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Development of Novel Fluorescent Oligonucleotide Probes for Use in Nucleic Acid Sequence Analysis

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A Thesis submitted for the Degree of Doctor of Philosophy

Department of Chemistry

January 2000

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF SCIENCE

CHEMISTRY

Doctor of Philosophy

DEVELOPMENT OF NOVEL FLUORESCENT OLIGONUCLEOTIDE PROBES FOR USE IN NUCLEIC ACID SEQUENCE ANALYSIS

by Lynda Jane Brown

DNA and RNA labelled probes are used for target recognition in complex systems of nucleic acids. The development of two novel probe systems (heterogeneous and homogeneous assay) for use in nucleic acid sequence analysis is described.

The homogeneous assay proposed required two probes, one terminating in an enzyme (3', β-D-galactosidase) and the other terminating in a fluorophore modified with β-D-galactosyl groups (enzyme substrates, 5') to remove its fluorescent properties. Synthesis of each probe is outlined. Fluorescence properties of the two probes hybridised adjacent to one another on a target nucleic acid was investigated.

The heterogeneous assay described involved a resin-bound nucleic acid probe synthesised with a stem and loop structure. The stem consisted of two short annealed arm sequences, one terminating in a quencher and the other in a fluorophore. In buffer only, the resin-bound probe was 'closed', quenching occurred and the clear glass resin was non-fluorescent. Introduction of a target nucleic acid complementary to the loop region causes the probe to 'open' and the glass beads became brightly fluorescent.

For Richard

Contents

Declaration		i
Abstract		ii
Contents		iv
Abbreviations		viii
Acknowledgements		xii
Chapter 1	**Introduction to DNA and its Detection**	1
1.1	**Nucleic acid background**	1
1.1.1	Primary Structure	1
1.1.2	Secondary Structure	2
1.1.3	The Genetic Code	3
1.2	**Detecting DNA**	4
1.2.1	DNA Diagnostics	5
1.2.2	Classical Methods	6
1.2.3	Non-Isotopic Labelling	7
	1.2.3.1 The Signal Emitting Moiety	8
	1.2.3.2 The Spacer	9
	1.2.3.3 The Reactive Group	9
1.3	**Labelling Strategies**	11
1.3.1	Indirect Labelling	11
1.3.2	Direct Labelling	12
	1.3.2.1 Radioactive Labels	13
	1.3.2.2 Enzyme Labels	13
	1.3.2.3 Chemiluminescent Labels	13
	1.3.2.4 Fluorescent Labels	14
	1.3.2.5 Lanthanide Labels	16
1.4	**Fundamentals of Fluorescence**	17
1.4.1	The Nature of Fluorescence	17
1.4.2	Fluorescence Quenching	18
1.4.3	Fluorescence Resonance Energy Transfer (FRET)	19
1.4.4	Time Resolved Fluorescence	20
1.4.5	Fluorescence Spectrometry	22
1.5	**Molecular Biological Methods Utilised in Fluorescence Assay**	23

1.5.1 Polymerase Chain Reaction 23
1.5.1.1 Hot Start PCR 24
1.5.2 Fluorescence *In Situ* Hybridisation 25
1.53 UV Thermal Melting 26
1.6 Practical Applications of Fluorescent Probe Systems 27
1.6.1 Taqman Assay 27
1.6.2 Molecular Beacons 28
1.6.3 Scorpion Primers 30
1.6.4 Invader Assay 32
1.6.5 PNA Probes 33
1.6.6 SUNRISE Primers 33
1.6.7 DNA Chip Technology 34
Chapter 2 Development of Novel Homogeneous Assay Systems 35
2.1 Introduction 35
2.2 Alkaline Phosphatase Approach 37
2.2.1 Introduction 37
2.2.2 Solid Phase Strategy 38
2.2.2.1 *4,4'-Dimethoxytrityl Ether* 38
2.2.2.2 *p-Methoxybenzyl Ether* 40
2.2.2.3 *t-Butyldimethyl silyl Ether* 41
2.2.2.4 *Levulinyl Esters* 41
2.2.2.5 *Disiloxyl Linker* 41
2.2.3 Solution Phase Strategy 45
2.2.4 Conclusions 46
2.3 β-D-Galactosidase Approach 47
2.3.1 Introduction 47
2.3.2 Synthesis of Signal Probe 47
2.3.3 Evaluation of the Signal Probe 52
2.3.4 β-Galactosidase-Oligonucleotide Enzyme Probe 1 53
2.3.4.1 Synthesis of MBS Oligonucleotide 55
2.3.4.2 Conjugation to β-Galactosidase 56
2.3.5 Enzyme Probe 1 Activity 58

2.3.6 SDS Page Electrophoresis 59
2.3.7 Assay Evaluation 60
2.3.8 Enzyme Probe 1 Hybridisation 63
2.3.8.1 UV Thermal Melting 64
2.3.8.2 Dot Blot Assay 65
2.3.9 Factors Influencing Formation and Stability of Hybrids 66
2.3.10 β-Galactosidase-Oligonucleotide Enzyme Probe 2 68
2.3.11 Enzyme Probe 2 Hybridisation 68
2.3.11.1 UV Thermal Melting 68
2.3.11.2 Dot Blot Assay 69
2.3.11.3 Molecular Beacon Approach 69
2.3.12 Assay Evaluation 71
2.4 Conclusions 74
Chapter 3 Heterogeneous Assay 76
3.1 Introduction 76
3.2 Molecular Beacons on Beads 76
3.2.1 Introduction 76
3.2.2 Resin Derivatisation 78
3.2.3 Heterogeneous Hybridisation 79
3.2.4 Molecular Beacons on Controlled Pore Glass 81
3.2.5 Molecular Beacons Immobilised on Alternative Supports 84
3.2.5.1 Polystyrene Beads 85
3.2.5.2 Tentagel™ Beads 86
3.2.5.3 Macroporous Beads 86
3.2.6 Conclusions and Future Work 87
3.3 Heterogeneous Capture Assay 89
3.3.1 Principle 89
3.3.2 Results 89
3.3.3 Conclusions 91
Chapter 4 Enzyme Labile Linkers 92
4.1 Introduction 92
4.2 β-Galactose Linker 94

4.2.1 Principle and Preparation 94
4.2.2 Oligonucleotide Synthesis and Linker Cleavage 97
4.2.3 Conclusions 97
4.3 Peptoid Linkers 98
4.3.1 Phenylalanine Linker 1 98
4.3.1.1 Principle and Preparation 98
4.3.1.2 Oligonucleotide Assembly and Linker Testing 99
4.3.1.3 Conclusions 102
4.3.2 Phenylalanine Linker 2 102
4.3.2.1 Principle and Preparation 102
4.3.2.2 Linker Cleavage 104
4.3.2.3 Conclusions 106
4.3.3 Lysine Linker 107
4.3.3.1 Principle and Preparation 107
4.3.3.2 Linker Cleavage 109
4.3.3.3 Conclusions 111
Chapter 5 Experimental 112
5.1 Preparation of Compounds 112
5.1.1 General Methods 112
5.1.2 List of Compounds 114
5.1.3 Experimental 117
5.2 Preparation of Resins 166
5.2.1 General Methods 166
5.2.2 Resin Derivatisation 166
5.3 Preparation of Synthetic Oligonucleotides 169
5.3.1 General Methods 169
5.3.2 Oligonucleotide Sequences 170
5.4 Molecular Biology 174
5.4.1 General Methods 174
5.4.2 Experimental 175
Chapter 6 Publication
Chapter 7 References 185

Abbreviations

Å	Angstrom unit (=10^{-10} m)
A	adenine
a	attomole
ABI	Applied Biosystems
Abs	absorbance
Ac	acetyl
AMPS	ammonium persulphate
Aq	aqueous
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BSA	bovine serum albumin
*t*Bu	tertiary butyl
C	cytosine
^{13}C	carbon [NMR]
$CDCl_3$	deuteriated chloroform
CPG	controlled pore glass
CPG-LCAA	controlled pore glass-long chain alkyl amino
Cy	cyanine (dye)
δ	chemical shift (parts per million)
DAB	diaminobenzimide tetrahydrochloride
DABCYL	4-(4'-dimethylaminophenylazo)benzoic acid
DBU	1,8-diazabicyclo(5.4.0)undec-7-ene
DCC	dicyclohexylcarbodiimide
Dde	4,4-dimethyl-2,6-dioxocyclohex-1-y(idene)ethane
DDQ	2,3-dichloro-5,6-dicyano-1,4-benzoquinone
DIPEA	diisopropylethylamine
DIC	disopropylcarbodimide
DIG	digoxigenin
DMAP	4-dimethylaminopyridine
DMF	*N,N*-dimethylformamide
DMSO	dimethylsulphoxide

DMT	4,4'-dimethoxytrityl
DNA	deoxyribonucleic acid
DNP	2,4-dinitrophenyl
DVB	divinylbenzene
dNTP	2'-deoxynucleoside triphosphate
ε	molar extinction coefficient
EDANS	5-(2'-aminoethyl)aminonapthalene-1-sulfonic acid
EDC	dimethylaminopropyl-3-ethylcarbodiimide
EDTA	ethylenediamine tetracetic acid
ES	electrospray (mass spectrometry)
EtOH	ethanol
EtOAc	ethyl acetate
F	fluorophore
FAM	5(6)carboxyfluorescein
FAB	fast atom bombardment
FISH	fluorescence *in situ* hybridisation
Fmoc	fluorenylmethyloxycarbonyl
βgal	β-galactosidase
G	guanine
^{1}H	proton [NMR]
Hepes	4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid
HEX	hexachlorinated fluorescein
hr	hour
HRP	horseradish peroxidase
HPLC	high performance liquid chromatography
Hz	Hertz
mer	nucleotides in length
MBS	maleimidobenzoyl-*N*-hydroxysuccinimide ester
MeCN	acetonitrile
MeOH	methanol
mp	melting point
NBT	nitroblue tetrazolium chloride

NHS	*N*-hydroxysuccinimide ester
NMP	*N*-methyl pyrrolidone
NMR	nuclear magnetic resonance
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PC	personal computer
PCR	polymerase chain reaction
PEG	polyethylene glycol
Ph	phenyl
Phe	phenylalanine
PNA	peptide nucleic acid
ppm	parts per million
PS	polystyrene
Piv	pivaloyl
Q	quencher
RNA	ribonucleic acid
RP	reverse phase
SDS	sodium dodecyl sulphate
SSC	trisodium citrate
T	thymidine
TBAF	tetrabutylammonium fluoride
TBS	*t*-butyldimethylsilyl
TCA	trichloroacetic acid
Temed	*N,N,N',N'*,-tetramethylethylenediamine
TET	tetrachlorinated fluorescein
THF	tetrahydrofuran
TG	tentagel
tlc	thin layer chromatography
Tm	melting temperature
Tris	tris(hydroxymethyl)aminomethane
U	uridine

UV	ultraviolet
Vis	visible
Xgal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Acknowledgements

To my supervisor, Prof. Tom Brown, thank you for the ideas, the support and the encouragement over the years, without which the research would not have been possible.

My gratitude also to Nycomed Amersham, not only for their generous financial support of my research, but for the continued advice and inspiration of my two industrial supervisors Dr. Alan Hamilton and Dr. Jon Cummins. The remainder of the project funding was provided by the BBSRC.

A special thanks to Dr. Sarah Allinson and Dr. Richard Brown for their time spent proof-reading this entire document, and to all the past and present members of the Brown group I have had the pleasure of working alongside over the years.

Thank you to Dr. John Langley and Miss Julie Herniman for all their mass spectrometry expertise and assistance and to Mrs Joan Street for providing excellent NMR resources. Thank you to Mr. Jamie Nicol for preparation of the Methyl Red quencher, to Miss Cathy Richards for synthetic assistance, to Dr. Andrew Stuart (Oswel Research Products) for guidance in protein purification and to Dr. Catherine McKeen (Oswel Research Products) for the early experimental investigations.

To my family for their encouragement through the good times and the bad, an especial thanks to you for always being there.

And finally, to Richard for his continued love and support.

Chapter 1

Introduction to DNA and its Detection

1.0 Introduction to DNA and its Detection

1.1 Nucleic Acid Background

1.1.1 Primary Structure

In all organisms there are only two forms of nucleic acid, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) possessing the genetic information of life. Each of these high molecular weight acids is composed of individual monomeric structural units called nucleotides. Nucleotides are phosphate esters of nucleosides and are the building blocks of polymeric DNA. A nucleotide consists of a pentose sugar, a nitrogen-containing heterocyclic base and covalently bound phosphate groups. The bases of DNA and RNA are monocyclic pyrimidines; cytosine (C), thymine (T) and uracil (U) or bicyclic purines; adenine (A) and guanine (G). Nucleosides are assembled by the attachment of one of the heterocyclic bases to the 1' position of a sugar ring forming a glycosidic linkage. In RNA the sugar is ribose whereas in DNA the sugar is 2'-deoxyribose.

