)^+$, 895 $(M+K)^+$

HRMS: $(M+H)^+$ 857.3762 (calc. 857.3770)

m.p. 126-131 °C decomp.

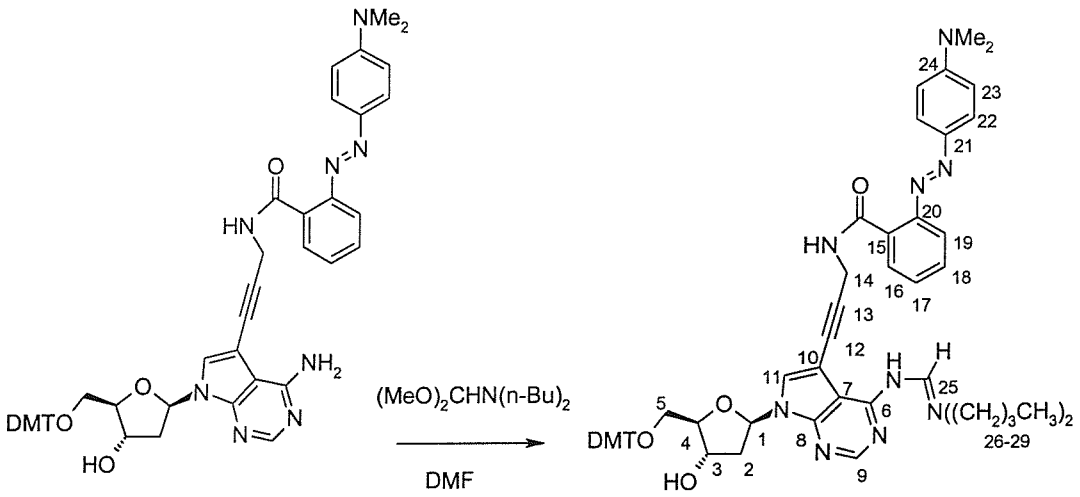
ε_{max} = 18.6 x 10^3 mol^{-1} dm^3 cm^{-1} @ 449nm / MeOH

δ^1H (300MHz, $CDCl_3$): 2.33-2.50 (m., 2H, $\underline{H}_2$), 2.68 (s., 6H, $N(C\underline{H}_3)_2$), 3.18-3.40 (m., 2H, $\underline{H}_5$), 3.66 (s., 6H, $OC\underline{H}_3$), 3.95-4.02 (m., 1 H, $\underline{H}_4$), 4.40-4.46 (m., 2H, $\underline{H}_{14}$), 4.41-4.50 (m., 1H, $\underline{H}_3$), 5.65 (b.s., 2H, $N\underline{H}_2$), 6.30 (d. J = 8Hz, 2H, $\underline{H}_{23}$), 6.59-6.79 (m., 1 H, $\underline{H}_1$), 6.69-6.80 and 7.10-7.35 (2m., 13H, DMT C$\underline{H}$), between 7.1 and 7.35 (s., 1H, $\underline{H}_{11}$), 7.35-7.50 (2m., each 1H $\underline{H}_{17}$ and $\underline{H}_{18}$), 7.38 (m., 1H, $\underline{H}_{16}$), 7.75 (d. J = 8 Hz, 2H, $\underline{H}_{22}$), 8.15 (s., 1H, $\underline{H}_9$), 8.39-8.46 (m., 1H, $\underline{H}_{19}$), 9.70-9.80 (m., 1H, $C_3O\underline{H}$)

$\delta^{13}C$ (75.42MHz, $CDCl_3$): 31.25 (C_{14}), 40.01 ($N\underline{Me}_2$), 41.02 (C_2), 55.34 ($H_3\underline{C}$-O), 64.02 ($\underline{C}_5$), 72.53 ($\underline{C}_3$), 83.48 ($\underline{C}_1$), 85.66 ($\underline{C}_4$), 86.75 ($\underline{C}_{13}$), 88.23 ($\underline{C}_{12}$), 95.81 (C_{10}), 103.60 ($\underline{C}_7$), 111.58 ($\underline{C}_{23}$), 113.35 (DMT $\underline{C}$H *ortho* to OMe), 116.05 ($\underline{C}_{22}$), 126.04 ($\underline{C}_{11}$), 126.19 ($\underline{C}_{16}$), 127.07, 128.08, 128.22, 129.64, 130.20, 131.41 and 132.28 (4 DMT $\underline{C}$H and $\underline{C}_{17}$, $\underline{C}_{18}$ and $\underline{C}_{19}$), 128.65 ($\underline{C}_{24}$), 135.68, 135.80, and 144.73 (DMT $\underline{C}_{quat}$), 143.25 ($\underline{C}_{20}$), 149.65 ($\underline{C}_6$), 150.53 ($\underline{C}_{21}$), 153.15 (C_9), 157.55 ($\underline{C}_8$), 158.69 (carbonyl), 166.44 ($\underline{C}_{15}$)

3.2.29

4-(Di-n-butylamino-methyleneamino)-7-[(O-(5-bis-(4-methoxyphenyl)-phenylmethyl)-2-deoxy-β-D-ribosyl)-5-(2-(dimethylamino-phenylazo)-benzoyl)-prop-2-ynyl]-pyrrolo[2,3-d]pyrimidine (66)



4-Amino-7-(O-(5-bis-(4-methoxyphenyl)-phenylmethyl)-2-deoxy-β-D-ribosyl)-5-[(2-(dimethylamino-phenylazo)-benzoyl)-prop-2-ynyl]-pyrrolo[2,3-d]pyrimidine **(65)** (800 mg, 0.934 mmol) was dissolved in anhydrous DMF (60.0 mL) and stirred at ambient temperature. N,N-Di-n-butylformamide dimethylacetal (2.00 eq., 1.87 mmol, 379 mg, 0.436 mL) was added dropwise and the reaction was stirred at ambient temperature for 48 hours after which time TLC analysis

showed complete reaction to a single product. The reaction mixture was diluted with Et_2O (250 mL) and shaken with water (3 x 100 mL) and the organic phase was dried over Na_2SO_4 (1.0 g) and concentrated *in vacuo*. The resulting solid was purified by flash column chromatography on silica gel (pre-equilibrated with 1% Et_3N / n-hexane), eluting with a gradient of n-hexane / EtOAc. This afforded 4-(di-n-butylamino-methyleneamino)-7-(O-(5-bis-(4-methoxyphenyl)-phenylmethyl)-2-deoxy-β-D-ribosyl)-5-[(2-(dimethylamino-phenylazo)-benzoyl)-prop-2-ynyl]-pyrrolo[2,3-d]pyrimidine **(66)** (709 mg, 76%) as a coloured (orange) solid after drying overnight *in vacuo* over P_2O_5. Analysis was concordant with the proposed structure.

R_F = 0.50 (EtOAc, u.v., anisaldehyde, 5% H_2SO_4 / EtOH (pink), NH_3 fumes (orange))

ES+; 996 $(M+H)^+$, 1018 $(M+Na)^+$

HRMS: $(M+H)^+$ 996.5131 (calc. 996.5131)

m.p. 143-149 °C decomp.

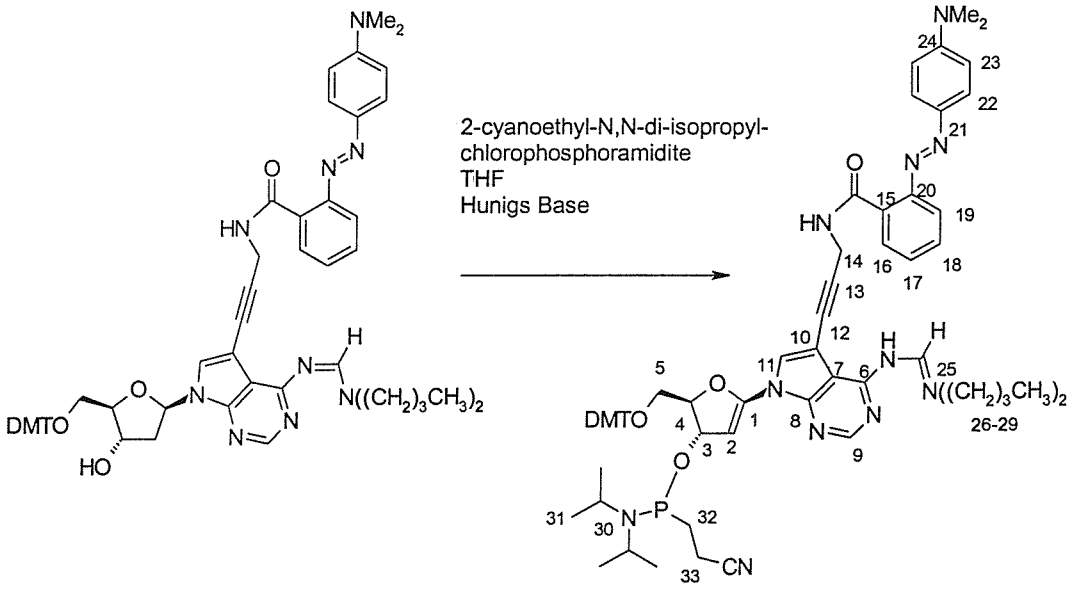
ε_{max} = 16.2 x 10^3 mol^{-1} dm^3 cm^{-1} @ 485nm / MeOH

δ^1H (300MHz, $CDCl_3$): 0.57 and 0.76 (2t., each 3H, J=9 Hz, $\underline{H}_{29}$ {Z/E pair}), 1.00-1.17 (m., 4H, $\underline{H}_{28}$), 1.28-1.49 (m., 4H, $\underline{H}_{27}$), 2.32-2.40 (m., 2H, $\underline{H}_2$), 2.50 (s., 6H, $N(C\underline{H}_3)_2$), 3.07-3.14 and 3.73-3.56 (2m., each 2H, $\underline{H}_{26}$ {Z/E pair}), 3.12-3.33 (m., 2H, $\underline{H}_5$), 3.62 (s., 6H, $OC\underline{H}_3$), 4.00-4.03 (m., 1H, $\underline{H}_4$), 4.40-4.60. (m., 2H, $\underline{H}_{14}$ and m., 1H, $\underline{H}_3$), 5.6 (b.s., 1H, O$\underline{H}$), 6.07 (d. J=8Hz, 2H, $\underline{H}_{23}$), 6.60-6.70 (m., 1H, $\underline{H}_1$), 6.65-6.72 and 7.10-7.35 (2m., 13H, DMT C$\underline{H}$), between 7.1 and 7.35 (s., 1H, $\underline{H}_{11}$), 7.30-7.50 (3m., each 1H, $\underline{H}_{16}$, $\underline{H}_{17}$ and $\underline{H}_{18}$), 7.75 (d. J=8 Hz, 2H, $\underline{H}_{22}$), 7.74 and 7.80 (2s., each 0.5H, $\underline{H}_{25}$ {Z/E pair}), 8.28-8.32 (m., 1H, $\underline{H}_{19}$), 8.30 (s., 1H, $\underline{H}_{11}$), 8.52 (s., 1H, $\underline{H}_9$), 9.99 (b.s., 1H, N$\underline{H}$)

$\delta^{13}C$ (75.42MHz, $CDCl_3$): 0.00 and 12.65 (C_{29}), 18.74 and 19.09 (C_{28}), 28.24 and 29.97 (C_{27}), 30.61 (C_{14}), 38.60 ($N\underline{C}H_2$), 39.57 (C_2), 44.48 and 50.69 (C_{26}), 54.15 ($H_3\underline{C}$-O), 63.04 ($\underline{C}_5$), 71.67 ($\underline{C}_3$), 77.45 ($\underline{C}_{12}$), 82.02 ($\underline{C}_4$), 84.28 ($\underline{C}_1$), 84.67 ($\underline{C}_{13}$), 85.61 (-$\underline{C}(Ar)_3$), 96.81 (C_{10}), 110.22 ($\underline{C}_7$), 110.38 ($\underline{C}_{23}$), 112.21 (DMT $\underline{C}$H *ortho* to OMe), 114.60 ($\underline{C}_{22}$), 125.43 ($\underline{C}_{11}$), 125.90 ($\underline{C}_{16}$), 126.45, 126.91, 127.05, 128.32, 129.03, 130.39, 130.91 (4 DMT $\underline{C}$H and $\underline{C}_{17}$, $\underline{C}_{18}$ and $\underline{C}_{19}$), 127.53 ($\underline{C}_{24}$), 1354.51 134.63, and 142.07 (DMT $\underline{C}_{quat}$), 143.59 ($\underline{C}_{20}$), 149.31 ($\underline{C}_6$), 151.47 ($\underline{C}_{21}$), 152.00 (C_9), 155.06 (C_{25}), 157.56 ($\underline{C}_8$), 160.96 (carbonyl), 164.68 ($\underline{C}_{15}$)

3.2.30

4-(Di-n-butylamino-methyleneamino)-7-[O-(5-bis-(4-methoxyphenyl)-phenylmethyl)-O-(3-(2-cyanoethyl-N,N-diisopropyl-phosphoramidite)]-2-deoxy-β-D-ribosyl)-5-[(2-(dimethylamino-phenylazo)-benzoyl)-prop-2-ynyl]-pyrrolo[2,3-d]pyrimidine (67)



4-(Di-n-butylamino-methyleneamino)-7-(O-(5-bis-(4-methoxyphenyl)-phenylmethyl)-2-deoxy-β-D-ribosyl)-5-[(2-(dimethylamino-phenylazo)benzoyl)-prop-2-ynyl]-pyrrolo[2,3-d]pyrimidine **(66)** (425 mg, 0.437 mmol) was dissolved in

anhydrous THF (3.00 mL) and stirred under a headspace of argon. DIPEA (4.00 eq., 1.75 mmol, 0.305 mL) was added, followed by 2-cyanoethyl-N,N-di-isopropyl chlorophosphoramidite (1.10 eq., 0.481 mmol, 0.114 mL) and the reaction was stirred at ambient temperature for 1 hour after which time TLC analysis showed *ca.* 5% of the substrate remained unreacted. Further N,N-di-isopropyl chlorophosphoramidite (0.02 mL) was added and TLC analysis showed that the reaction was complete after stirring for a further 15 minutes. The reaction mixture was diluted with DCM (60 mL) and shaken with sat $KCl_{(aq)}$. The organic phase was dried over Na_2SO_4 (5.0 g) under a headspace of argon with transfer by cannula and then removed from the drying agent, also by cannular. The drying agent was similarly washed with anhydrous DCM (10 ml) and the combined organic solutions were concentrated *in vacuo*. The resulting solid was dissolved in anhydrous DCM (2.0 ml) and precipitated by adding dropwise to stirred n-hexane (300ml) @ -78°C. The precipitate was collected by filtration through a glass wool plug directed by a wide-bore cannula. This solid was recovered by dissolving in anhydrous DCM (2.0 ml) followed by concentration *in vacuo* to give a solid The solid was dried *in vacuo* over P_2O_5 for 5 hours, dissolved in anhydrous MeCN (3.0 ml), filtered through a Gelman acrodisc filter (0.45 μm) to remove particulate matter and dried *in vacuo* over P_2O_5 for 5 hours. This afforded 4-(di-n-butylamino-methyleneamino)-7-[O-(5-bis-(4-methoxyphenyl)-phenylmethyl)-O-(3-(2-cyanoethyl-N,N-diisopropyl)-phosphoramidite)]-2-deoxy-β-D-ribosyl)-5-[(2-(dimethylamino-phenylazo)-benzoyl)-prop-2-ynyl]-pyrrolo[2,3-d]pyrimidine **(67)** (0.52 g, 0.437 mmol, 100%) as a red solid. Analysis was concordant with the proposed structure.

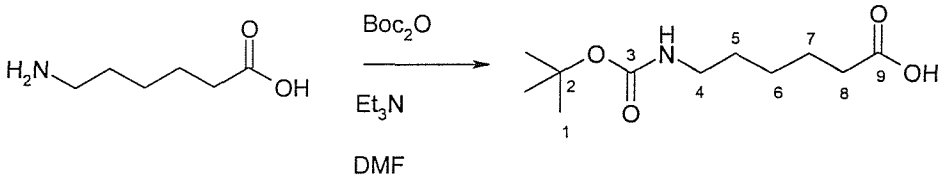
R_F = 0.6, 0.7 (EtOAc / hexane (1:2), u.v., anisaldehyde, 5% H_2SO_4 / EtOH (pink), NH_3 fumes (orange))

ES+; 1197 $(M+H)^+$

δ^1H (300MHz, $CDCl_3$): 0.57 and 0.76 (2t., each 3H, J=9 Hz, $\underline{H}_{29}$ {Z/E pair}), 1.00-1.17 (m., 4H, $\underline{H}_{28}$ and 4H, $\underline{H}_{31}$), 11.15-1.25 (m., 2H, $\underline{H}_{32}$), 1.28-1.49 (m., 4H, $\underline{H}_{27}$), 1.50-1.60 (m., 2H, $\underline{H}_{30}$), 2.32-2.40 (m., 2H, $\underline{H}_2$), 2.50 (s., 6H, $N(C\underline{H}_3)_2$), 3.07-3.14 and 3.73-3.56 (2m., each 2H, $\underline{H}_{26}$ {Z/E pair}), 3.12-3.33 (m., 2H, $\underline{H}_5$), 3.30-3.45 (m., 4H, $\underline{H}_{32}$), 3.62 (s., 6H, $OC\underline{H}_3$), 4.00-4.03 (m., 1H, $\underline{H}_4$), 4.40-4.60. (m., 2H, $\underline{H}_{14}$ and m., 1H, $\underline{H}_3$), 5.6 (b.s., 1H, O$\underline{H}$), 6.07 (d. J=8Hz, 2H, $\underline{H}_{23}$), 6.60-6.70 (m., 1H, $\underline{H}_1$), 6.65-6.72 and 7.10-7.35 (2m., 13H, DMT C$\underline{H}$), 7.1-7.35 (s., 1H, $\underline{H}_{11}$), 7.30-7.50 (3m., each 1H, $\underline{H}_{16}$, $\underline{H}_{17}$ and $\underline{H}_{18}$), 7.75 (d. J = 8 Hz, 2H, $\underline{H}_{22}$), 7.74 and 7.80 (2s., each 0.5H, $\underline{H}_{25}$ {Z/E pair}), 8.28-8.32 (m., 1H, $\underline{H}_{19}$), 8.30 (s., 1H, $\underline{H}_{11}$), 8.52 (s., 1H, $\underline{H}_9$), 9.99 (b.s., 1H, N$\underline{H}$)

3.2.31

6-*t*-Butoxycarbonylamino-hexanoic acid (69)



6-Aminohexanoic acid **(68)** (10.0 g, 76.3 mmol) was charged to a reaction flask and stirred under a head space of nitrogen. Anhydrous triethylamine (10.6 mL, 76.3 mmol, 1.00 eq) was added and the resulting solution was stirred at ambient temperature. A solution of Boc anhydride (16.3 g, 76.3 mmol, 1.00 eq) in anhydrous DMF (60 mL) was added with vigorous magnetic stirring and the resulting solution was stirred at ambient temperature for 18 hours. After that time TLC analysis indicated that the reaction was complete. The reaction mixture was concentrated *in vacuo* and residual DMF was displaced by co-evaporation with toluene (3 x 25 mL). The residual oil was dissolved in DCM (250 mL) and washed by extraction with sat. $KCl_{(aq)}$ (150 mL) then with 5% citric acid solution (100 mL). The organic phase was dried over Na_2SO_4 (10 g) and concentrated *in vacuo* to give a colourless oil. This was purified by flash column chromatography

on silica gel, eluting with 5% MeoH / DCM affording 6-*t*-butoxycarbonylamino-hexanoic acid **(69)** (10.5 g, 64%) as a colourless oil. Analysis was concordant with the proposed structure.