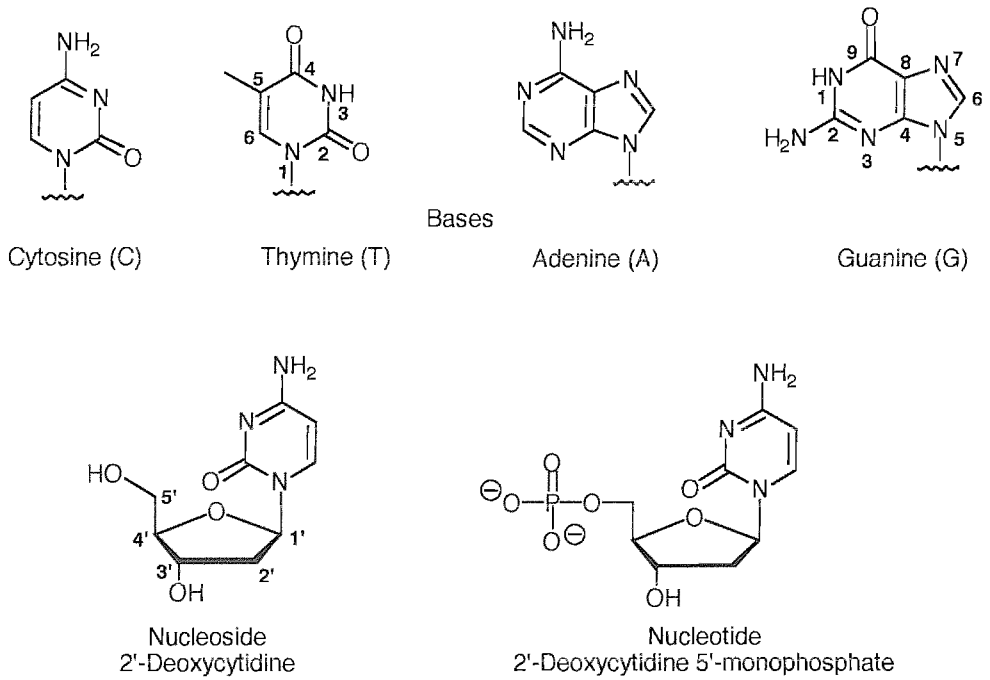


Figure 1.1 – Structure of four heterocyclic bases, a nucleoside and nucleotide

The addition of one or more phosphate groups to the 5', 3' or 2' position of the pentose ring gives rise to nucleoside mono-, di- or triphosphates. In DNA and RNA, deoxyribonucleotides or ribonucleotides respectively are joined into a polymer by the covalent linkage of phosphate groups of the 5' hydroxyl of one ribose and the 3' hydroxyl of the next. This linkage is known as the phosphodiester bond and it is this that gives the nucleic acid direction. At physiological pH each phosphate group is deprotonated (hence the term 'acid') causing nucleic acids to be highly charged molecules.

1.1.2 Secondary Structure

The characteristic secondary structure of DNA is known as the "double helix". James Watson and Francis Crick first deduced the structure of this highly organised duplex in 1953[1,2] making use of the X-ray fibre diffraction data of Rosemary Franklin[3,4] and Maurice Wilkins[5,6] and the information contained in Chargaff's rules (see later). The structure is described as two separate, antiparallel chains of DNA wound around each other following a helical pathway. The coiling produces a double helix that is right-handed; the negatively charged sugar-phosphate chain forms the external backbone.

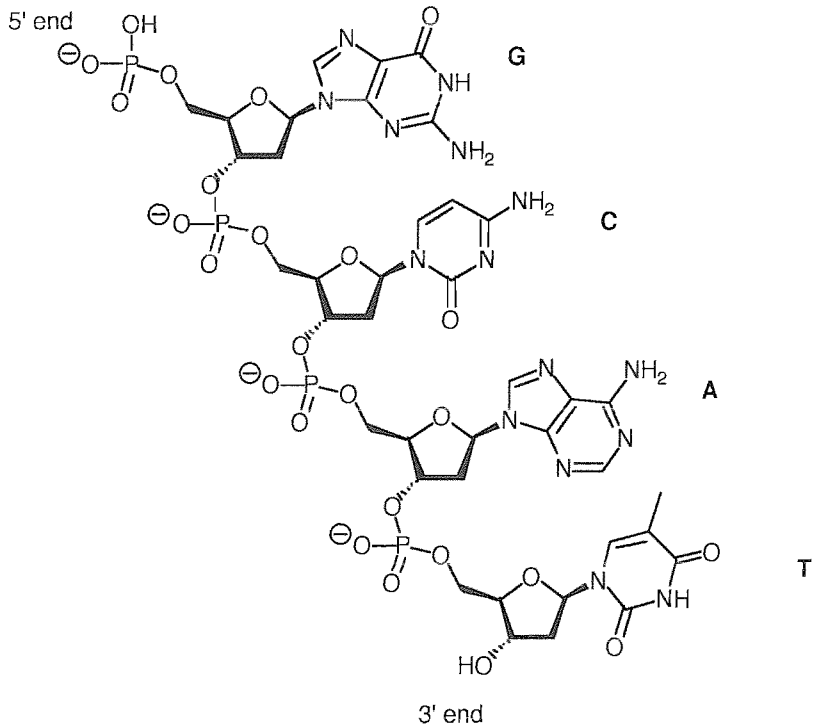


Figure 1.2 – Phosphodiester bonds and covalent structure of a DNA strand

The planar heterocyclic bases form the core of the helix by stacking one above the other providing considerable stability to the helix. This conformation leads to the formation of major and minor grooves that follow the coiled path of the molecule. The two strands are held together by "base-pairs" formed by hydrogen bonds between individual bases on facing strands and around 10 base pairs make up one turn of the helix.

Under the influence of early work by Avery, MacLeod and McCarty[7], Erwin Chargaff in the early 1950's published rules indicating the amount of adenine always equalled the amount of thymine and the same was true for guanine and cytosine in DNA[8]. This feature is explained, as the two strands are complementary in terms of sequence. The base pairs are always purine-pyrimidine pairs, where A only pairs with T and G only pairs with C, therefore the sequence of one strand directly defines the sequence of the other.

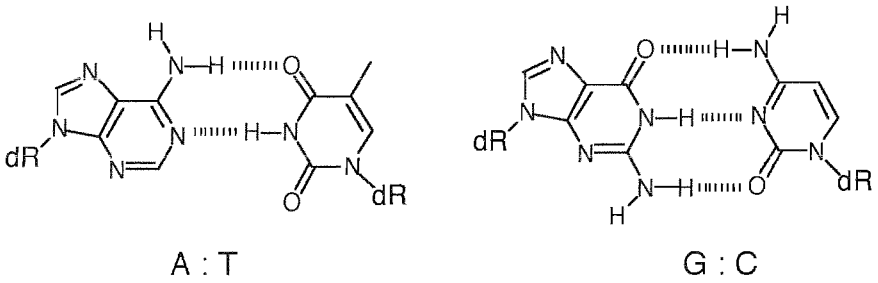


Figure 1.3 – Hydrogen bonding between the DNA base pairs

1.1.3 The Genetic Code [9]

The human genome contains approximately 10^5 genes, encoded by about 3% to 5% of the total 3 x 10^9 base pairs in human DNA. The DNA sequence is found on 23 chromosomes; 22 pairs of autochromosomes and 1 pair of sex chromosomes. A gene is simply a segment of a DNA molecule that codes for the synthesis of a protein, and in eukaryotes is divided into coding regions (exons) and non-coding regions (introns). Each gene can consist of up to 2 million base pairs.

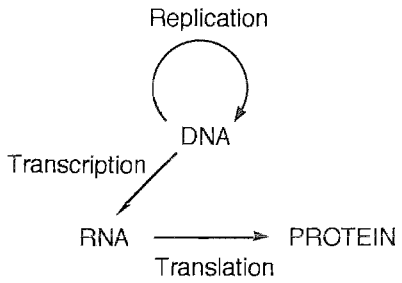


Figure 1.4 – Central dogma of molecular biology

The central dogma of molecular biology describes the flow of the genetic information from DNA through RNA to a final protein product. The sequence in which amino acids in a protein are joined together during protein synthesis is ultimately determined by the sequence of nucleotide bases in a gene encoding that protein. Each amino acid is coded by a codon (groups of three nucleotides). There are 64 non-overlapping codons which make up the "genetic code", 61 coding for amino acids and 3 stop codons. As there are twenty amino acids, the code is degenerate, some amino acids being coded by more than one triplet. The code is universal and the basis of the heredity information of nucleic acids.

In order for the sequence of bases to be deciphered during protein synthesis the double-stranded DNA is transcribed into single-stranded mRNA. The information carried by the mRNA is then used as a template for the synthesis of protein molecules during translation in which other RNA molecules, principally tRNA and rRNA, act as key intermediates[10,11].

1.2 Detecting DNA

The fundamentals of using DNA diagnostically involves: (i) defining the nucleic acid sequences that are essential in the expression of proteins associated with disease; (ii) determination of the presence of these sequences in an individual. Nucleic acid hybridisation is the most powerful method for revealing and quantifying specific DNA or RNA. The association of complementary strands can be exploited in the detection of nucleic acids. A probe is a short piece of DNA or RNA that is labelled to allow its detection, subsequent to hybridisation. A suitable probe system should be highly specific, have low detection limits and be simple to prepare and purify[12-15].

1.2.1 DNA Diagnostics

Applications of routine nucleic acid screening depend upon the development of sensitive, rapid, accurate and economical procedures, where ideally PCR amplification is used in conjunction with appropriate probe technology.

Single nucleotide substitutions and insertions and deletion mutations can lead to mutant alleles or gene variants that are associated with genetic disease e.g. sickle cell anaemia[16], cystic fibrosis, phenylketonuria[17] and Huntington's disease. These defects can be screened for directly or by probing for linked genetic markers coinherited with the mutant gene[18]. Analysis is important in pre-natal diagnosis and in genetic counselling in affected families for subsequent pregnancies. Genetic information can be also be used to determine susceptibility in individuals to exogenous risks such as diet or environmental factors.

Infectious disease e.g. measles, rubella, HIV[19] Hepatitis A and B, salmonella and candida can be clinically diagnosed by the detection of pathogenic organisms by means of DNA probes. Selection of DNA sequence allows tailoring of a test to a particular disease, for example HIV screening of donated blood, or for differentiation between closely related organisms e.g. Herpes Simplex Virus types 1 and 2. Genes related to resistance to antibiotics for treatment of disease can also be screened.

Applications in forensic science based upon the identification of individuals at a genetic level are more accurate than traditional identification by blood types, fingerprints or physical characteristics. DNA can be extracted from body fluids and be used to deduce if individuals are related in paternity testing, or for compatibility in bone marrow transplants. Criminal investigation employs the technique of DNA fingerprinting which exploits the highly variable microsatellite sequences of the human genome to yield a "bar code" of an individual[20].

1.2.2 Classical Methods

The first DNA probes to be implemented were labelled radioactively. The labels are generally radioactive deoxyribonucleoside triphosphates incorporated directly into nucleic acid molecules by enzymatic methods. These methods employ include nick translation[21], random priming[22,23], polymerase chain reaction[24,25] and the 3' end modification by terminal transferase[26,27].

Labelling at the 5'end is carried out by phosphorylation using γ^{32}P-ATP and T4 polynucleotide kinase. The choice of labelling strategy is determined by the nature of the probe, DNA or RNA, double-stranded or single-stranded. The choice of isotope depends on the nature of the probe, the method of incorporation and the sensitivity and stability required. Isotopes such as ^{3}H, ^{125}I and ^{14}C are often used to label nucleoside triphosphates. The phosphodiester bond can be modified to contain ^{32}P or ^{35}S in the case of phosphothioates[28,29].

Radioisotope	^{32}P	^{125}I	^{35}S	^{3}H	^{14}C
Half-Life	14.3 days	60.2 days	87.9 days	12.3 years	5730 years

Figure 1.5 – The half-lives of commonly employed radioisotopes

An early example of the use of a radiolabelled DNA probe was described by Southern in 1975[28]. The technique he describes, known as Southern Blotting, allows the mapping of DNA fragments relative to restriction endonuclease sites. DNA is cut up into fragments by restriction enzymes; the fragments are separated electrophoretically and immobilised on to a nitro-cellulose membrane by blotting. ^{32}P labelled probes specific to sites on the target nucleic acid sequence are introduced and after stringent washing, any bands containing complementary sequences can be visualised by autoradiography.

Quantification of the amount of a specific DNA or RNA present in a sample can be deduced by a similar technique known as dot or slot blotting[30]. Here the nucleic acid is hybridised to a membrane as a dot (or slot) carefully washed, and then the intensity of

probe concentration remaining on the membrane is measured. The reading directly represents the amount of target sequence present in the sample.

Radiolabelling has the advantages of very high sensitivity. For example ^{125}I has a lower detection limit of 10amol, and radiolabels are easily incorporated. However, there are numerous conflicting disadvantages associated with this technique:

- hazardous handing; regulated by the Home Office
- short half-life; short shelf-life
- expensive
- limited signal emissions
- time consuming methodology
- waste disposal

1.2.3 Non-isotopic Labelling

In order to overcome the disadvantages associated with radioactive labels the search began for safe and convenient alternatives. The most common methods to emerge employed colourimetric[31,32], chemiluminescent[33], bioluminescent[34] or fluorescent[35,36] reporter groups. The labels allow the incorporation of more than one label molecule per oligonucleotide[37] and also permit the simultaneous detection of several DNA targets within one experiment. This has also enabled the development of automated DNA sequence analysis as a different coloured fluorophore can be used for each reaction specific for each of the four bases[38]. Combinatorial labelling of probes has been used to increase the number of target sequences that can be detected at the same time[39].