R_F = (10% MeOH / DCM, u.v., anisaldehyde, 5%H_2SO_4 / EtOH)

ES-: 249 and 251$(M+Cl)^-$

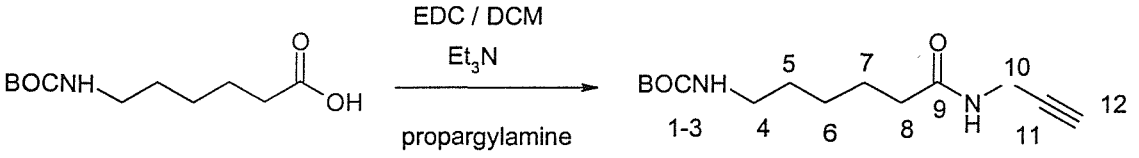
HRMS: $(M+H)^+$ 232.1547 (calc. 232.1549)

δ^1H (300MHz, $CDCl_3$): 1.30-1.60 (m., 4H, $\underline{H}_6$ and $\underline{H}_5$), 1.37 (s., 9H, $\underline{H}_1$), 1.50-1.52 (m., 2H, $\underline{H}_7$), 2.23-2.39 (m., 2H, $\underline{H}_4$), 3.00-3.20 (m., 2H, $\underline{H}_8$), 4.6 (b.s., 1H, NH), 5.7 (b.s., 1H, OH)

δ^{13}C (75.42MHz, $CDCl_3$): 24.50 (C_5), 26.35 (C_6), 28.54 (C_1), 29.82 (C_7), 34.11 (C_4), 40.50 (C_8), 79.42 (C_2), 179.06 (carbonyl)

3.2.32

6-*t*-Butoxycarbonylamino-prop-2-ynyl-hexanamide (70)



6-*t*-Butoxycarbonylamino-hexanoic acid **(69)** (10.0 g, 43.3 mmol) was dissolved in anhydrous DCM (100 mL) and stirred under a headspace of nitrogen at ambient temperature. EDC (9.09 g, 47.6 mmol, 1.10 eq) was added in one portion followed by anhydrous triethylamine (6.62 mL, 1.10 eq) and prop-2-ynylamine (3.26 mL, 1.10 eq). The resulting mixture was stirred at ambient temperature for 16 hours after which time TLC analysis showed that all of the substrate had reacted. The reaction mixture was filtered and silica gel (2.0 g)

was added to the filtrate prior to concentration *in vacuo* to give a free flowing solid. Initial purification by flash column chromatography on silica gel eluting with 10% methanol / DCM lead to an amorphous coloured solid. This was further purified by crystallisation from EtOAc affording 6-*t*-butoxycarbonylamino-prop-2-ynyl-hexanamide **(70)** (8.80 g, 76%) as a white, crystalline solid. Analysis was concordant with the proposed structure.

R_F = (10% MeOH / DCM, u.v., anisaldehyde, 5% H_2SO_4 / EtOH)

ES+: 291$(M+Na)^+$

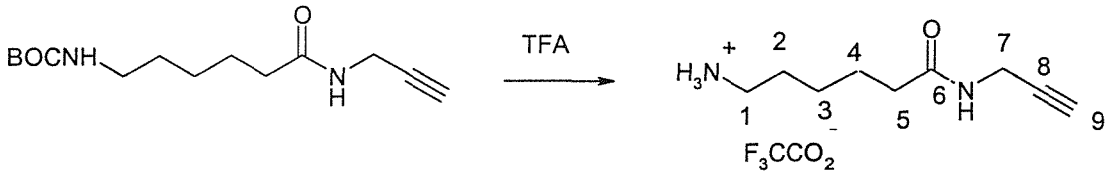
m.p. 150-152°C

δ^1H (300MHz, DMSO): 1.22-1.53 (m., 2, $\underline{H}_6$), 1.32-1.46 (m. 2H, $\underline{H}_5$), 1.37 (s., 9H, $\underline{H}_1$), 1.43-1.56 (m., 2H, $\underline{H}_7$), 2.03-2.17 (m., 2H, $\underline{H}_4$), 2.85-2.96 (m., 2H, $\underline{H}_8$), 3.43 (s., 1H, $\underline{H}_{12}$), 3.80 (s., 2H, $\underline{H}_{10}$), 6.7 (b.t., 1H, NH), 8.3 (b.t., 1H, NH)

δ^{13}C (75.42MHz, DMSO): 24.96 (C_5), 26.04 (C_6), 27.77 (C_{10}), 28.35 (C_1), 29.36 (C_7), 35.08 (C_4), 40.40 (C_8), 72.89 ($\underline{H}_{12}$), 77.38 ($\underline{H}_{11}$), 81.42 (C_2), 155.64 and 171.90 (carbonyl)

3.2.33

6-Trifluoroacetylammonium-prop-2-ynyl-hexanamide (71)



6-*t*-Butoxycarbonylamino-prop-2-ynyl-hexanamide **(70)** (8.50 g, 31.7 mmol) was stirred in TFA (20.0 mL) at 0°C with cooling on ice for 20 minutes after which

time TLC analysis showed approximately 10% unreacted substrate remained, with the gradual formation of a low running component in addition to the desired product. The reaction mixture was concentrated *in vacuo* at ambient temperature to give an oil which was triturated with diethyl ether to give a solid that was further purified by flash column chromatography on silica gel, eluting with 10% MeOH / DCM followed by crystallisation from EtOAc. This afforded 6-trifluoroacetylammonium-prop-2-ynyl-hexanamide **(71)** (5.71g, 64%) as a white, crystalline solid. Analysis was concordant with the proposed structure.

R_F = (10% MeOH / DCM, u.v., anisaldehyde, 5%H_2SO_4 / EtOH)

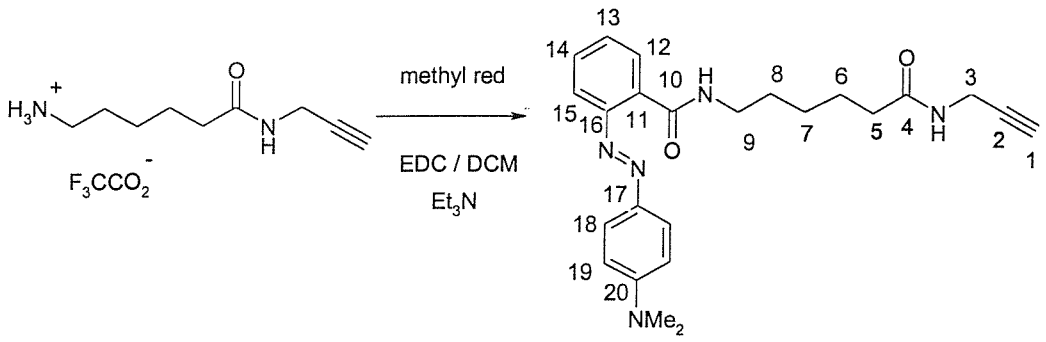
ES+: 169 $(M+H)^+$

m.p. 102-105°C

δ^1H (300MHz, DMSO): 1.22-1.53 (m., 2, $\underline{H}_3$), 1.45-1.70 (m. 4H, $\underline{H}_2$ and $\underline{H}_4$), 2.03-2.17 (m., 2H, $\underline{H}_1$), 2.73-2.88 (m., 2H, $\underline{H}_5$), 3.10 (s., 1H, $\underline{H}_9$), 3.85 (m., 2H, $\underline{H}_7$), 7.7 (b.s., 3H, NH), 8.3 (b.s., 1H, NH)

δ^{13}C (75.42MHz, DMSO): 24.67 (C_3), 25.54 (C_2), 26.84 (C_7), 27.79 (C_4), 34.83 (C_1), 38.70 (C_5), 72.89 ($\underline{H}_9$), 81.39 ($\underline{H}_8$), 158.35 (CF_3), 171.83 (carbonyl)

3.2.34
6-(2-(Dimethylamino-phenylazo)-benzoyl)-prop-2-ynyl-hexanamide (72)



6-Trifluoroacetylammonium-prop-2-ynyl-hexanamide **(71)** (3.00 g, 10.6 mmol), EDC (2.24 g, 11.7 mmol, 1.10 eq) and 2-(4-dimethylamino-phenylazo)-benzoic acid (methyl red) (3.15 g, 11.7 mmol, 1.10 eq) were charged to a reaction flask and stirred under a headspace of nitrogen at ambient temperature. Anhydrous DCM (30.0 mL) was added followed by anhydrous triethylamine (1.92 mL, 1.30 eq) and the resulting mixture was stirred for 15 hours under nitrogen at ambient temperature. At that stage TLC analysis showed that all of the substrate had reacted. The reaction mixture was filtered and the filtrate was concentrated *in vacuo* to give a solid. This was purified by flash column chromatography on silica gel eluting with 20% Et_2O / DCM affording 6-(2-(dimethylamino-phenylazo)-benzoyl)-prop-2-ynyl-hexanamide **(72)** (1.43 g, 32%) as an orange solid. Analysis was concordant with the proposed structure.

R_F = (10% MeOH / DCM, u.v., anisaldehyde, 5% H_2SO_4 / EtOH)

ES+: 420 $(M+H)^+$, 442 $(M+Na^+)$, 458 $(M+K)^+$

HRMS: $(M+H)^+$ 419.2318 (calc. 419.2321)

m.p. 97-105°C decomp.

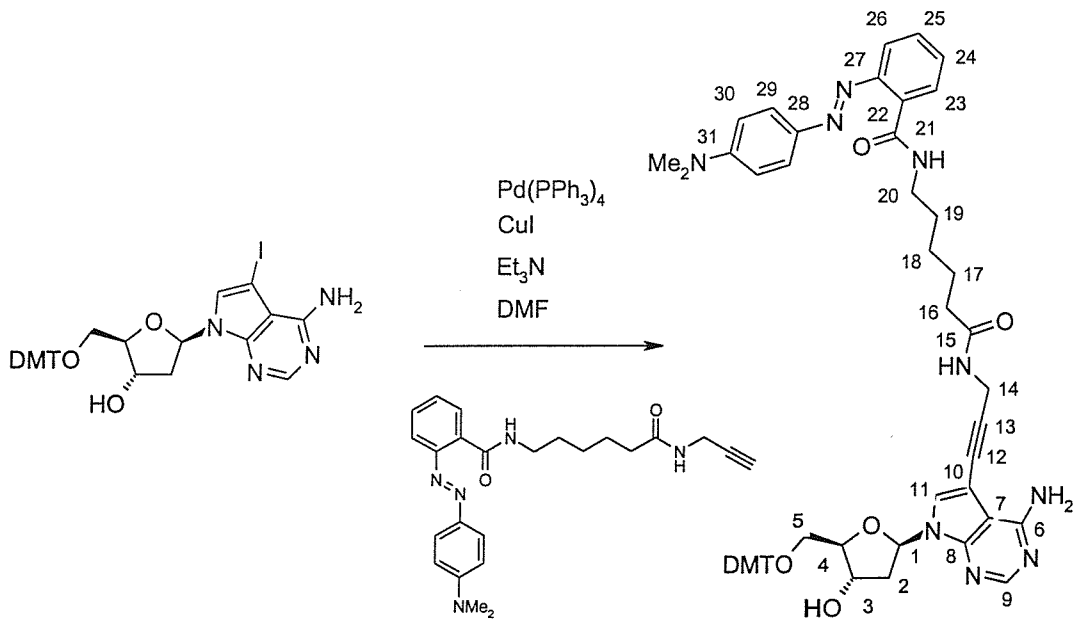
δ^1H (300MHz, DMSO): 1.20-1.42 (m., 2, $\underline{H}_7$), 1.48-1.53 (m., 4H, $\underline{H}_8$ and $\underline{H}_6$), 2.02-2.14 (m., 2H, $\underline{H}_9$), 3.10 (2s., 1H, $\underline{H}_1$ and 6H, $N\underline{Me}_2$), 3.25-3.40 (m., 2H, $\underline{H}_5$), 3.87

(m., 2H, $\underline{H}_3$), 6.81 (d., 2H, J= 8 Hz, $\underline{H}_{19}$), 7.42-7.58 (m., 2H, $\underline{H}_{12}$ and $\underline{H}_{13}$), 7.64-7.79 (m., 2H, $\underline{H}_{14}$ and $\underline{H}_{15}$), 8.28 (b.s., 3H, NH), 8.62 (b.s., 1H, NH)

δ^{13}C (75.42MHz, DMSO): 25.09 (C_7), 26.36 (C_8), 27.81 (C_3), 29.24 (C_6), 35.13 (C_9), 39.19 (C_5), 39.96 ($N\underline{Me}_2$), 72.89 ($\underline{H}_1$), 81.43 ($\underline{C}_2$), 72.89 ($\underline{H}_1$), 111.64 ($\underline{C}_{19}$), 115.75 ($\underline{C}_{18}$) , 125.34 ($\underline{C}_{12}$), 129.19 ($\underline{C}_{13}$), 129.24 ($\underline{C}_{14}$), 130.42 ($\underline{C}_{15}$), 134.24 ($\underline{C}_{20}$), 142.79 ($\underline{C}_{16}$) , 149.40 ($\underline{C}_{17}$) , 152.93 ($\underline{C}_{10}$) , 166.71 ($\underline{C}_{11}$), 171.92 ($\underline{C}_4$)

3.2.35

4-Amino-7-[(O-5-bis-(4-methoxyphenyl)-phenylmethyl)-2-deoxy-β-D-ribosyl]-5-(6-(2-(dimethylamino-phenylazo)-benzoyl)-prop-2-ynyl-hexanamido)-pyrrolo[2,3-d]pyrimidine (73)



4-Amino-7-[(O-5-bis-(4-methoxyphenyl)-phenylmethyl)-2-deoxy-β-D-ribosyl]-5-iodopyrrolo[2,3-d]pyrimidine **(62)** (0.869 g, 1.28 mmol) was charged to a reaction flask equipped with a vented septum and a stirrer bar, followed by CuI (49.0 mg, 0.256 mmol, 0.20 eq.) and $Pd(P(Ph)_3)_4$ (148 mg, 0.128 mmol.0.10 eq) and the mixture of solids was dried *in vacuo* over P_2O_5 for 16 hours. The vacuum was

broken with nitrogen, the mixture was stirred at ambient temperature under a headspace of nitrogen and anhydrous DMF (8.00 mL) was added followed by anhydrous triethylamine (1.78 mL, 12.8 mmol, 10 eq.) and 6-(2-(dimethylamino-phenylazo)-benzoyl)-prop-2-ynyl-hexanamide **(72)** (0.59 g, 1.41 mmol, 1.10 eq). The resulting mixture was stirred at ambient temperature for 16 hours after which time TLC analysis showed complete absence of the substrate. The reaction mixture was diluted with diethyl ether (25 mL), washed by extraction with water (2 x 15 mL) and the organic phase was dried over Na_2SO_4 (2.0 g) and concentrated *in vacuo*. The residual oil was purified by flash column chromatography on silica gel eluting with a gradient of 0 to 10% EtOAc in DCM to afford 4-amino-7-[(O-5-bis-(4-methoxyphenyl)-phenylmethyl)-2-deoxy-β-D-ribosyl]-5-(6-(2-(dimethylamino-phenylazo)-benzoyl)-prop-2-ynyl-hexanamido)-pyrrolo[2,3-d]pyrimidine **(73)** (903 mg, 73%) as a red solid. Analysis was concordant with the proposed structure.

R_F = 0.50 (EtOAc / MeOH / NH_4OH (5:1:1), u.v., anisaldehyde, 5% H_2SO_4 / EtOH)

ES+: 970 $(M+H)^+$, 992 $(M+Na^+)$

HRMS: $(M+Na)^+$ 992.4412 (calc. 992.4430)

m.p. 100-108°C decomp.

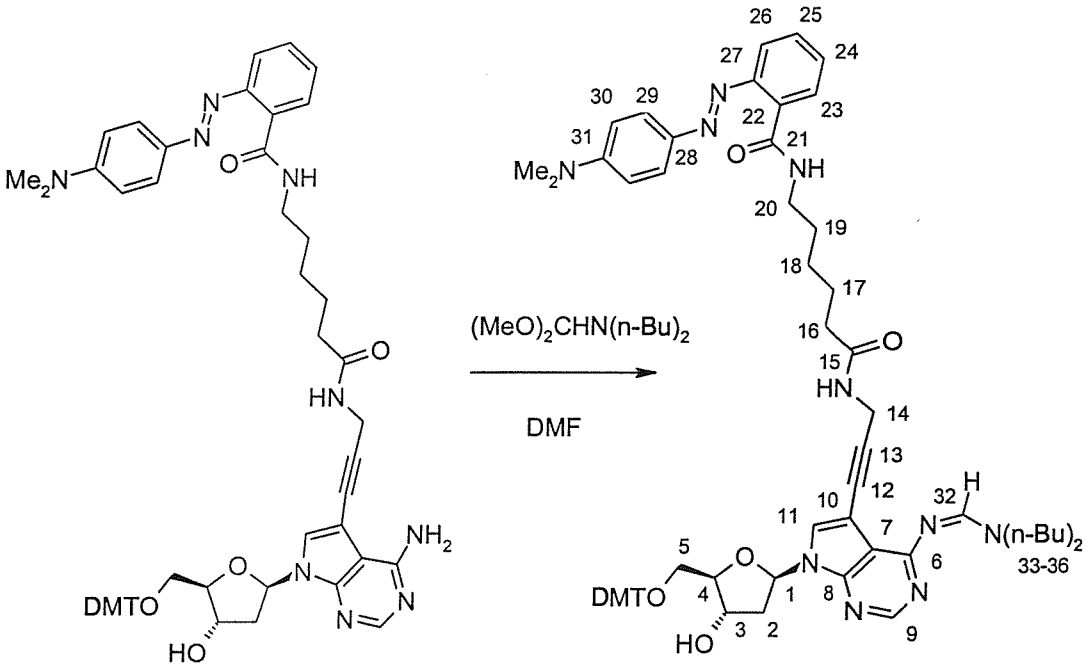
δ^1H (300MHz, DMSO): 1.25-1.42 (m., 2H, $\underline{H}_{18}$), 1.48-1.63 (m., 4H, $\underline{H}_{19}$ and $\underline{H}_{17}$), 2.02-2.17 (m., 2H, $\underline{H}_{20}$), 2.03-2.15 and 2.18-2.35 (2m., 2H, $\underline{H}_2$), 3.05 (s., 6H, N$\underline{Me}_2$), 3.10-3.24 (m., 2H, $\underline{H}_5$), 3.25-3.40 (m., 2H, $\underline{H}_{16}$), 3.70 (s., 6H, O$\underline{Me}$), 3.90-4.05 (m., 1 H, $\underline{H}_4$), 4.10-4.20 $\underline{H}_{14}$), 4.30-4.42 (m., 1H, $\underline{H}_3$), 5.35 (b.s., 1H, C_3-O$\underline{H}$), 6.45-6.55 (m., 1 H, $\underline{H}_1$), 6.81-6.95 (m., 4H, DMT C$\underline{H}$ *ortho* to ether, 2H, $\underline{H}_{30}$), 7.10-7.40 (m., 9H, DMT C$\underline{H}$, 2H, $\underline{H}_{29}$) 7.60 (s., 1H, $\underline{H}_9$), 7.62-7.79 (m., 2H, $\underline{H}_{23}$

and $\underline{H}_{24}$), 7.65-7.81 (m., 2H, $\underline{H}_{25}$ and $\underline{H}_{26}$), 8.10 (s., 1H, $\underline{H}_{11}$), 8.42 and 8.59 (2b.t., each 1H, NH)

δ^{13}C (75.42MHz, DMSO): 25.12 (C_{18}), 26.42 (C_{19}), 29.23 (C_{14}), 30.78 (C_{17}), 35.29 (C_{20}), 39.14 (C_{16}), 39.90 ($N\underline{Me}_2$), 40.41 (C_2), 55.07 ($O\underline{Me}$), ($\underline{C}_{10}$), 64.26 ($\underline{C}_5$), 70.69 ($\underline{C}_3$), 75.02 ($\underline{H}_{13}$), 85.52 ($\underline{C}_{14}$), 82.78 ($\underline{C}_1$), 85.54 (-$\underline{C}(Ar)_3$), 89.32 ($\underline{C}_4$), 95.02 ($\underline{C}_{11}$), 102.44 ($\underline{C}_7$), 111.62 ($\underline{C}_{30}$), 113.20 (DMT $\underline{C}$H *ortho* to ether), 115.75 ($\underline{C}_{29}$), 125.34 ($\underline{C}_{23}$), 126.70, 127.77, 127.89 and 129.80 (DMT $\underline{C}$H), 129.22 ($\underline{C}_{24}$), 126.70 ($\underline{C}_{25}$), 130.43 ($\underline{C}_{26}$), 134.18 ($\underline{C}_{31}$), 135.58, 135.68 and 145.02 (DMT $\underline{C}_{quat}$), 142.79 ($\underline{C}_{27}$), 146.41 (C_9), 149.45 ($\underline{C}_6$), 149.49 ($\underline{C}_{28}$), 152.91 ($\underline{C}_{21}$), 157.59 ($\underline{C}_8$), 166.69 ($\underline{C}_{22}$), 172.42 ($\underline{C}_{15}$)