A label is a molecule that can be attached to a protein or nucleic acid and is capable of releasing a signal. Ideally a label for a DNA probe would possess the following properties:

- attached to DNA or RNA under mild conditions with a simple, cheap and reproducible protocol
- attachment should not significantly alter the signal generating properties of the label or effect hybridisation properties of oligonucleotide

- detectable at low concentrations using simple instrumentation
- produce a hybridisation signal which is distinguishable from that of an unreacted signal
- stable to hybridisation conditions e.g. elevated temperatures, detergents and solvents[40]
- the corresponding reagent is highly specific for its target
- chemically cheap and variable to permit synthetic modification of functionality (changes to physical properties such as solubility, emission characteristics and charge)
- allow simultaneous detection of several labels in one experiment
- multiple addition of the label to a single system
- stable to long term storage
- easily disposed of

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

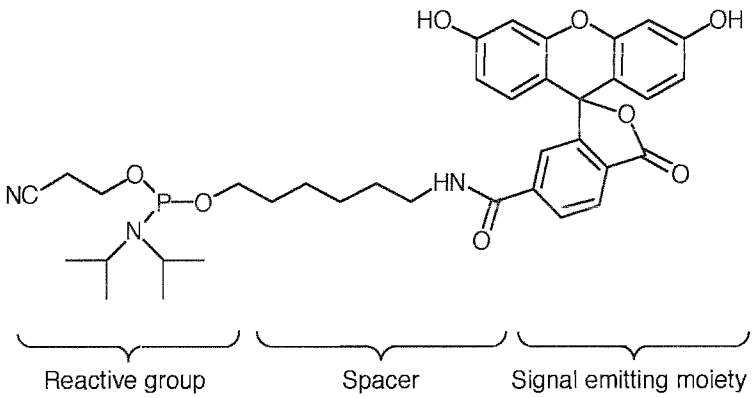


Figure 1.6 – Components of a label

1.2.3.1 The Signal Emitting Moiety

In direct labelling strategies this is generally a luminescent label, but can be an enzyme for example alkaline phosphatase directly conjugated to an oligonucleotide probe[41]. The label molecule has to either exhibit a satisfactory quantum yield in aqueous solution or turnover a signal-generating substrate. Indirect methods employ either enzymes or haptens (small foreign molecules that can be attached to a macromolecule to which antibodies can be raised) as the label, and the signal is generated sequentially. Enzymes include horseradish peroxidase conjugated to an antibody[42] and a common hapten system is based on the highly specific binding between biotin and strepavidin[43].

1.2.3.2 The Spacer

Many simple aliphatic and aromatic molecules have been used to separate the luminescent moiety from the labelled substance[44]. An important prerequisite of the spacer is that it does not interfere with the luminescent properties of the label. This bridging group can be used to change the hydrophobicity or hydrophilicty of the molecule, and alter the flexibility of the label relative to the labelled substance thereby assisting hybridisation.

1.2.3.3 The Reactive Group

In general terms, this can be any group capable of reacting with a nucleic acid, under mild conditions, to form a covalent linkage. There are a few examples of non-covalent labelling of nucleic acids such as the intercalation of ethidium bromide[45] in double-stranded DNA, however covalent coupling has proved to be more popular and versatile. Examples of some significant labelling reactions are described:

(i) Free amino groups on DNA or nucleosides can couple with N-hydroxysuccinimide esters, isothiocyanates or activated carboxyl groups of luminescent dyes yielding stable thiourea or amide bonds. The amino groups are either those present on the heterocyclic bases or generated by the derivatisation of 5' or 3' end of DNA. These reactions are usually mild and can be carried out in aqueous solution (a and b, figure 1.7)[46-50].

(ii) The nucleic acid is synthesised with a free thiol at one terminus. This undergoes Michael addition with α,β-unsaturated ketones attached to the luminescent label (c, figure 1.7)[51].

(iii) Labels are functionalised with azido groups which upon activation form highly reactive nitrene intermediates that react with many different types of chemical bonds in the nucleic acids. However this results in non-specific labelling of the nucleic acid[52,53].

(iv) Labelling of nucleoside triphosphates by the reaction of activated sites on the label and free amino groups attached to the purine or pyrimidine bases at convenient sites. The triphosphates are enzymatically incorporated into the DNA probe by methods such as nick translation or PCR (d, figure 1.7)[54].

(v) Labels are synthesised as phosphoramidites that can be incorporated into DNA during solid phase oligonucleotide synthesis. One or more of these monomers can be placed internally or at the 3' or 5' terminus of the oligonucleotide (e, figure 1.7)[32,35,55]

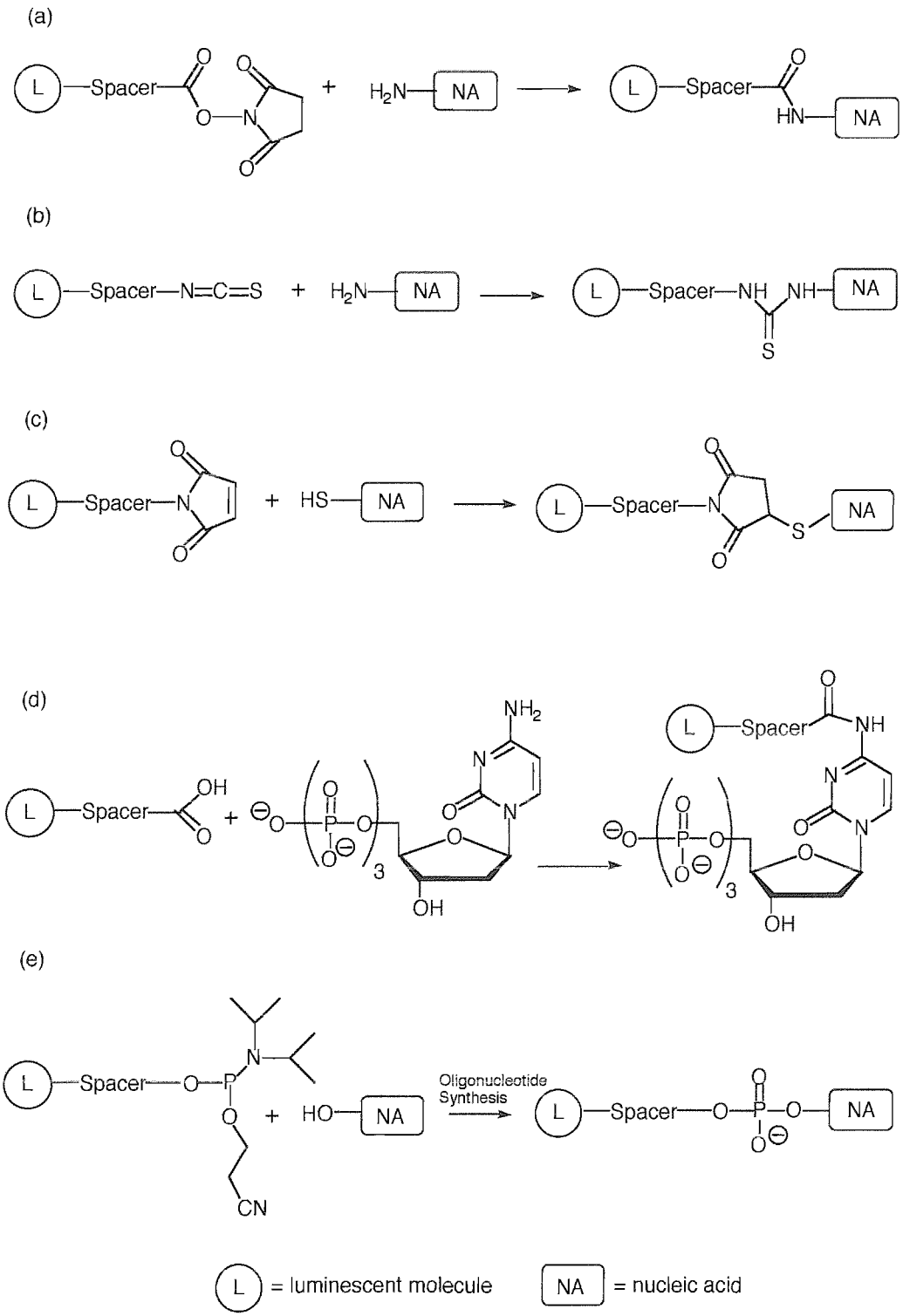


Figure 1.7- Popular methods of label incorporation into nucleic acids

1.3 Labelling Strategies

1.3.1 Indirect Labelling

Indirect labelling requires a DNA probe labelled with a small molecule that is detectable by a highly specific binding protein. A classic case is the incorporation of a hapten into a probe that is recognised by an antibody. The antibody is either covalently linked to a luminescent molecule or conjugated to an enzyme (alkaline phosphatase, horseradish peroxidase). The antibody will bind tightly to the hapten and its presence is detected directly, or in the case of an enzyme case by the turnover of a substrate that results in a colourimetric, chemiluminescent or fluorescent product. A more complicated, yet more sensitive method involves the binding of a primary antibody to the hapten of the probe, followed by binding of a secondary antibody that recognises the first antibody. In this case it is the secondary antibody which is labelled for detection. Antibodies that recognise specific haptens are produced in mammals by exposure to the hapten coupled to a protein carrier for immunisation.

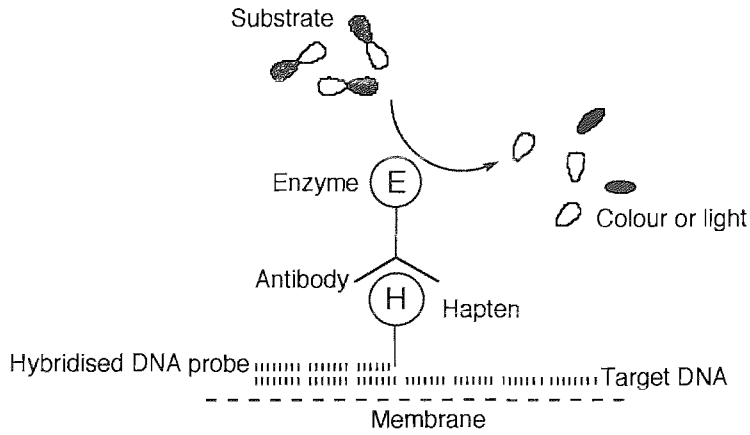


Figure 1.8 – Indirect labelling strategy

The most commonly studied hapten is biotin[56]. Linking to DNA occurs by the reaction of reagents such as *N*-biotinyl-6-aminocaproic acid *N*-hydroxysuccinimide ester or biotin hydrazide[57] (figure 1.9) with an amino group on DNA, or by enzymatic incorporation of biotinylated nucleotides. Reporter groups are attached to strepavidin and avidin, proteins (not antibodies) that bind very tightly to biotin, and hybridisation can be detected. Biotin

has the disadvantage that it is present at high levels in certain tissues, leading to high background signals. However, amplification of signal occurs in the presence of extra biotin as there are four possible binding sites on avidin only one of which is occupied by the biotin attached to the probe. Other widely applied indirect detection systems include digoxigenin (DIG) in combination with anti-DIG antibodies, and dinitrophenyl group (DNP) with anti-DNP antibodies[58,59].

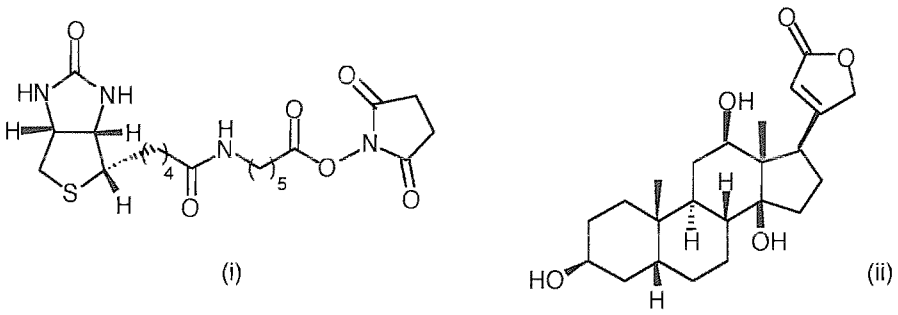


Figure 1.9 - i, *N*-biotinyl-6-aminocaproic acid *N*-hydroxysuccinimide ester, ii, digoxigenin

1.3.2 Direct Labelling

Direct labelling occurs when a reporter molecule is bound immediately to a nucleic acid probe. The specific molecular recognition of a nucleic acid probe annealing to its complementary target sequence enables rapid direct detection[60]. The label must not interfere with the hybridisation process and must be stable to hybridisation conditions. The nucleic acid probe can be a long polynucleotide or a short synthetic strand. Synthetic probes are advantageous as they can be easily prepared in large quantities, and the probe length can be used to control the melting temperature of the probe-target duplex. Alteration of the hybridisation temperature can be used to favour formation of the desired duplex over mismatch hybrids, and can allow the assay to proceed at lower temperatures and with shorter annealing times.

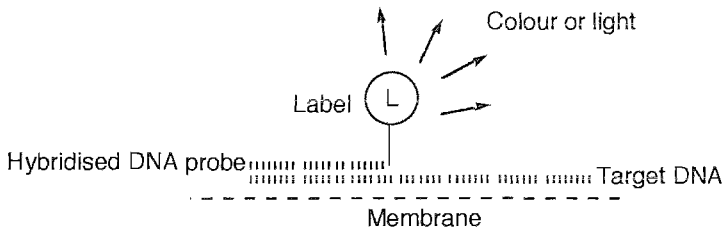


Figure 1.10 – Direct labelling strategy

1.3.2.1 Radioactive Labels

These are commonly employed and exhibit high signal sensitivities especially when the probe is multiply labelled. However radiolabels are associated with numerous disadvantages (section 1.2.2).