3.2.36

4-(Di-n-butylamino-methyleneamino)-7-[(O-5-bis-(4-methoxyphenyl)-phenylmethyl)-2-deoxy-β-D-ribosyl]-5-(6-(2-(dimethylamino-phenylazo)-benzoyl)-prop-2-ynyl-hexanamido)-pyrrolo[2,3-d]pyrimidine (74)



4-Amino-7-[(O-5-bis-(4-methoxyphenyl)-phenylmethyl)-2-deoxy-β-D-ribosyl]-5-(6-(2-(dimethylamino-phenylazo)-benzoyl)-prop-2-ynyl-hexanamido)-pyrrolo[2,3-d]pyrimidine **(73)** (768 mg, 0.792 mmol) was charged to a reaction flask equipped with a vented septum and a stirrer bar and was dried *in vacuo* over P_2O_5 for 3 hours. The vacuum was broken with nitrogen and the solid was stirred at ambient temperature under a headspace of nitrogen. Anhydrous DMF (8.00 mL), was added followed by N,N-di-n-butylformamide dimethylacetal (1.60 g, 7.88 mmol, 10 eq.) and the resulting solution was stirred at ambient temperature for 16 hours. At that time TLC analysis showed that all of the substrate had reacted. The reaction mixture was concentrated *in vacuo* and residual DMF was removed by co-evaporation with toluene (3 x 10 mL). The residual solid was purified by flash column chromatography on silica gel, eluting with a gradient of 50 to 100% EtOAc / Et_2O, through to 10% MeOH / EtOAc. This afforded 4-(di-n-butylamino-methyleneamino)-7-[(O-5-bis-(4-methoxyphenyl)-phenylmethyl)-2-deoxy-β-D-ribosyl]-5-(6-(2-(dimethylamino-phenylazo)-benzoyl)-prop-2-ynyl-hexanamido)-pyrrolo[2,3-d]pyrimidine **(74)** (660 mg, 75%) as an orange foam. Analysis was concordant with the proposed structure.

R_F = 0.34 (EtOAc, u.v., anisaldehyde, 5% H_2SO_4 / EtOH)

ES+: 1109 $(M+H)^+$

m.p. 99.5-103 °C decomp.

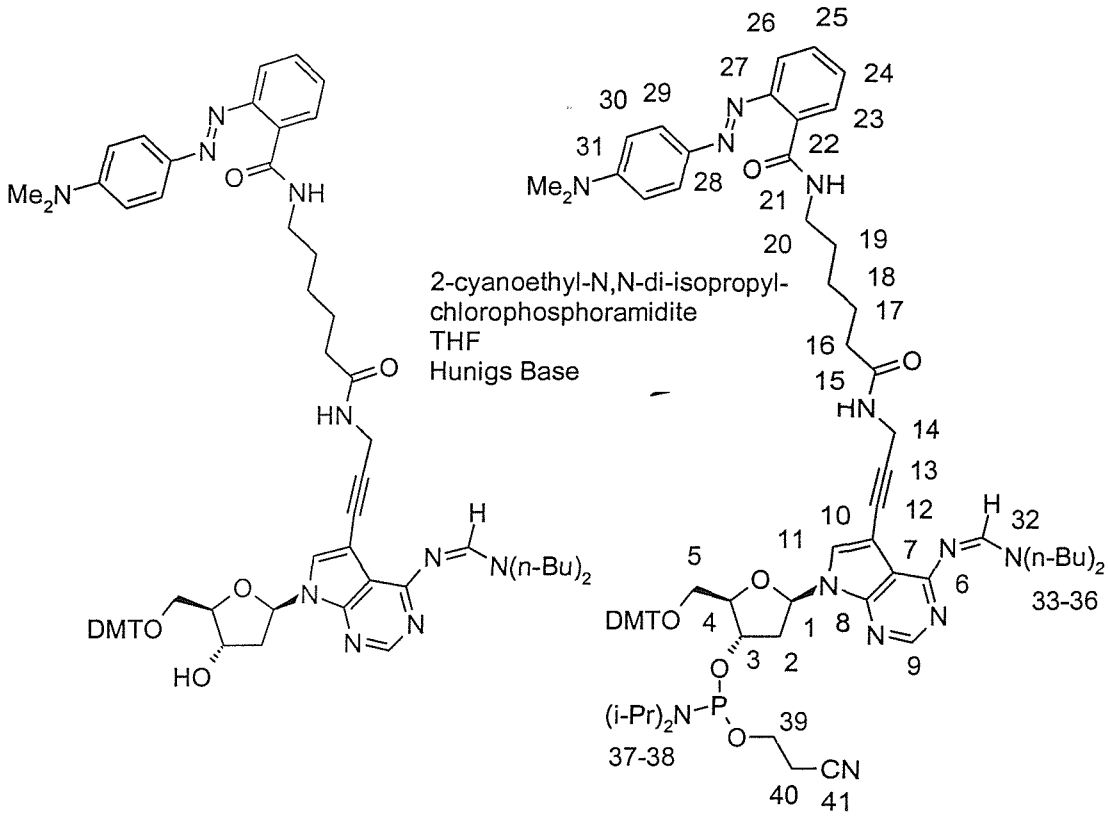
δ^1H (300MHz, DMSO): 0.85-1.02 (m., 6H, $\underline{H}_{36}$), 1.25-1.48 (m., 2H, $\underline{H}_{18}$, 4H, $\underline{H}_{35}$), 1.50-1.80 (m., 8H, $\underline{H}_{19}$, $\underline{H}_{17}$ and $\underline{H}_{34}$), 2.02-2.17 (m., 2H, $\underline{H}_{20}$), 2.15-2.40 (m., 2H, $\underline{H}_2$), 3.05-3.24 (m., 2H, $\underline{H}_5$), 3.05 (s., 6H, N$\underline{Me}_2$), 3.25-3.40 (m., 2H, $\underline{H}_{16}$), 3.30-3.60 (m., 4H, $\underline{H}_{33}$), 3.72 (s., 6H, O$\underline{Me}$), 3.90-4.00 (m., 1 H, $\underline{H}_4$), 4.10-4.20 (m., 2H, $\underline{H}_{14}$), 4.30-4.42 (m., 1H, $\underline{H}_3$), 5.42 (b.d., 1H, C_3-O$\underline{H}$), 6.55-6.64 (m., 1 H, $\underline{H}_1$), 6.74-6.88 (m., 2H, $\underline{H}_{30}$ and 4H, DMT C$\underline{H}$ *ortho* to ether), 7.10-7.40 (m., 9H, DMT C$\underline{H}$), 7.62-7.79 (m., 3H, $\underline{H}_{23}$, $\underline{H}_{32}$, and $\underline{H}_{24}$), 7.65-7.81 (m., 2H, $\underline{H}_{25}$ and $\underline{H}_{26}$), 8.35

(s., 1H, $\underline{H}_9$), 8.25 and 8.59 (2b.t., each 1H, NH), 8.85 (s., 1H, $\underline{H}_{11}$). The multiplicity of some signals is due to Z/E configuration around the formamidine bond.

δ^{13}C (75.42MHz, DMSO): 13.69 and 13.87 (C_{36}), 198.31 and 19.72 (C_{35}), 25.14 (C_{18}), 26.47 (C_{19}), 28.90 and 29.10 (C_{34}), 29.30 (C_{14}), 30.66 (C_{17}), 35.14 (C_{20}), 39.19 (C_{16}), 39.89 (N$\underline{Me}_2$), 44.67 (C_2), 55.04 (O$\underline{Me}$), ($\underline{C}_{10}$), 64.34 ($\underline{C}_5$), 70.79 ($\underline{C}_3$), 76.71 ($\underline{H}_{13}$), 85.49 (-$\underline{C}$(Ar)$_3$), 82.82 ($\underline{C}_1$), 86.52 ($\underline{C}_4$), 96.82 ($\underline{C}_{11}$), ($\underline{C}_7$), 111.62 ($\underline{C}_{30}$), 113.20 (DMT $\underline{C}$H *ortho* to ether), 115.72 ($\underline{C}_{29}$), 125.34 ($\underline{C}_{23}$), 127.76, 127.90, 128.21 and 129.23 (DMT $\underline{C}$H), 129.77 ($\underline{C}_{24}$), 126.69 ($\underline{C}_{25}$), 130.44 ($\underline{C}_{26}$), 134.16 ($\underline{C}_{31}$), 135.57, 135.72 and 145.02 (DMT $\underline{C}_{quat}$), 142.79 ($\underline{C}_{27}$), 149.40 ($\underline{C}_6$), 151.02 ($\underline{C}_{28}$), 152.91 ($\underline{C}_{21}$), 156.38 (C_9), 158.08 ($\underline{C}_8$), 166.66 ($\underline{C}_{22}$), 171.77 ($\underline{C}_{15}$). Multiplicity of some signals is due to Z/E configuration around the formamidine bond.