1.3.2.2 Enzyme Labels

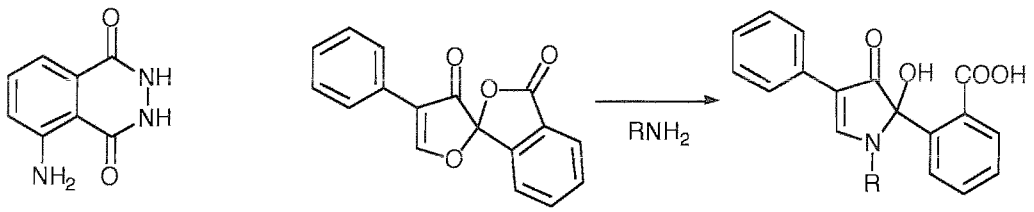
Enzymes are covalently attached to nucleic acids. The enzymes can be detected by the turnover of a substrate to produce colour or light. Typical systems use alkaline phosphatase[41,61] with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) or horseradish peroxidase[62] with diaminobenzimide tetrahydrochloride (DAB). The conditions of the assay must not cause the enzyme to be denatured; therefore the activity of the enzyme must be monitored. These labels offer the highest sensitivities with low background signals and visualisation is rapid.

1.3.2.3 Chemiluminescent Labels

Chemilumescence is the emission of light as a result of a chemical reaction. The enthalpy of the reaction causes an atom to be promoted to a vibronically excited state, when it decays a photon is emitted.

$$C + \text{Reactant} \xrightarrow[\text{Catalyst}]{} Pr^* \longrightarrow Pr + h\nu$$

The emitter is chemically different from the original molecule therefore one molecule can only produce one photon. However in some cases the product is capable of fluorescing but may only be transiently stable. Chemiluminescent molecules have been used to detect nucleic acids, for example luminol is a chemiluminescent substrate for horseradish peroxidase, and has been used in conjugation with a biotin-strepavidin system[63]. Fluorescamine is intrinsically non-fluorescent but reacts in milliseconds with primary aliphatic amines to yield a fluorescent derivative (figure 1.11).



Luminol Fluorescamine

Figure 1. 11 – Chemiluminescent molecules

1.3.2.4 Fluorescent Labels

Fluorescent molecules absorb light of a specific wavelength and emit light of lower energy and longer wavelength. Attachment of these molecules to nucleic acids permits direct detection if monitored at the correct emission wavelength. In principle, fluorescence measurements offer extremely high sensitivities, however in practice the sensitivity can be limited by light scattering, background fluorescence and quenching effects. Many fluorescent dyes are commercially available and their excitation and emission wavelengths are represented in figure 1.12

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

The most commonly employed fluorescent moieties include 6-carboxyfluorescein (FAM)[35,60,64,65], its tetra and hexachlorinated analogues (TET and HEX) and carboxy-X-rhodamine (ROX)[66]. These have proved popular due to their high absorption and emission wavelengths and the range of dyes will allow the detection of several targets within one experiment[67]. On the negative side, the broad nature of their emission peaks complicates the simultaneous detection of multiple sequences. They are also sensitive to pH[68] and susceptible to photobleaching. The inadequacies of these dyes has led to the development of alternatives such as the CyDyes™ and the BODIPY™ spectral range dyes.

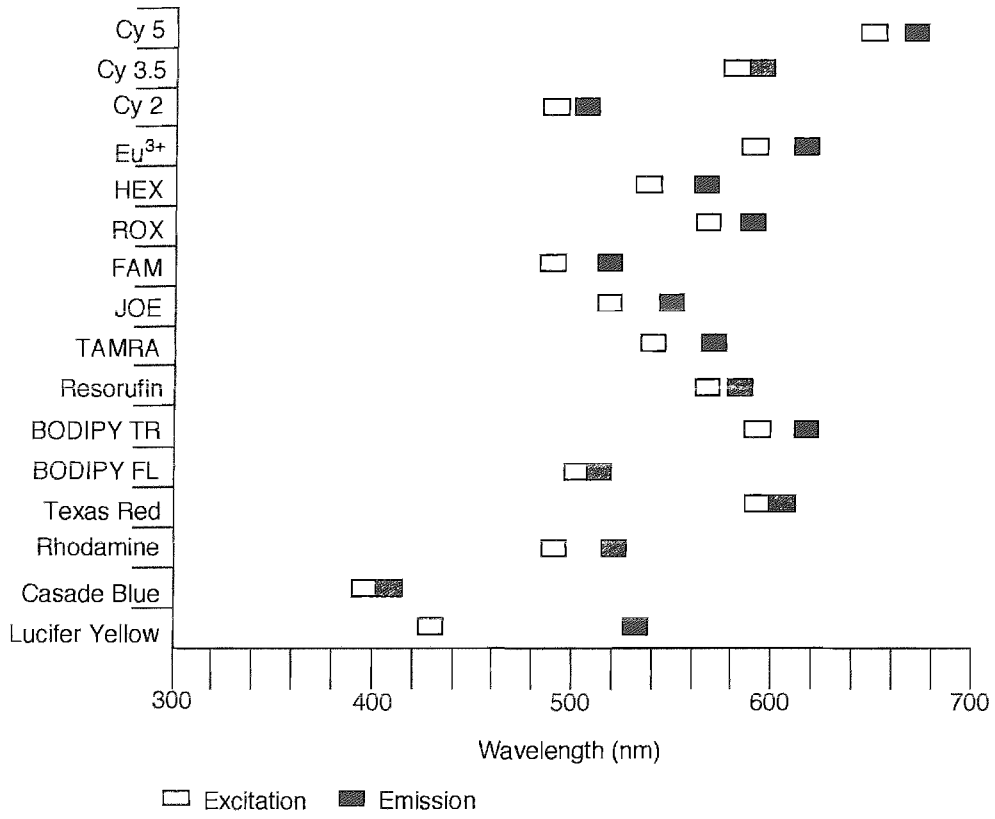
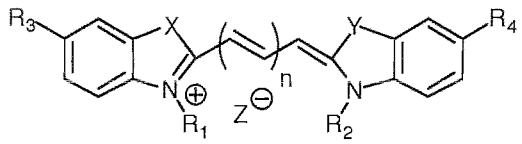


Figure 1.12 – Excitation and emission maxima of a range of fluorescent dyes

Cyanine Dyes (CyDye™)

These covalent labelling agents, introduced by Amersham Life Science[69], emit in the far-red region of the visible spectrum and have the general structure shown in figure 1.13. These dyes have many of the properties of ideal fluorescent probes including high extinction coefficients, high quantum yields, and excellent photostability. The spectral properties (excitation and emission maxima 500-750nm) are selected by the appropriate choice of heterocyclic nuclei (X and Y) and the length of the polymethine chain (n). The groups R_1 - R_4 are variable, providing the desired functionality, charge, reactivity and solubility. Cyanine dyes are popular, as they are commercially available and pH insensitive. They can be used to detect proteins[70,71], antibodies, peptides and nucleic acids[72,73].



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

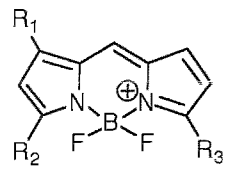
n = 1,2,3,.....

R_1 - R_4 = Substituent

Figure 1.13 – Generic structure of a cyanine dye

BODIPY™ dyes

These versatile dyes which have been patented by Molecular Probes Inc. span the visible spectrum by alteration of the substituents R_1 and R_2 which can either increase or decrease the level of conjugation[74]. The substituents can be used to alter the solubility and the dyes are pH insensitive (figure 1.14). The improved spectral characteristics of these dyes have been exploited in automated DNA sequencing. It is reported that the improved sensitivity allows a reduction in reagent consumption by 33%, compared to conventional dye primers[75].



R_1-R_2 = Substituent

R_3 = Linker

Figure 1.14 – Generic structure of a BODIPY dye

1.3.2.5 Lanthanide Labels

Lanthanides form chelates that are highly fluorescent with large Stokes shifts and extremely long lifetimes[76-78]. DNA has been directly labelled with lanthanide chelates by incorporation of labelled deoxynucleoside triphosphates by nick translation, random priming, PCR and ruthenium phosphoramidites. Hurskainen *et al.* describe a method using the amino groups on cytidine, which undergo a transamination reaction in the presence of sodium bisulfite diamine. The free primary aliphatic amine groups react with an

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

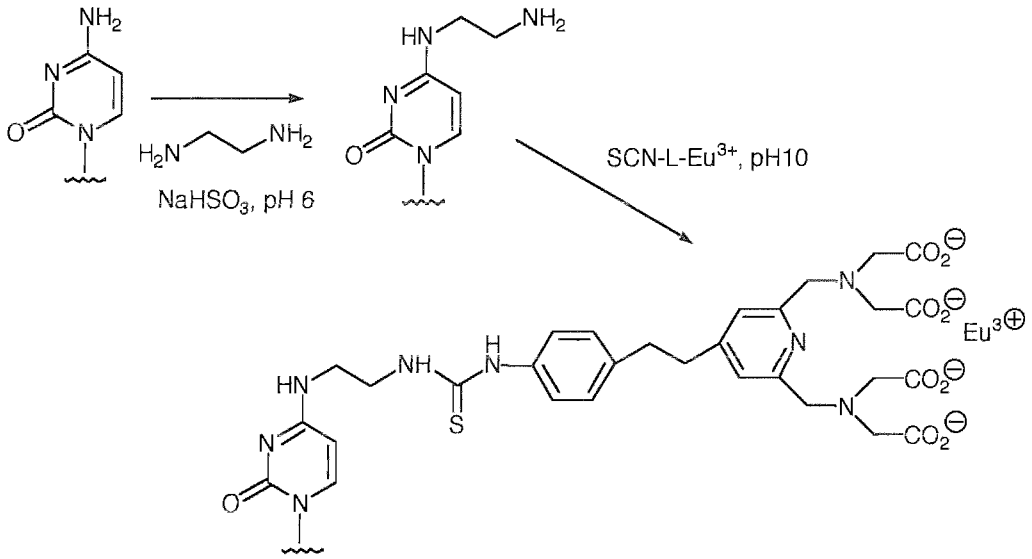


Figure 1.15 – Labelling of cytosine with a europium chelate

1.4 Fundamentals of Fluorescence

1.4.1 The Nature of Fluorescence[79,80]

Fluorescence is the property of some atoms and molecules to absorb photons of incident light of a particular wavelength (excitation wavelength) and after a short period (fluorescence lifetime) re-emit a lower energy photon (emission wavelength). The process begins when a fluorophore absorbs a photon from an external light source, which causes the promotion of an electron from its ground state (S_0) to the first excited singlet state (S_1). The excited molecule then undergoes a conformational change and is subject to collisions with surrounding molecules. These interactions cause discharge of thermal energy from the molecule to its environment (internal conversion). After a short fluorescence lifetime the electron falls back to the ground state and a photon of lower energy, longer wavelength is emitted (fluorescence). Each fluorophore can repeat the cyclic process many times before

photobleaching prevails. The 'shift' in energy or wavelength from excitation to emission is called the Stokes shift and this is fundamental to the sensitivity of fluorescence measurements, a large Stokes shift leads to low background signals.

The fluorescence quantum yield is the ratio of the number of fluorescence photons emitted to the number of photons absorbed. It is a measure of the emission efficiency of a fluorophore. Physical measurements can be complicated by background signals that may originate from endogenous sample constituents.

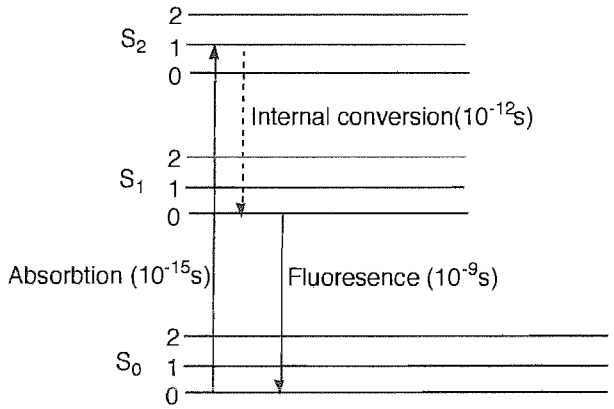


Figure 1.16 – Jablonski energy level diagram

1.4.2 Fluorescence Quenching[81,82]

The fluorescence energy of an excited fluorophore can be transferred non-radiatively to a molecule in close proximity resulting in the loss of fluorescence emission. No photons are emitted and the phenomenon is known as quenching. There are two main quenching processes both requiring molecular contact between fluorophore and quencher:
(i) Collisional (or dynamic); quencher molecules collide with the excited fluorophore and energy is dissipated as heat, significantly reducing the fluorescence lifetime of the fluorophore. Collisional quenchers include molecular oxygen, acrylamide, nitromethane, purines, pyrimidines and some olefins.
(ii) Static (or complex); a complex is formed between the fluorophore and quencher which is non-fluorescent. Alternatively resonance energy transfer (section 1.4.3) quenches the fluorophore.

Collisional quenching only affects the excited states of the fluorophore and therefore no changes in the absorption spectra are observed. In contrast, ground state complex formation will result in perturbation of the absorption spectrum of the fluorophore.

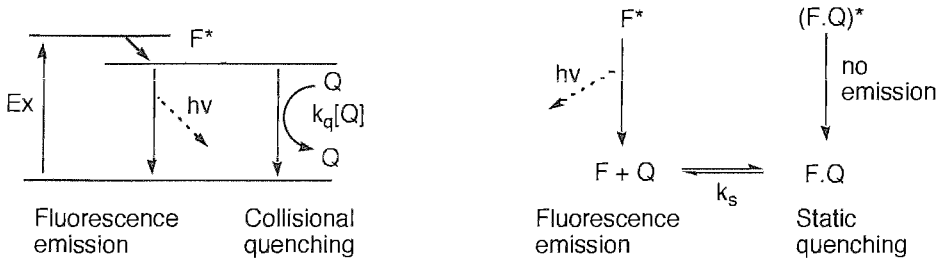


Figure 1.17 – Principles of collisional and static quenching

1.4.3 Fluorescence Resonance Energy Transfer (FRET)

An excited fluorophore (donor, D) may transfer its energy to a neighbouring chromophore or fluorophore (acceptor A) non-radiatively through induced dipole-induced dipole interactions. This occurs when the dipoles are in an approximately parallel orientation. The criterion for occurrence is the overlap of the emission spectra of the donor and the excitation spectrum of the acceptor. Förster in the late 1940's first proposed a theory[83], which was later confirmed by Stryer and co-workers[84], describing long-range molecular interactions by resonance energy transfer. His theory related the interchromophore distance (r) and the spectroscopic properties of the chromophore. The rate of energy transfer between the donor and the acceptor is inversely proportional to the sixth power of the distance ($1/r^6$). The optimum distance (r) for non-radiative transfer of energy is between 10-100Å and it is this distance that governs the efficiency of the process. The extent of energy transfer can be measured because the fluorescence of the donor (both intensity and lifetime) is significantly reduced, and the acceptor, if it is fluorescent, increases in emission (sensitised emission).