3.2.37

4-(Di-*n*-butylamino-methyleneamino)-7-[(O-5-bis-(4-methoxyphenyl)-phenylmethyl)-2-deoxy-β-D-ribosyl-O-3-(2-cyanoethyl-N,N-di-isopropyl-phosphoramidite)]-5-(6-(2-(dimethylamino-phenylazo)-benzoyl)-prop-2-ynyl-hexanamido)-pyrrolo[2,3-d]pyrimidine (75)



4-(Di-*n*-butylamino-methyleneamino)-7-[(O-5-bis-(4-methoxyphenyl)-phenylmethyl)-2-deoxy-β-D-ribosyl]-5-(6-(2-(dimethylamino-phenylazo)-benzoyl)-prop-2-ynyl-hexanamido)-pyrrolo[2,3-d]pyrimidine **(74)** (560 mg, 0.505 mmol) was charged to a reaction flask equipped with a stirrer bar and a vented septum and was dried *in vacuo* over P_2O_5 for 3 hours. The vacuum was broken with nitrogen and anhydrous THF (6.00 mL) was added with stirring under nitrogen, followed by DIPEA (196 mg, 1.52 mmol, 3.00 eq.) and 2-cyanoethyl N,N-di-isopropyl chlorophosphoramidite (131 mg, 0.556 mmol, 1.10 eq). The resulting solution was stirred at ambient temperature for 1 hour after which time TLC analysis indicated that the reaction was complete. The reaction mixture was diluted with degassed EtOAc (25.0 mL) {solvents degassed by displacement with nitrogen at ambient temperature} and washed by extraction with sat. $KCl_{(aq)}$ under nitrogen. The organic phase was transferred *via* a cannula and dried over Na_2SO_4 (4.0 g). The dry organic solution was recovered from the drying agent by cannula transfer and the drying agent was washed with degassed EtOAc (10

mL) using the same technique. The combined organic solutions were concentrated *in vacuo* to give a red oil. This was purified by flash column chromatography on silica gel eluting with 20% diethyl ether in EtOAc {eluents degassed as above} to afford a red solid. This was dissolved in anhydrous MeCN (4.00 mL) under nitrogen and the resulting solution was clarified by passing it through a Gelman acrodisc filter (0.45 μm) to remove particulate matter and was then concentrated *in vacuo* affording 4-(di-*n*-butylamino-methyleneamino)-7-[(O-5-bis-(4-methoxyphenyl)-phenylmethyl)-2-deoxy-β-D-ribosyl-O-3-(2-cyanoethyl-N,N-di-isopropyl-phosphoramidite)]-5-(6-(2-(dimethylamino-phenylazo)-benzoyl)-prop-2-ynyl-hexanamido)-pyrrolo[2,3-d]pyrimidine **(75)** (556 mg, 84%) as a red oil. Analysis was concordant with structure.

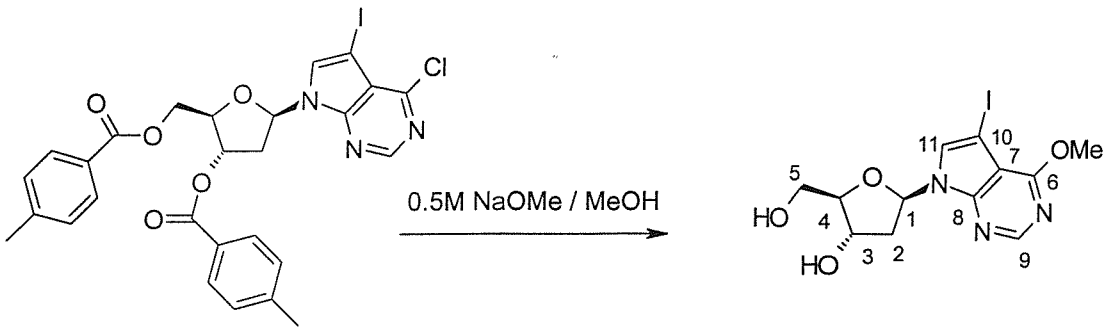
R_F = 0.71 (EtOAc, u.v., anisaldehyde, 5%H_2SO_4)

ES+: 1310 $(M+H)^+$

δ^1H (300MHz, $CDCl_3$): 0.78-0.90 (m., 6H, $\underline{H}_{36}$), 1.00-1.07 and 1.10-1.20 (m., 12H, $\underline{H}_{38}$), 1.25-1.48 (m., 4H, $\underline{H}_{35}$, m., 2H, $\underline{H}_{18}$ and m., 2H, $\underline{H}_{40}$), 1.50-1.80 (m., 8H, $\underline{H}_{19}$, $\underline{H}_{17}$ and $\underline{H}_{34}$), 1.98-2.11 (m., 2H, $\underline{H}_{20}$), 2.30-2.70 (m., 2H, $\underline{H}_2$ and 2m., 2H, $\underline{H}_{37}$), 3.05 (s., 6H, N$\underline{Me}_2$), 3.25-3.40 (m., 2H, $\underline{H}_{16}$ and m., 2H, $\underline{H}_5$), 3.30-3.50 (m., 4H, $\underline{H}_{33}$ and m., 2H, $\underline{H}_{39}$), 3.72 (s., 6H, O$\underline{Me}$), 4.12-4.24 (m., 1 H, $\underline{H}_4$ and m., 2H, $\underline{H}_{14}$), 4.55-4.66 (m., 1H, $\underline{H}_3$), 5.65 and 5.70 (2 b.t., each 0.5H, NH), 6.50-6.63 (m., 1 H, $\underline{H}_1$), 6.74-6.88 (m., 2H, $\underline{H}_{30}$ and 4H, DMT C$\underline{H}$ *ortho* to ether), 7.10-7.52 (m., 11H, DMT C$\underline{H}$, $\underline{H}_{23}$, and $\underline{H}_{24}$), 7.65-7.81 (m., 3H, $\underline{H}_{32}$, $\underline{H}_{25}$ and $\underline{H}_{26}$), 8.24, 8.26, 8.34 and 8.35 (4s., each 0.25H, $\underline{H}_9$), 8.72 (s., 1H, $\underline{H}_{11}$), 8.95 (b.t., 1H, NH). Duplicity of some signals is due to diasteromeric effect of tetrahedral P^{III}. Detailed structure of relevant multiplets also shows Z/E pairs for the formamidine.

3.2.38

4-Methoxy-7-(2-deoxy-β-D-ribosyl)-5-iodo-pyrrolo[2,3-d]pyrimidine (76)



5-Chloro-7-(2-deoxy-3,5-di-O-*p*-toluoyl-β-D-ribosyl)-5-iodopyrrolo[2,3-d]pyrimidine **(59)** (9.00 g, mmol) was dissolved in 0.50M NaOMe / MeOH (90.0 mL) and stirred under reflux for 1 hour, after which time TLC analysis indicated that all of the substrate had reacted. The reaction mixture was allowed to cool to ambient temperature, silica gel (4.00g) was added and the mixture was concentrated *in vacuo* to give a free flowing solid. The title compound was obtained by flash silica column chromatography eluting with 5% MeOH / DCM. This afforded 4-methoxy-7-(2-deoxy-β-D-ribosyl)-5-iodo-pyrrolo[2,3-d]pyrimidine **(76)** (0.553 g, 89%) as an amorphous white solid. Analysis was concordant with the proposed structure.

R_F = 0.40 (10% MeOH / DCM, u.v., anisaldehyde)

ES+: 392 $(M+H)^+$

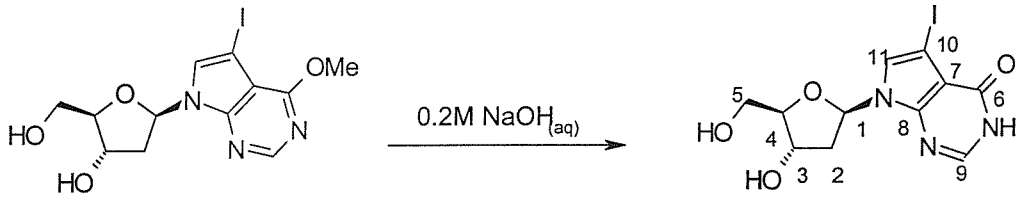
m.p. 160-163°C

δ^1H (300MHz, DMSO): 2.09-2.20 and 2.45-2.62 (2m., 2H, $\underline{H}_2$), 3.45-3.70 (m., 2H, $\underline{H}_5$), 3.80-3.90 (m., 1 H, $\underline{H}_4$), 4.04 (s., 3H, OMe), 4.33-4.40 (m., 1H, $\underline{H}_3$), 5.05 (t., 1 H, C_5-O$\underline{H}$), 5.4 (d., 1 H, C_3-O$\underline{H}$), 6.55-6.62 (m., 1 H, $\underline{H}_1$), 790 (s., 1H, $\underline{H}_{11}$), 8.45 (s., 1H, $\underline{H}_9$)

$\delta^{13}C$ (75.42MHz, DMSO): 39.94 (C_2), 53.83 (O<u>Me</u>), 51.45 (<u>C_{10}</u>), 61.83 (<u>C_5</u>), 70.94 (<u>C_3</u>), 83.17 (<u>C_1</u>), 87.62 (<u>C_4</u>), 106.72 (<u>C_7</u>), 129.23 (<u>C_{11}</u>), 151.12 (C_9), 151.36 (<u>C_6</u>), 162.29 (<u>C_8</u>)

3.2.39

7-(2-Deoxy-β-D-ribosyl)-5-iodo-pyrrolo[2,3-d]pyrimidin-5-one (77)



5-Methoxy-7-(2-deoxy-β-D-ribosyl)-5-iodo-pyrrolo[2,3-d]pyrimidine **(76)** (6.10 g, 15.6 mmol) was suspended in 0.2 M $NaOH_{(aq)}$ (60.0 mL) and heated under reflux for 2 hours after which time TLC analysis showed approximately 50% conversion of the substrate. Further $NaOH_{l(s)}$ (0.50 g, 12.5 mmol) was added and the resulting mixture was stirred under reflux for a further 1 hour. At that point TLC analysis showed that there was still approximately 20% unreacted substrate present. Further $NaOH_{l(s)}$ (0.50 g, 12.5 mmol) was added and the mixture was stirred under reflux for a further 1 hour. At that point TLC analysis showed that all of the substrate had reacted. The reaction mixture was allowed to cool to ambient temperature and silica gel (2.00g) was added. The mixture was concentrated *in vacuo* and ground to give a free-flowing solid. This was purified by flash column chromatography on silica gel affording 7-(2-deoxy-β-D-ribosyl)-5-iodo-pyrrolo[2,3-d]pyrimidin-5-one **(77)** (4.37 g, 74%) as a white, crystalline solid. Analysis was concordant with the proposed structure.

R_F = 0.12 (10% MeOH / DCM u.v., anisaldehyde)

ES+: 378 $(M+H)^+$

HRMS: 376.9868 (calc. 376.9873)

m.p. 220-225°C decomp.

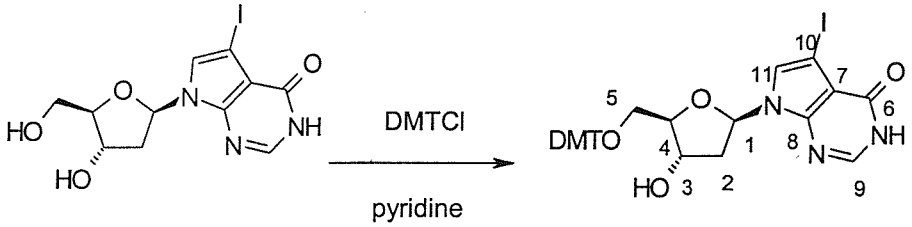
ν_{MAX} /cm^{-1} (DCM solution cell): 1739 C=O str

δ^1H (300MHz, DMSO): 2.09-2.20 and 2.35-2.50 (2m., 2H, $\underline{H}_2$), 3.45-3.70 (m., 2H, $\underline{H}_5$), 3.75-3.85 (m., 1 H, $\underline{H}_4$), 4.30-4.45 (m., 1H, $\underline{H}_3$), 5.05 (b.s., 1 H, C_5-O$\underline{H}$), 5.4 (b.s., 1 H, C_3-O$\underline{H}$), 6.43-6.54 (m., 1 H, $\underline{H}_1$), 7.55 (s., 1H, $\underline{H}_9$), 7.95 (s., 1H, $\underline{H}_{11}$)

δ^{13}C (75.42MHz, DMSO): 40.19 (C_2), 55.28 ($\underline{C}_{10}$), 61.81 ($\underline{C}_5$), 70.77 ($\underline{C}_3$), 82.99 ($\underline{C}_1$), 87.45 ($\underline{C}_4$), 107.88 ($\underline{C}_7$), 125.45 ($\underline{C}_{11}$), 144.53 (C_9), 147.11 ($\underline{C}_6$), 157.61 ($\underline{C}_8$)

3.2.40

7-(O-(5-Bis-(4-methoxyphenyl)-phenylmethyl)-2-deoxy-β-D-ribosyl)-5-iodo-pyrrolo[2,3-d]pyrimidin-5-one (78)



7-(2-Deoxy-β-D-ribosyl)-5-iodo-pyrrolo[2,3-d]pyrimidin-5-one **(77)** (4.22g, 10.8 mmol) and DMTCl (4.02 g, 11.9 mmol, 1.10 eq) were charged to a flask equipped with a vented septum and a magnetic stirrer bar. The mixture of solids was dried *in vacuo* over P_2O_5 for 2 hours, the vacuum was broken with argon and the flask contents were stirred under a headspace of argon. The mixture was cooled to 0°C (ice bath) and ice-cold anhydrous pyridine (50.0 mL) was added in one portion with vigorous magnetic stirring. The reaction mixture was

allowed to warm to ambient temperature and stirred for 3 hours after which time TLC analysis indicated that all of the substrate had reacted. The reaction mixture was diluted with DCM (100 mL), washed by extraction with sat. $NaHCO_3$ (50 mL), dried over Na_2SO_4 (5.00g) and concentrated *in vacuo* to give a coloured oil. Residual pyridine was displaced by co-evaporation with toluene (2 x 20 mL) which lead to a coloured solid. This was purified by crystallisation from EtOAc / Et_2O to afford 7-(O-(5-bis-(4-methoxyphenyl)-phenylmethyl)-2-deoxy-β-D-ribosyl)-5-iodo-pyrrolo[2,3-d]pyrimidin-5-one **(78)** (6.66g, 91%) as a white, crystalline solid. Analysis was concordant with the proposed structure.

R_F = 0.44 (10% MeOH / DCM, u.v., anisaldehyde, 5% H_2SO_4 / EtOH)

ES+: 702 $(M+Na)^+$

HRMS: $(M+H)^+$ 680.1278 (calc. 680.1258)

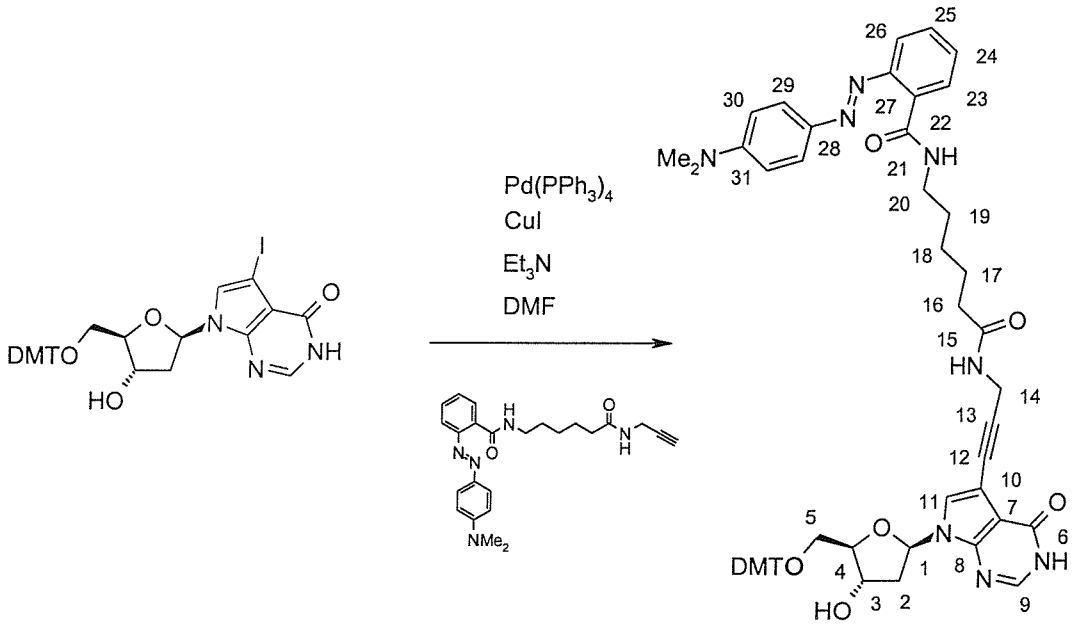
m.p. 132-140°C decomp.

δ^1H (300MHz, DMSO): 2.20-2.30 and 2.50-2.62 (2m., 2H, $\underline{H}_2$), 3.05-3.28 (m., 2H, $\underline{H}_5$), 3.70 (s., 6H, O$\underline{Me}$), 3.90-4.00 (m., 1H, $\underline{H}_4$), 4.35-4.45 (m., 1H, $\underline{H}_3$), 5.40 (d., 1H, C_3-O$\underline{H}$), 6.43-6.54 (m., 1H, $\underline{H}_1$), 6.83-6.95 (m., 4H, DMT C$\underline{H}$ *ortho* to ether),7.10-7.40 (m., 9H, DMT C$\underline{H}$) 7.40 (s., 1H, $\underline{H}_9$), 7.95 (s., 1H, $\underline{H}_{11}$), 12.25 (bs., 1H, N$\underline{H}$)

$\delta^{13}C$ (75.42MHz, DMSO): 39.98 (C_2), 55.14 (O$\underline{Me}$), 55.61 ($\underline{C}_{10}$), 64.17 ($\underline{C}_5$), 70.75 ($\underline{C}_3$), 83.13 ($\underline{C}_1$), 85.64 (-$\underline{C}(Ar)_3$), 85.72 ($\underline{C}_4$), 108.22 ($\underline{C}_7$), 113.27 (DMT $\underline{C}$H *ortho* to ether), 125.40 ($\underline{C}_{11}$), 126.75, 127.79, 127.95 and 129.80 (DMT $\underline{C}$H), 135.56, 135.66 and 144.96 (DMT $\underline{C}_{quat}$), 144.70 (C_9), 147.31 ($\underline{C}_6$), 157.77 ($\underline{C}_8$)

3.2.41

7-(O-(5-Bis-(4-methoxyphenyl)-phenylmethyl)-2-deoxy-β-D-ribosyl)-5-(6-(2-(dimethylamino-phenylazo)-benzoyl)-prop-2-ynyl-hexanamido)-pyrrolo[2,3-d]pyrimidin-5-one (79)



7-(O-(5-Bis-(4-methoxyphenyl)-phenylmethyl)-2-deoxy-β-D-ribosyl)-5-iodo-pyrrolo[2,3-d]pyrimidin-5-one **(78)** (0.869 g, 1.28 mmol) was charged to a flask followed by CuI (49.0 mg, 0.256 mmol, 0.20 eq.) and $Pd(PPh_3)_4$ (148 mg, 0.128 mmol, 0.10 eq.). The mixture of solids was dried *in vacuo* over P_2O_5 for 16 hours and the vacuum was broken with nitrogen. The mixture was stirred at ambient temperature under a headspace of nitrogen and anhydrous DMF (8.00 mL) was added followed by anhydrous triethylamine (1.78 mL, 12.8 mmol, 10.0 eq.) and 6-(2-(dimethylamino-phenylazo)-benzoyl)-prop-2-ynyl-hexanamide **(72)** (0.59 g, 1.41 mmol, 1.10 eq.). The resulting mixture was stirred at ambient temperature for 16 hours after which time TLC analysis showed complete absence of the substrate. The reaction mixture was diluted with diethyl ether (25 mL) and washed by extraction with water (2 x 15 mL). The organic extract was dried over Na_2SO_4 (2.0 g) and concentrated *in vacuo*. The residual oil was purified by flash column chromatography on silica gel eluting with a gradient of 0% to 10% EtOAc

in DCM to afford 7-(O-(5-bis-(4-methoxyphenyl)-phenylmethyl)-2-deoxy-β-D-ribosyl)-5-(6-(2-(dimethylamino-phenylazo)-benzoyl)-prop-2-ynyl-hexanamido)-pyrrolo[2,3-d]pyrimidin-5-one **(79)** (597 mg, 48%) as a red solid. Analysis was concordant with the proposed structure.