FRET has been used in many biochemical and structural biological applications as a qualitative or quantitative tool. The subject has been extensively reviewed with references to applications for peptide and protein interactions and the use of FRET as a spectroscopic

ruler[84-87]. FRET is an extremely powerful tool for probing nucleic acid structure[88], sequence[89] and hybridisation[90]. Examples include examining triple helical DNA[91] and the structure of a four-way DNA junction at varying salt concentrations[92].

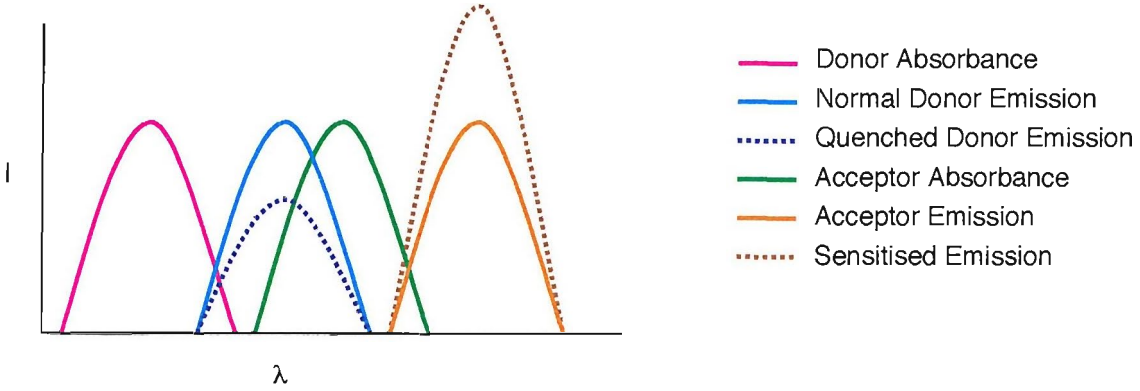


Figure 1.18 – Illustration of quenched donor emission and sensitised acceptor emission

1.4.4 Time Resolved Fluorescence[93,94]

Fluorescent measurements are plagued by background signals arising from Raman and Rayleigh scattering or the emission of organic fluorophores within biological samples (350-600nm). This can limit the sensitivities of the commonly employed short-lived fluorescent dyes (10^{-9} to 10^{-10}s). However, the use of fluorophores with long fluorescent lifetimes permits selective detection of signals, by delaying measurements until all other species have decayed. For an interference-free measurement of a signal, the label lifetime must be at least ten times longer (>100ns) than the decay of background signals (≈10ns).
It was the advent of rare-earth metal ion complexes used as labels that transformed the technology of time-resolved fluorescence. The chelates of Eu (III)[77], Tb (III)[78], Sm (III) and Ru (III) with aromatic chromophores, all have long fluorescent lifetimes (1μs to 1ms), large Stokes shifts (>200nm), narrow emission bandwidths (10nm) and long emission wavelengths.

Luminescence of these chelates is not strictly fluorescence or phosphorescence. The organic ligand absorbs energy and is promoted from S_0 to the excited S_1 state, where it rapidly loses energy (intersystem crossing) and falls to the excited T_1 state. From this level

the molecule can either return to S_0 ground state (phosphorescence) or transfer energy intramolecularly to the 4f energy level of the central metal ion. If this phenomenon occurs the metal ion moves into its own S_1 state and can decay to S_0 emitting radiation (ion fluorescence). When a sample is excited by short light pulses short-lived fluorophores will quickly dissipate to zero. Time-resolved fluorescent measurements are taken after this period eliminating unwanted background signals.

DELFIA® (dissociation enhanced lanthanide fluorescence immunoassay, Wallac Oy, Turku, Finland) is a commercially available assay format that uses a non-luminescent lanthanide chelate (e.g. diazaphenyl-ethylenediamine tetraacetic acid-Eu (III)) as a label for an antibody or nucleic acid. The final step of the assay involves lowering the pH to induce dissociation of the lanthanide complex and capture of the lanthanide in a solubilising micelle. The micelle contains reagents capable of forming a luminescent chelate with the ion (e.g. fluorinated aromatic β-diketones) enabling detection[95].

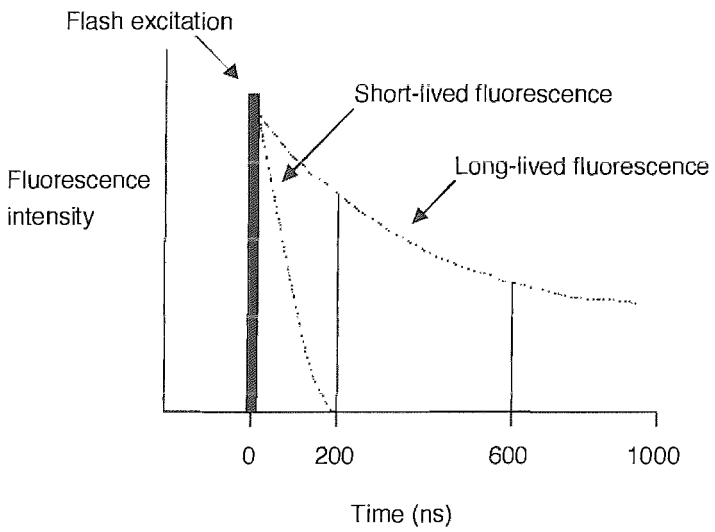


Figure 1.19 – Principle of time-resolved fluorescence

Enzymes as either labels or analytes can be detected by the turnover of a substrate to release a product that can chelate with lanthanide ion and form a luminescent complex. An example of this is the glucose oxidase substrate 1,10 phenanthroline-2,9-dicarboxylic acid dihydrazide that gives the detectable chelate Eu (III)-phenanthroline-dicarboxylic acid[96].

1.4.5 The Fluorescence Spectrometer

The basic task performed by a fluorimeter is delivery of excitation energy to the fluorescencing species, and separation of the weaker emitted light from the brighter excitation light[97]. This ensures only emitted light is detected and a sensitive and defined image is generated. Most modern spectrometers consist of four main elements; an excitation source, wavelength selection devices, the sample cell, and a detector. The basic construction can be seen in figure 1.20. Light emerges from the source (e.g. xenon or mercury lamps) and enters the excitation monochromator. In modern spectrometers, monochromators are based on diffraction gratings, not prisms, allowing the selection of a narrow band of light by rotation of the concave grating. Typical monochromators have entrance and exit slits, which are variable. Large slit widths produce increased signal levels but with higher signal: noise ratios, smaller slit widths give higher resolution but at the expense of light intensity. The chosen wavelength of light travels to the sample, the emitted light generated then passes through the emission monochromator to a photomultiplier. Here, incident photons hitting the photocathode are accelerated through a series of dynodes towards an anode, this causes the signal to be amplified and the final output is proportional to the emitted light intensity (figure 1.20).

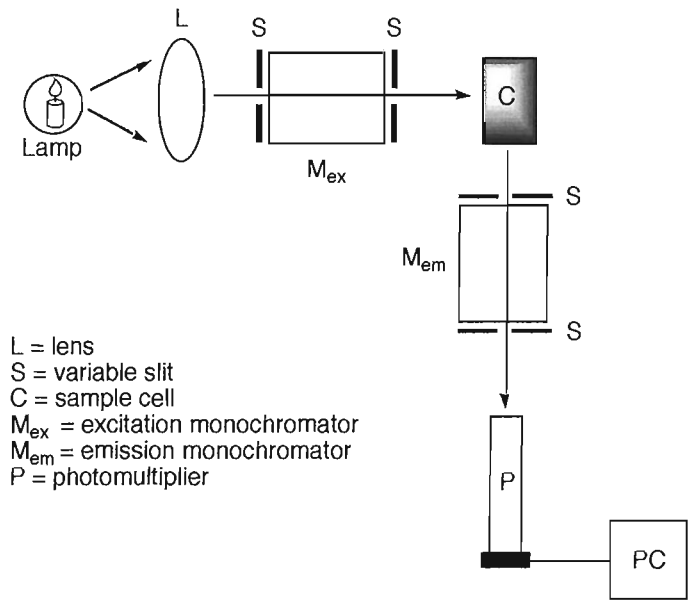


Figure 1.20 – Typical set-up of a fluorescence spectrometer

To measure the excitation spectrum of a fluorophore, the emission monochromator is set at a desired wavelength (usually the emission maximum) and the excitation monochromator is scanned through the absorption bands. To measure the emission spectrum the appropriate excitation wavelength is chosen and wavelength scanned with the emission monochromator to determine the distribution of light emitted by the sample.

Fluorescence spectroscopy is a highly sensitive technique; signals can originate from sources other than those of the fluorophore of interest. Interference can arise due to Rayleigh and Raman scattering, background fluorescence of solvents, light leaks in the instrumentation or stray scatter due to particulates. Acquisition of spectra is complicated by the wavelength-dependent nature of the source, monochromators and photomultipler tubes all playing an important part in instrument design[98].

1.5 Molecular Biological Methods Utilised in Fluorescence Assay

1.5.1 Polymerase Chain Reaction

The polymerase chain reaction (PCR) is a technique used widely in molecular biology[99] to amplify a section of target DNA that is flanked by two known genetic sequences. Two short oligonucleotides are prepared (usually synthetically) and are designed such that each is complementary to one end of the target strand. These oligonucleotides are known as primers, and are typically 18-30 bases in length, with similar (%G+C) content to ensure similar annealing temperatures. The region of the template bound by the primers is amplified by a series of cycles. In the first cycle the duplex target is dissociated into two single stands by heating to 95°C, subsequent cooling to approximately 55°C allows the primers to anneal with their 3' ends pointing towards each other. The temperature is now increased to 72°C, the optimum temperature for activity of the thermostable *Taq* polymerase. This polymerase is isolated from the thermophilic bacterium *Thermus aquaticus* and has a half-life over two hours at 95°C so is suited well to the experiment[100]. The polymerase, in the presence of Mg^{2+}, uses nucleotide triphosphates to extend the primers along the length of the target producing two new double-stranded sections of DNA.

The second cycle begins again by heating to 95°C effecting the denaturation of the newly synthesised molecules. Each single strand now acts as a template for primer annealing and extension. During the second cycle primers annealing to newly synthesised molecules can only be extended as far as the first primer, affording molecules of the correct length. All of the following cycles amplify the correct length products, which soon outnumber the original target molecules. In practice n cycles will amplify a particular sequence 2^n times[24,101].

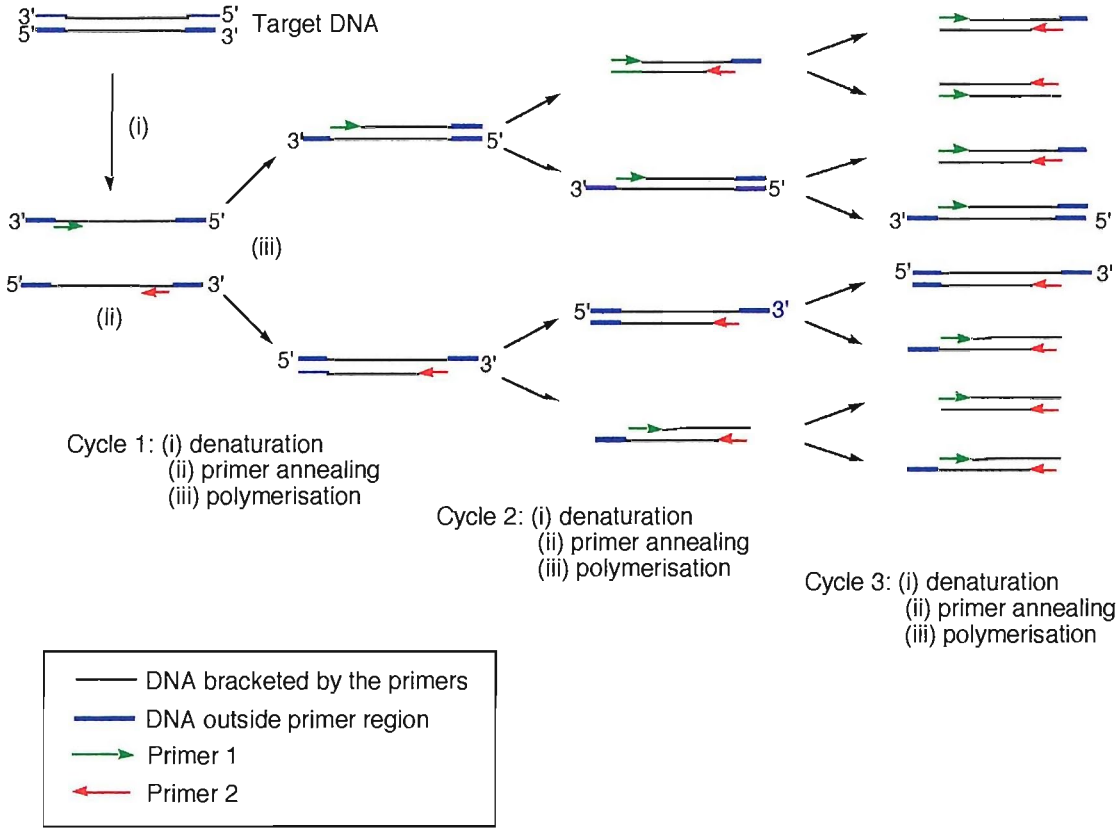


Figure 1.21 – The first three cycles of a polymerase chain reaction

1.5.1.1 Hot Start PCR

Hot start PCR is designed to alleviate some of the problems associated with background signals in PCR such as primer-dimer formation and non-specific priming. This technique is carried out manually. Initially the reaction mixture lacks a crucial component such as magnesium ions or Taq polymerase. Heating during the first cycle ensures there is no binding, at this point the tube can be opened and the missing component added. PCR now

continues as normal. The major disadvantage of this technique is the possibility of contamination during the addition of the missing component to the open reaction tube. Alternatives include adding a reagent that can bind to the polymerase and inhibit its action (e.g. an antibody) which is denatured in the first heating cycle[102], or enclosing an essential component in wax, again released upon heating.

1.5.2 Fluorescence *In Situ* Hybridisation (FISH)[103]

Fluorescence *in situ* hybridisation is an essential tool in genetic analysis as it allows the identification of the presence and location of cellular DNA or RNA within morphological preserved chromosome preparations with sensitivity and ease. The principle lies in the annealing of a labelled probe to its complementary strand in fixed cells or tissues followed by detection of the label (radioactive or luminescent). FISH is different to other probe systems as the target is embedded in a complex matrix that can hinder probe access and destabilise probe: target hybrids formed. The probe (DNA or RNA) is usually prepared by one of four methods: nick-translation; random priming; PCR or chemical synthesis and is labelled during each process. The length of the probe is dependent on the application, but the typical length is between 200 bp and 1kb. Longer probes increase non-specific background but short probes can be difficult to detect due to insufficient hybridisation, high probe concentrations decrease the signal: noise ratio. It is important that the target is accessible to the probe and must be retained *in situ*, not degraded by nucleases. Visualisation limits span from an entire chromosome to a 40kb chromosomal section. FISH employs both indirect and direct labelling strategies, however methods generating signal amplification have proved most popular.

FISH has been used in toxicological studies to monitor the effect of exposure to radiation on chromosomal aberrations (structural and numerical)[104]. Chromosome painting[105,106] by combinatorial or ratio labelling of specific probes has led to the painting of all twenty four different human chromosomes being labelled with distinct colours which can provide a general screening test for chromosome abnormalities. Other applications include monitoring changes in aneuploidy in sperm (a major cause of birth defects) and genetic mapping by the use of probes labelled with multiple colours allows gene order along a

chromosome to be determined[107]. Complex and unusual structural rearrangements in genes that lead to tumours can be assessed by comparative genomic hybridisation (CGH). Two probes are generated one specific for normal DNA and the other for tumour DNA, each are labelled differently. Equimolar amounts of the probes are introduced into the test cells; the nature of the signal is a measure of the extent of hybridisation of one probe over another[108].

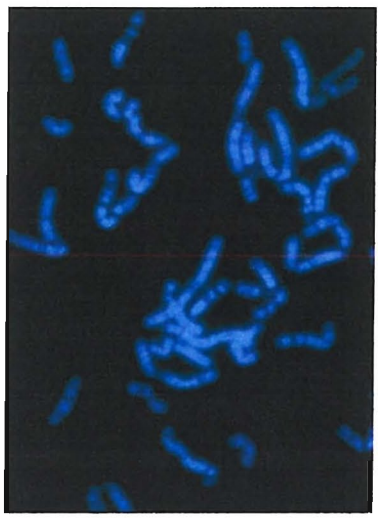


Figure 1.22 - Human metaphase chromosomes stained with DAPI (Image from Violette Paragas and Jeff Pollack, Molecular Probes, Inc.)

1.5.3 UV Thermal Melting

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

Heating double stranded nucleic acids causes denaturation by disrupting the ordered stacking of the bases through breakdown of hydrogen bonds. The process can be conveniently monitored by an increase in UV absorbance as the double strands unwind to

single strands. The thermal denaturation of dsDNA is co-operative indicating that the ends and the AT-rich internal regions destabilise adjacent regions of the helix. This leads to a progressive and concerted melting of the whole structure at a well-defined temperature, corresponding to the mid-point of a smooth transition. This temperature is known as the melting temperature (Tm). UV melting experiments can provide both quantitative and qualitative data about the nature, purity and degree of hybridisation of a sample.

1.6 Practical Examples of Fluorescent Probe Systems

1.6.1 Taqman™ Assay

The Taqman™ assay is a widely used format for the detection of accumulation of PCR-specific products[109,110]. A linear probe is assembled consisting of an oligonucleotide labelled with a fluorogenic donor (5' FAM) and acceptor (internal or 3' TAMRA). Irradiation of the intact probe causes excited emission by the donor, which is quenched by the process of FRET (section 1.4.3). The probe can hybridise to the complementary template and during polymerisation the probe will be cleaved due to the inherent 5'→ 3' nucleolytic activity of Taq DNA polymerase. Cleavage causes separation of the donor and acceptor that resulting in an increase in fluorescent intensity, due to loss of quenching. Measurement of the rise in fluorescent intensity directly indicates the generation of probe-specific amplicons.

The design and performance of the probe requires three main considerations:
(i) Degree of quenching; dependent on choice and separation of the fluorophore and quencher pair, the conformational flexibility and purity of the probe.

(ii) Hybridisation of the probe; internal quenchers can disrupt base pairing and reduce Tm, a 2-3°C reduction in Tm has been reported for two internal TAMRA molecules[109].

(iii) Efficiency of probe cleavage by Taq polymerase; dependant on the accessibility of the probe to the enzyme and the complementarity between the probe and template.

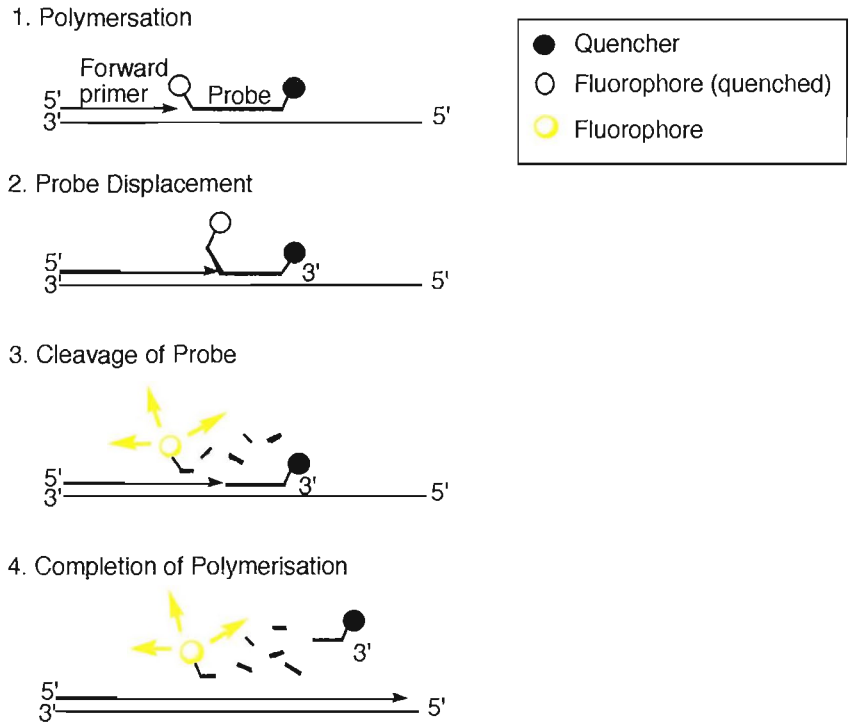


Figure 1.23 – The Taqman™ assay

The Taqman format has applied to be rapid and accurate detection of hepatitis C virus in 48 patient samples[111] and detection of salmonella in food samples in less than 20 hours producing no false negatives or false positive results[112]. The assay has also been used to develop a test for parental DNA for a deletion sequence linked to the genetic syndrome cutaneous malignant melanoma[113].

1.6.2 Molecular Beacons

Tyagi and Kramer have described the recent discovery of a novel probe technology for the detection of specific nucleic acids in homogeneous solution[114]. These probes, named 'Molecular Beacons', fluoresce only upon hybridisation to their target. They produce sensitive, real-time signals that indicate the degree of hybridisation of the probe to a target nucleic acid. The essential feature of the probe is their stem and loop structure. The loop portion is an oligodeoxynucleotide sequence that probes for its complementary target nucleic acid in solution. The stem is constructed of two short oligodeoxynucleotide arms

either side of the loop, one arm is terminally labelled with a fluorophore and the other with a quencher. Ideally the quencher should be non-fluorescent. Annealing of the arms causes intermolecular energy transfer from the fluorophore to the quencher and this energy is dissipated as heat. In this conformation the probe is non-fluorescent. Hybridisation of the loop to its target causes a conformational change, the stem arms dissociate, and the fluorophore and quencher are no longer in close proximity; fluorescence is restored.

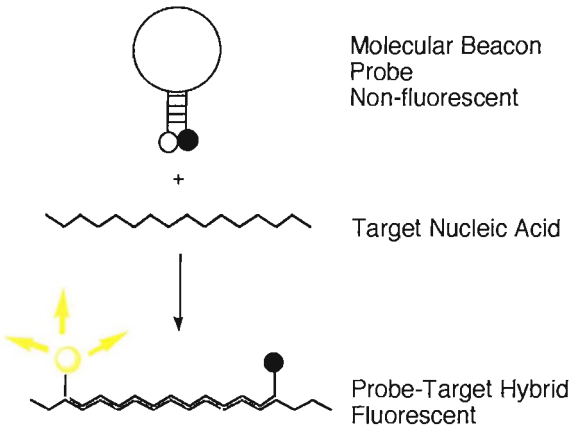


Figure 1.24 – Principles of a molecular beacon assay

The original molecular beacons synthesised by Kramer's group utilised 5-(2'-aminoethyl)aminonapthalene-1-sulfonic acid (EDANS) as a fluorophore and 4-(4'-dimethylaminophenylazo) benzoic acid (DABCYL) as the quencher. DABCYL was later shown to be an efficient quencher (98%) of a series of spectral dyes (emission maxima 475-615nm) due to Van der Waal contact of the fluorophore and quencher permitting direct energy transfer[115].

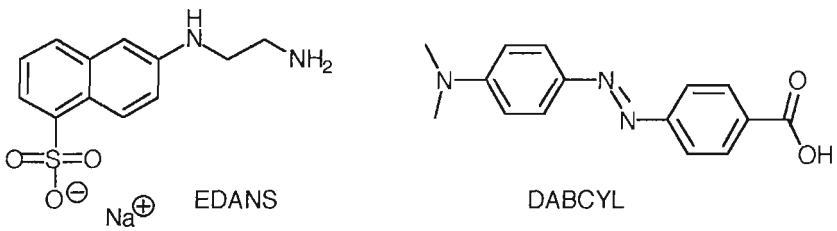


Figure 1.25 – Structure of a commonly employed fluorophore and quencher pair

Molecular beacons must be designed so that the arm sequences are unrelated to the target and are long enough to form a stem (>4 bp) but not so long that dissociation is difficult (<12 bp). In addition, hybridisation of the arm sequences in the hairpin loop must produce a weaker interaction than target-to-probe annealing.

Molecular beacons have been used to detect and quantify PCR amplicon concentration by the intensity of fluorescence at the annealing stage in each cycle. They are particularly suited to this application, exhibiting fast hybridisation kinetics and allowing sealed tube experiments. The probes exhibit allelic discrimination, only binding to their perfectly complementary targets and have the ability to recognise single base mutations. These principles have allowed the use of multicoloured probes in a single experiment[115,116].

Practical examples of their application to real systems include detection of the single point mutation of cytosine to thymidine in the methylenetetrahydrofolate reductase (MTHFR) gene. This mutation has been related to an increased risk of cardiovascular disease and neural tube defects. Molecular beacons specific for the wild type or mutant sequence demonstrated high levels of specificity for their target[117]. A mutant form arising from a conservative substitution (V641) in the coding region of the CCR2 genotype has a protective effect against HIV-1 disease progression. A molecular beacon assay that accurately discriminated between the wild type labelled with fluorescein, and mutant form labelled with hexachlorinated fluorescein has been described[118].

In addition the probes have been used for the detection of mRNA[119,120], in surface-immobilised hybridisation studies[121], as PNA-DNA hybrid probes[122], sequence analysis of amplified segments of *M.tuberculosis* DNA[123] and in the spectral genotyping of the alleles of the β-chemokine receptor 5 (CCR5)[19].

1.6.3 Scorpion Primers

A recently described technology combines a highly specific probing region with a primer for a PCR reaction[65]. The assay is unimolecular and therefore kinetically and

thermodynamically favoured. The probe region is chemically attached to the 5' end of one of the PCR primers and is blocked against copying by a hexaethylene glycol spacer. The primer binds to the target and is extended by polymerisation. The newly synthesised strand contains a sequence complementary to the probe sequence, annealing occurs and fluorescence is detected. The principal superiority of the assay is its unimolecular nature, inferring zero–order kinetics increasing the efficiency of the probing process (the probe is held in close proximity to its target) and reducing the time-scale of hybridisation. Intermolecular systems (Taqman™[109] and molecular beacons[124]) are limited by the formation of alternative intra-strand secondary structures at the assay temperature and the competition of re-annealing complementary PCR amplicons.