R_F = 0.1 (EtOAc, u.v., anisaldehyde, 5%H_2SO_4 / EtOH)

ES+: 971 $(M+H)^+$, 993 $(M+Na^+)$

HRMS: $(M+Na)^+$ 993.4245 (calc. 993.4270)

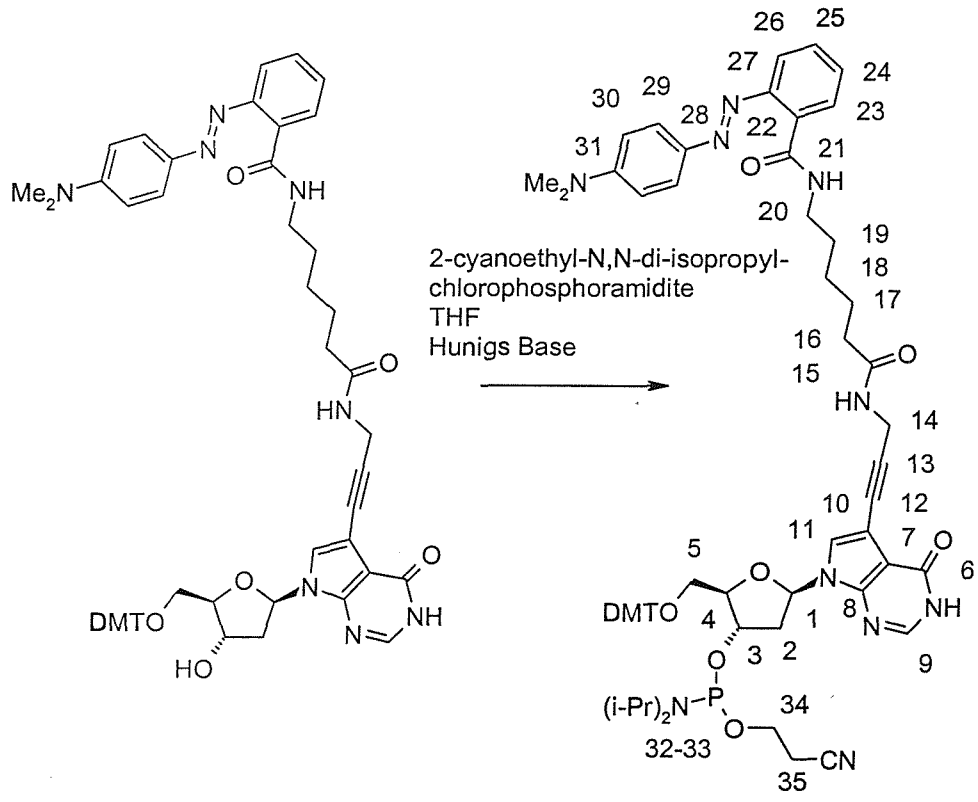
m.p. 143-155°C decomp.

δ^1H (300MHz, DMSO): 1.20-1.42 (m., 2H, $\underline{H}_{18}$), 1.48-1.63 (m. 4H, $\underline{H}_{19}$ and $\underline{H}_{17}$), 2.05-2.13 (m., 2H, $\underline{H}_{20}$), 2.20-2.30 and 2.50-2.62 (2m., 2H, $\underline{H}_2$), 3.05-3.24 (m., 2H, $\underline{H}_5$), 3.10 (s., 6H, N$\underline{Me}_2$), 3.25-3.40 (m., 2H, $\underline{H}_{16}$), 3.70 (s., 6H, O$\underline{Me}$), 3.85-4.00 (m., 1H, $\underline{H}_4$), 3.90-4.05 (m., 2H, $\underline{H}_{14}$), 4.30-4.42 (m., 1H, $\underline{H}_3$), 5.35 (d., 1 H, C_3-O$\underline{H}$), 6.40-6.50 (m., 1H, $\underline{H}_1$), 6.83-6.95 (m., 4H, DMT C$\underline{H}$ *ortho* to ether, 2H, $\underline{H}_{30}$), 7.10-7.40 (m., 9H, DMT C$\underline{H}$, 1H, $\underline{H}_9$, 2H, $\underline{H}_{29}$), 7.62-7.79 (m., 2H, $\underline{H}_{23}$ and $\underline{H}_{24}$), 7.70-7.83 (m., 2H, $\underline{H}_{25}$ and $\underline{H}_{26}$), 7.95 (s., 1H, $\underline{H}_{11}$), 8.32 and 8.59 (2b.t., each 1H, NH), 12.20 (b.s., 1H, N$\underline{H}$)

δ^{13}C (75.42MHz, DMSO): 25.11 (C_{18}), 26.45 (C_{19}), 28.76 (C_{14}), 29.28 (C_{17}), 35.20 (C_{20}), 39.20 (C_{16}), 39.91 (N$\underline{Me}_2$), 39.46 (C_2), 55.07 (O$\underline{Me}$), 59.86 ($\underline{C}_{10}$), 64.26 ($\underline{C}_5$), 70.64 ($\underline{C}_3$), 75.14 ($\underline{H}_{13}$), 85.52 ($\underline{C}_{14}$), 83.11 ($\underline{C}_1$), 85.54 (-$\underline{C}(Ar)_3$), 87.44 ($\underline{C}_4$), 98.94 ($\underline{C}_{11}$), 107.87 ($\underline{C}_7$), 111.63 ($\underline{C}_{30}$), 113.21 (DMT $\underline{C}$H *ortho* to ether), 115.74 ($\underline{C}_{29}$), 125.33 ($\underline{C}_{23}$), 126.72, 127.75, 127.90 and 129.76 (DMT $\underline{C}$H), 129.23 ($\underline{C}_{24}$), 129.84 ($\underline{C}_{25}$), 130.40 ($\underline{C}_{26}$), 134.26 ($\underline{C}_{31}$), 135.50, 135.75 and 144.02 (DMT $\underline{C}_{quat}$), 142.79 ($\underline{C}_{27}$), 145.02 (C_9), 147.06 ($\underline{C}_6$), 149.39 ($\underline{C}_{28}$), 152.92 ($\underline{C}_{21}$), 157.51 ($\underline{C}_8$), 166.71 ($\underline{C}_{22}$), 171.82 ($\underline{C}_{15}$)

3.2.42

7-[(O-(5-Bis-(4-methoxyphenyl)-phenylmethyl)-2-deoxy-β-D-ribosyl-O-3-(2-cyanoethyl-N,N-diisopropyl-phosphoramidite)]-5-(6-(2-(dimethylamino-phenylazo)-benzoyl)-prop-2-ynyl-hexanamido)-pyrrolo[2,3-d]pyrimidin-5-one (80)



7-(O-(5-Bis-(4-methoxyphenyl)-phenylmethyl)-2-deoxy-β-D-ribosyl)-5-(6-(2-(dimethylamino-phenylazo)-benzoyl)-prop-2-ynyl-hexanamido)-pyrrolo[2,3-d]pyrimidin-5-one **(79)** (453 mg, 0.467 mmol) was charged to a reaction flask equipped with a stirrer bar and a vented septum and the mixture was dried *in vacuo* over P_2O_5 for 3 hours. The vacuum was broken with nitrogen and anhydrous THF (5.00 mL) was added with stirring under nitrogen, followed by DIPEA (181 mg, 1.40 mmol, 3.00 eq.) and 2-cyanoethyl-N,N-di-isopropyl

chlorophosphoramidite (122 mg, 0.514 mmol, 1.10 eq.). The mixture was stirred at ambient temperature for 1 hour after which time TLC analysis indicated that the reaction had proceeded to completion. The reaction mixture was diluted with degassed EtOAc (25.0 mL) {degassed by displacement with nitrogen at ambient temperature} and washed by extraction with similarly degassed sat. $KCl_{(aq)}$ under a headspace of nitrogen. The organic phase was transferred *via* a cannula to Na_2SO_4 (4.0 g) and shaken under a nitrogen atmosphere. The dry organics were recovered from the drying agent by cannula transfer and the drying agent was washed with degassed EtOAc (10 mL) using the same technique. The combined organic solutions were concentrated *in vacuo* to give a red oil. This was purified by flash column chromatography on silica gel eluting with EtOAc {eluent degassed as above} to afford a red solid. This was dissolved in anhydrous MeCN (4.00 mL) under nitrogen and the resultant was passed through a Gelman acrodisc filter (0.45 μm) to remove particulate matter and concentrated *in vacuo* affording 7-[(O-(5-bis-(4-methoxyphenyl)-phenylmethyl)-2-deoxy-β-D-ribosyl-O-3-(2-cyanoethyl-N,N-diisopropyl-phosphoramidite)]-5-(6-(2-(dimethylamino-phenylazo)-benzoyl)-prop-2-ynyl-hexanamido)-pyrrolo[2,3-d]pyrimidin-5-one **(80)** (342 mg, 62%) as an orange solid. Analysis was concordant with the proposed structure.

R_F = 0.25 (EtOAc, u.v., anisaldehyde, 5% H_2SO_4 / EtOH)

ES+: 1171 $(M+H)^+$, 1193 $(M+Na^+)$

δ^1H (300MHz, $CDCl_3$): 1.02-1.25 (m., 12H, $\underline{H}_{33}$), 1.25-1.40 (m., 2H, $\underline{H}_{18}$), 1.65-1.82 (m. 4H, $\underline{H}_{19}$ and $\underline{H}_{17}$), 1.65-1.80 (m., 2H, $\underline{H}_{32}$), 2.10-2.20 (m., 2H, $\underline{H}_{20}$), 2.35 and 2.58 (2t., each 1H, J=9Hz, $\underline{H}_{35}$), 2.35-2.54 (2m., 2H, $\underline{H}_2$), 3.05 (s., 6H, N$\underline{Me}_2$), 3.17-3.30 (m., 2H, $\underline{H}_5$), 3.45-3.80 (m., 2H, $\underline{H}_{16}$, 2H, $\underline{H}_{34}$), 3.75 (s., 6H, O$\underline{Me}$), 4.10-4.25 (m., 2H, $\underline{H}_{14}$, 1H, $\underline{H}_4$), 4.57-4.70 (m., 1H, $\underline{H}_3$), 6.35 (b.t., 1H, NH), 6.40-6.50 (m., 1 H, $\underline{H}_1$), 6.62-6.80 (m., 2H, $\underline{H}_{30}$, 4H, DMT C$\underline{H}$ *ortho* to ether), 7.10-7.40 (m., 9H, DMT C$\underline{H}$, 2H, $\underline{H}_{29}$, 1H, $\underline{H}_9$), 7.62-7.79 (m., 3H, $\underline{H}_{23}$, $\underline{H}_{24}$ and

$\underline{H}_{25}$), 7.95 (2s., 1H, $\underline{H}_{28}$), 8.25 (2s., 1H, $\underline{H}_{11}$), 9.05 (b.t., 1H, NH), 11.80 (bs., 1H, N$\underline{H}$)

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