Scorpions have shown high specificity in single tube genotyping experiments of the two main variants of the Hereditary Haemochromatosis gene: C282Y, the major disease causing mutation and H63D, a prevalent polymorphism of uncertain clinical relevance. Scorpions have also been shown to accurately distinguish between matched and mis-matched allelic polymorphs of exon 10 of the BRCA2 gene.

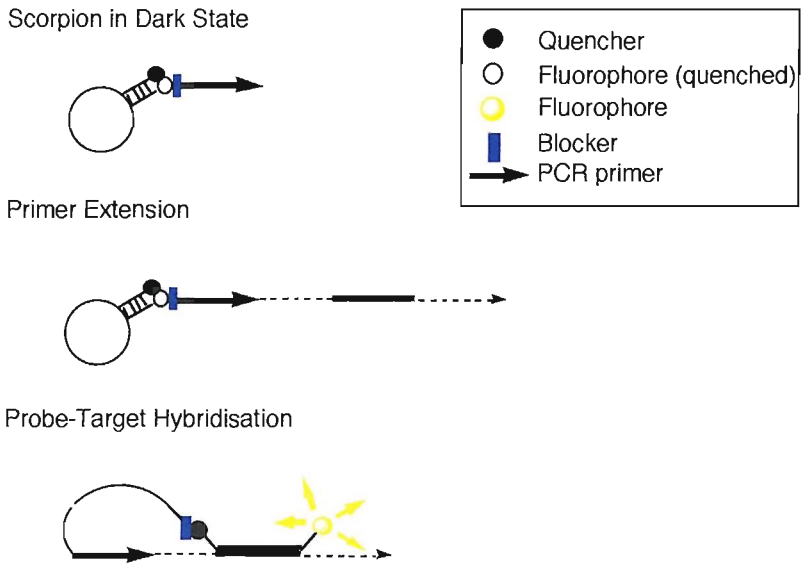


Figure 1.26 – Stages of a Scorpion detection assay

1.6.4 Invader Assay

Third wave technologies have described an isothermal, quantitative assay that requires no PCR and generates amplified signals upon probe-target hybridisation[125]. Termed "Invader Assay" the system is comprised of two probes that hybridise adjacent on a target nucleic acid. The probes are designed so that the 3' end of the upstream Invader probe overlaps the 5' end of the downstream, labelled, signal probe. This invasion of the signal probe-target duplex region causes the displacement (by at least one base) of a portion of the 5' signal probe, creating a branched structure with a single stranded flap. The junction between the flap and the partially invaded duplex is recognised and cleaved by the Cleavase™ enzyme[126].

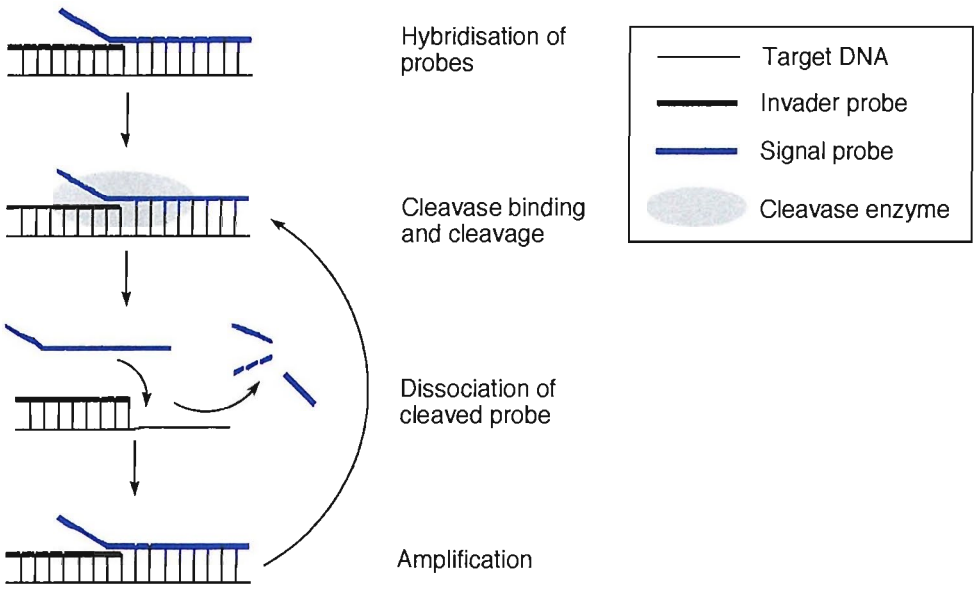


Figure 1.27 – Third wave Invader assay format

Detection can be effected by direct gel analysis, FRET or mass spectrometry. The relatively high temperature of the assay causes the cleaved signal probe to dissociate and another intact signal probe to anneal, whilst the invader probe remains bound to the target. Providing the signal probe is in excess there will be an amplification of signal. It has been shown, using Human cytomegalovirus genome as a target, that cleavage generates a signal

that is proportional to the number of copies of target DNA present. Carry-over contamination, false-positives and background signals are all alleviated as signal is only procured in the presence of a target[127].

1.6.5 PNA Probes

PNA oligomers are not substrates for DNA, RNA or protein modifying enzymes, and will hybridise to DNA and RNA by normal Watson and Crick base pairing[128]. They bind strongly under conditions in which normal nucleic acids hybrids are disfavoured, for example at low salt concentrations. PNA probes are assembled by standard FMOC or BOC chemistry and can be labelled directly with fluorescent dyes[122], enzymes, or visualised indirectly with haptens such as biotin and dinitrophenol. PNA probes have high specificity, affinity and stability at low salt concentrations in comparison with DNA probes. DNA probes often fail to distinguish certain regions of highly structured rRNA. However PNA probes detect these highly structured rRNA regions with significantly higher fluorescent signals, exhibiting improved performance at high temperatures and low salt concentrations.

1.6.6 SUNRISE Primers

An amplification system has been developed called SUNRISE™. The basis is the incorporation of fluorescence energy-transfer labelled primers into PCR products. These primers have specific target sequences at the 3'end and hairpin structures at the 5' end that are non-fluorescent prior to PCR. Incorporation of the primer during PCR causes the hairpin to be disrupted and fluorescence is detectable. The fluorescent signal intensifies with increasing number of PCR cycles as more primers are incorporated[129].

Following this a Universal SUNRISE Primer was reported to allow the incorporation of an identical hairpin primer into any target nucleic acid[130]. An initial PCR step incorporates a primer that carries an oligonucleotide tail (15 bases) that is complementary to the sequence of the universal hairpin primer. Following incorporation of the tail, the hairpin primer takes over and is unwound in the same manner as before.

The primers have been successfully applied to a closed tube amplification detection format to distinguish between the normal (WG4) and mutant (RG4) alleles of the β(3)-adrenergic receptor gene[131].

1.6.7 DNA Chip Technology

DNA chip technology utilises high-density microscopic arrays of nucleic acids immobilised on solid surfaces for biochemical analysis. DNA or RNA samples from biological sources are labelled enzymatically by the incorporation of labelled primers or nucleoside triphosphates during PCR amplification. Hybridisation of the targets to the microarrays and subsequent detection can be used for polymorphism detection, DNA sequencing and genotyping. For example analysis of the CFTR gene was carried out by the preparation of a microplate loaded with 428 features to identify mutations in exon 11, and a microarray containing 96,600 20mers was used to identify mutations over the entire 3.45 kb of exon 11[132].

The oligonucleotide arrays are often prepared on glass due to its inert chemical properties, low intrinsic fluorescence and the ability to derivitise its surface. The strategies for the preparation of these libraries using photolithography and micro-spotting, has been excellently reviewed by B.Lemieux *et al.*[133] and E.M. Southern[134]

Chapter 2

Development of Novel Homogeneous Assay Systems

2.0 Development of Novel Homogeneous Assay Systems

The preceding chapter introduced the reagents, techniques and strategies employed in the design of systems to probe genetic sequences. The following chapter aims to record the work carried out in the design, synthesis and testing of a novel two-probe assay.

2.1 Introduction

The aim of this project was to further develop the use of fluorescence as a detection method for DNA sequences. When designing a novel assay the incorporation of the following features was considered to be beneficial:

(i) Homogeneous assay (all components in solution) is advantageous over its heterogeneous counterpart[135] (solid and solution phase components) as it does not rely on the hybridisation of a probe to an insoluble support and therefore displays faster hybridisation kinetics. In homogenous assay formats there is no requirement for washing steps as the signal is generated only when the labelled probe is hybridised to the target.

(ii) Enzyme-linked probes are highly specific for their substrates and provide high detection sensitivities, similar to those attained using radioisotopes[61].

(iii) Isothermal signal generation simplifies an assay by reducing the number of steps performed.

(iv) In certain cases, the use of more than one probe in an assay increases the specificity for a particular target as two or more adjacent nucleic acid sequences can be probed.

An assay was proposed (figure 2.1) that required a probe labelled at the 5' end that is initially non-fluorescent and complementary to a known sequence on a target nucleic acid (signal probe). This probe is 'dark', as the fluorescent label it bears has been chemically modified with masking groups to remove its fluorescent characteristics. The masking

groups are substrates that can be cleaved from the label by an enzyme-catalysed reaction. A second probe consists of the conjugate formed when an enzyme is linked to the 3' end of an oligonucleotide (enzyme-probe). The enzyme-probe is complementary to a sequence downstream (further along the target DNA) of the upstream signal probe sequence on the target DNA. There will be little or no fluorescence from either the signal probe or enzyme-probe individually in solution. When the two probes are mixed there may be a small amount of background fluorescence detected due to the enzymatic hydrolysis occurring when the two probes meet in solution. However, in the presence of a target nucleic acid the two probes can hybridise adjacent to each other, bringing the 3' enzyme and the 5' substrate molecules into close contact. Cleavage of the masking groups by the enzyme will release a strong fluorescent signal, which can be detected.

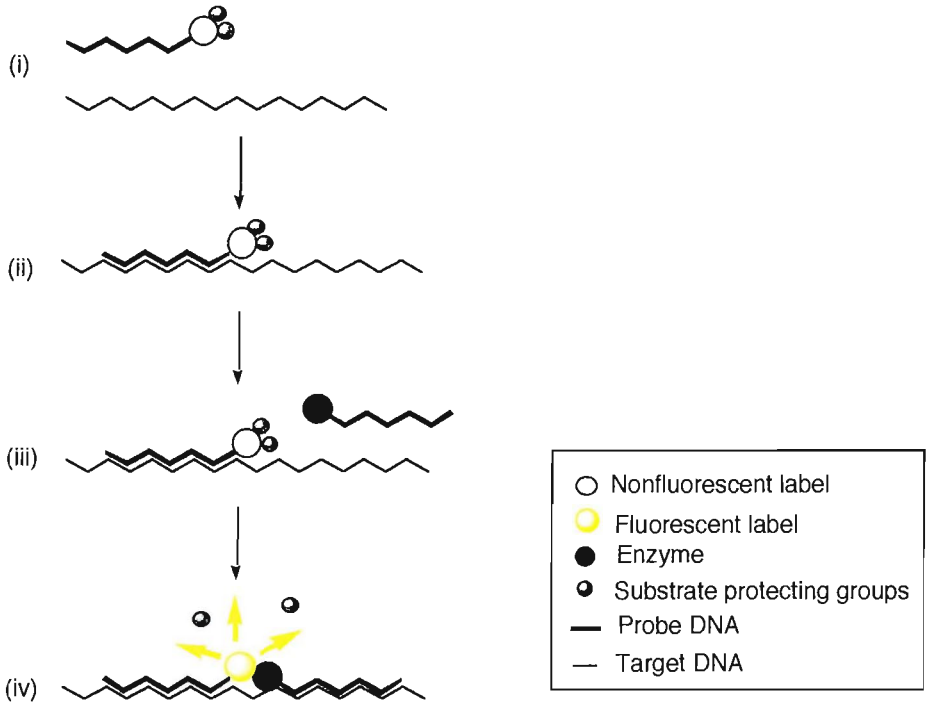


Figure 2. 1- (i) An oligonucleotide is labelled with a fluorophore (5') which has been chemically modified to remove its fluorescent properties (signal probe), the probe is introduced to the target nucleic acid. (ii) The probe hybridises to its complementary sequence on the target nucleic acid. (iii) An oligonucleotide labelled with a enzyme (3') is introduced (enzyme-probe). (iv) The oligonucleotide-enzyme conjugate hybridises adjacent to the signal probe, the enzyme cleaves the substrate groups attached to the fluorophore and fluorescence is visualised.

The assay is desirable as is homogeneous, isothermal, requires two probes and exploits enzyme detection. The close proximity of the substrate and enzyme is caused only by hybridisation of the two probes adjacent to one another on a target; consequently fluorescence is only generated in the presence of a target nucleic acid. The assay format is sensitive, specific and the fluorescent signal generated will be directly proportional to the number of enzyme-probes hybridised.

Fluorescein is a well-known dye that will only fluoresce when its phenolic hydroxyl groups are unsubstituted. This allows the lone pairs on the oxygen to participate in the highly conjugated delocalised system and stabilise the lowest excited state. When the phenolic hydroxyl groups are unprotected the wavelength of emission is in the visible region and fluorescence is observed. However, protection of the hydroxyl groups masks this fluorescence by shifting the wavelength of emission into the ultraviolet region. A non-fluorescent probe can be created by attachment of a phenol-protected fluorescein monomer to the 5'end of an oligonucleotide.

2.2 Alkaline Phosphatase Approach

2.2.1 Introduction

Alkaline phosphatase catalyses the hydrolysis of phosphomonoesters to produce inorganic phosphate and the corresponding alcohol. It is a relatively small enzyme with a molecular weight of about 89 kDa and is dimeric in structure. Alkaline phosphatase has been applied to indirect[136] and direct labelling strategies[61,62] in nucleic acid hybridisation probe technology with detection reported at the level of one attomole[137]. Indirect procedures described in the literature involve attachment to a hapten or to an antibody specific for a hapten, in both cases detection is achieved by enzymatic turnover of a substrate releasing light or colour. Probe assembly in direct methodologies involves either covalently attaching the enzyme or the substrate to an oligonucleotide. Hybridisation is measured by the increase in colour or light caused by turnover of the substrate such as 5-bromo-4-chloro-3-indolyl phosphate / nitroblue tetrazolium (BCIP/NBT). The chemistry involved in

conjugation of alkaline phosphatase to oligonucleotides is routine[138] presenting a convenient starting point for assay development.

Preparation of a signal probe required the attachment of a label modified with substrates for alkaline phosphatase (phosphate esters). Fluorescein diphosphate was chosen as a suitable label modified with a reactive spacer for attachment to an oligonucleotide (figure 2.2). Several synthetic approaches to prepare the signal probe are described in the following sections.

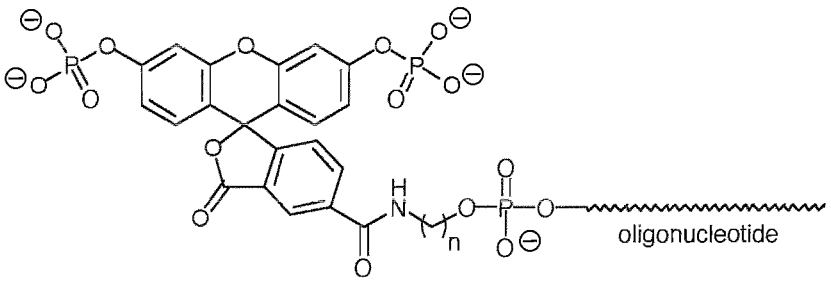


Figure 2.2 – Fluorescein diphosphate signal probe

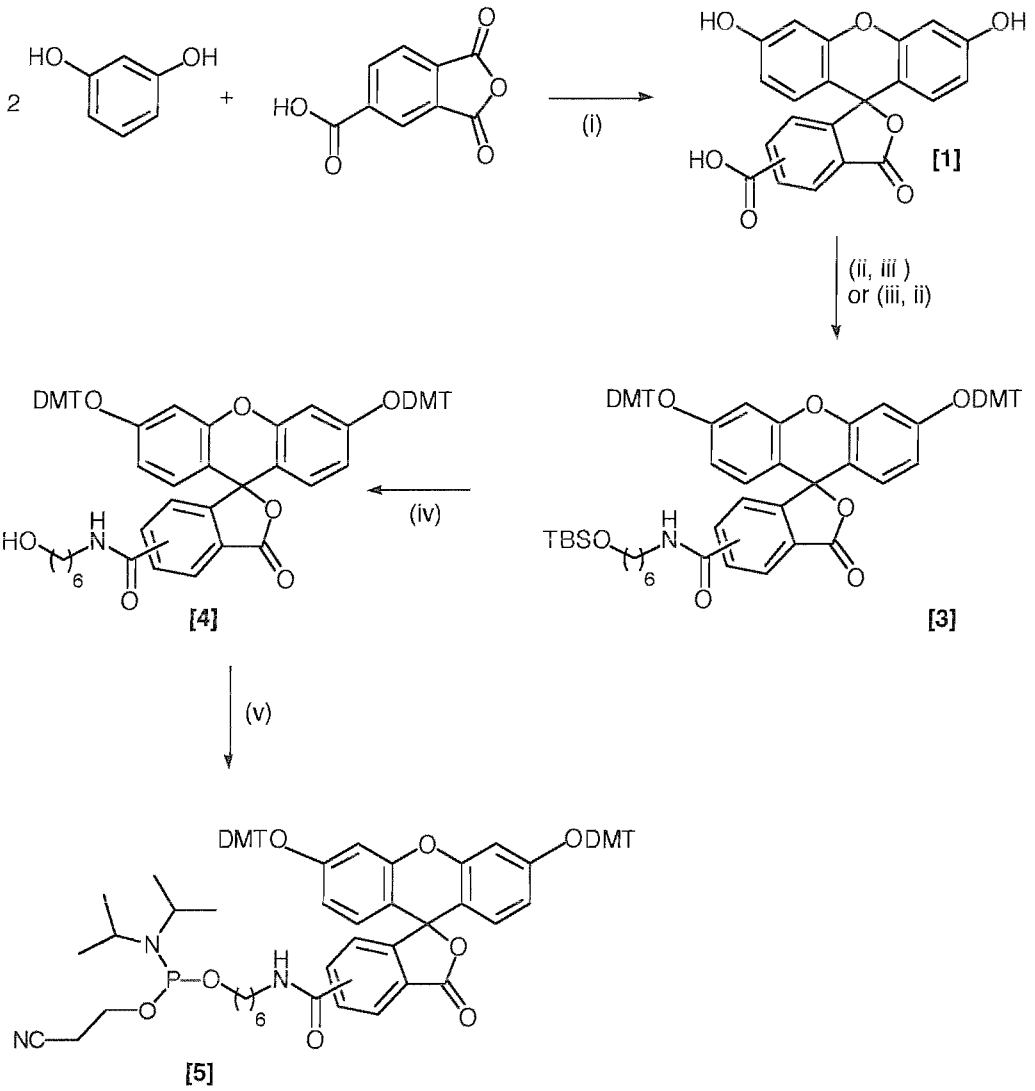
2.2.2 Solid Phase Strategy

This approach involved synthesising a fully protected phosphoramidite monomer of fluorescein that was compatible with standard solid phase DNA synthesis[139]. The monomer could be coupled to the 5' end of a resin bound oligonucleotide on a DNA synthesiser, and following selective removal of the fluorescein protecting groups, on-resin phosphorylation of the phenolic hydroxyls could be attempted. The choice of protecting groups for the phenolic hydroxyls of fluorescein was imperative, as their selective removal required orthogonality between these protecting groups and those of the heterocyclic bases. In addition any deprotection reagents applied had to leave the oligonucleotide-resin linkage intact allowing subsequent on-resin phosphorylation.

2.2.2.1 4,4'-Dimethoxytrityl Ether

4,4'-Dimethoxytrityl ethers are acid labile and repeatedly removed from the 5' hydroxyl of nucleosides during solid phase DNA synthesis, inferring compatibility for protection of the

phenolic hydroxyls of fluorescein. Research in the Oswel laboratory had previously completed the synthesis of the fluorescein monomer (**[5]**, figure 2.3) by a stepwise route (ii, iii, and iv). The synthesis was also achieved by the alternative route shown in figure 2.3 (ii, iv, iii).



Reagents and conditions: i, $ZnCl_2$, 190°C, 1 hr, 70%; ii, $NH_2(CH_2)_6OTBS$ **[2]**, EDC, pyridine, rt, 18hr, 66%; iii, 4,4'-dimethoxytrityl chloride, pyridine, reflux, 8hrs, 43%; iv, 1.0M TBAF in THF, rt, 4.5hrs, 46%; v, 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite, DIPEA, THF, rt, 1hr, 95%

Figure 2.3 - Synthesis of DMT protected fluorescein monomer **[5]**

A short polythymidine oligonucleotide was synthesised (TTTTTT, **[6]**), using standard methods, and the phosphoramidite monomer **[5]** coupled to the 5' terminus. Subsequent

treatment with trichloroacetic acid in CH_2Cl_2 (3%) was expected to release the 4,4-dimethoxytrityl cation. Normally, the highly visible bright orange cation is carried away by solvent flow. Acid treatment of the monomer turned the resin orange indicating release of the cation. However, all attempts to separate the cation from the resin by repeated washing or addition of known efficient trityl cation scavengers, (pyrrole and triethylsilane[140]) failed. It was concluded that the cation was strongly, ionically associated with the phenolic anion (figure 2.4), a phenomenon that can occur when nucleophilic species remain bound to the resin[141]. The tight association of the DMT cation led to the search for other suitable hydroxyl protecting groups.

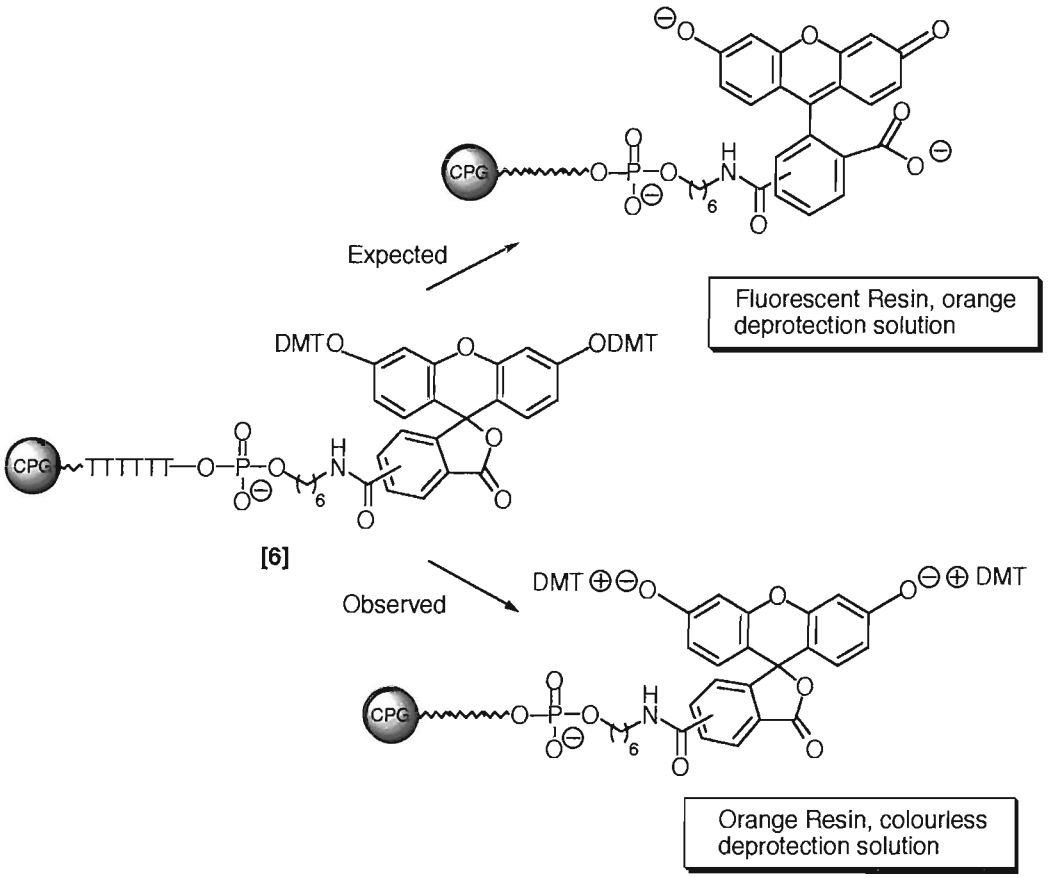


Figure 2.4 - Expected and observed results: acidic deprotection of resin-bound labelled oligonucleotide

2.2.2.2 p-Methoxybenzyl Ether (PMB)

p-Methoxybenzyl ethers are cleaved oxidatively using 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) in water[142], or by ceric ammonium nitrate in aqueous acetonitrile to release alcohols. A range of reagents and solvents were investigated to protect 5(6)

carboxyfluorescein as a PMB ether. These included using sodium hydride, triethylamine and diisopropylethylamine in THF, DMF and pyridine. Reactions in THF proved problematic due to poor solubility of carboxyfluorescein. Reactions in DMF and pyridine displayed good solubility but consistently gave low yields of the desired product and higher yields of the triply protected product (PMB ethers and PMB ester).

2.2.2.3 t-Butyldimethysilyl Ether (TBS)

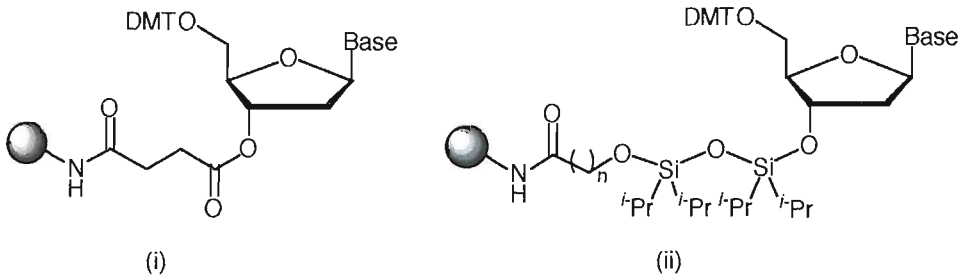
TBS ethers are relatively acid and base stable but labile to fluoride ions. 5(6) carboxyfluorescein was reacted with *t*-butyldimethylsilyl chloride and dimethylaminopyridine in pyridine to give the desired compound. However, subsequent purification and handling revealed a high degree of instability to acid, indicated by the constant regeneration of fluorescence.

2.2.2.4 Levulinyl Esters

Levulinyl groups have been used to protect of hydroxyl functions of oligonucleotides[143]. The esters are cleaved by 0.5M hydrazine hydrate in pyridine/acetic acid/water[144] and under these conditions the oligonucleotide-support remains intact. Reactions of levulinyl anhydride with 5(6) carboxyfluorescein produced a product that exhibited instability in the presence of amines. Introduction of the linker amine **[2]** or the use of tetrabutylammonium fluoride caused the appearance of fluorescence, indicating deprotection had occurred.

2.2.2.5 Disiloxyl linker

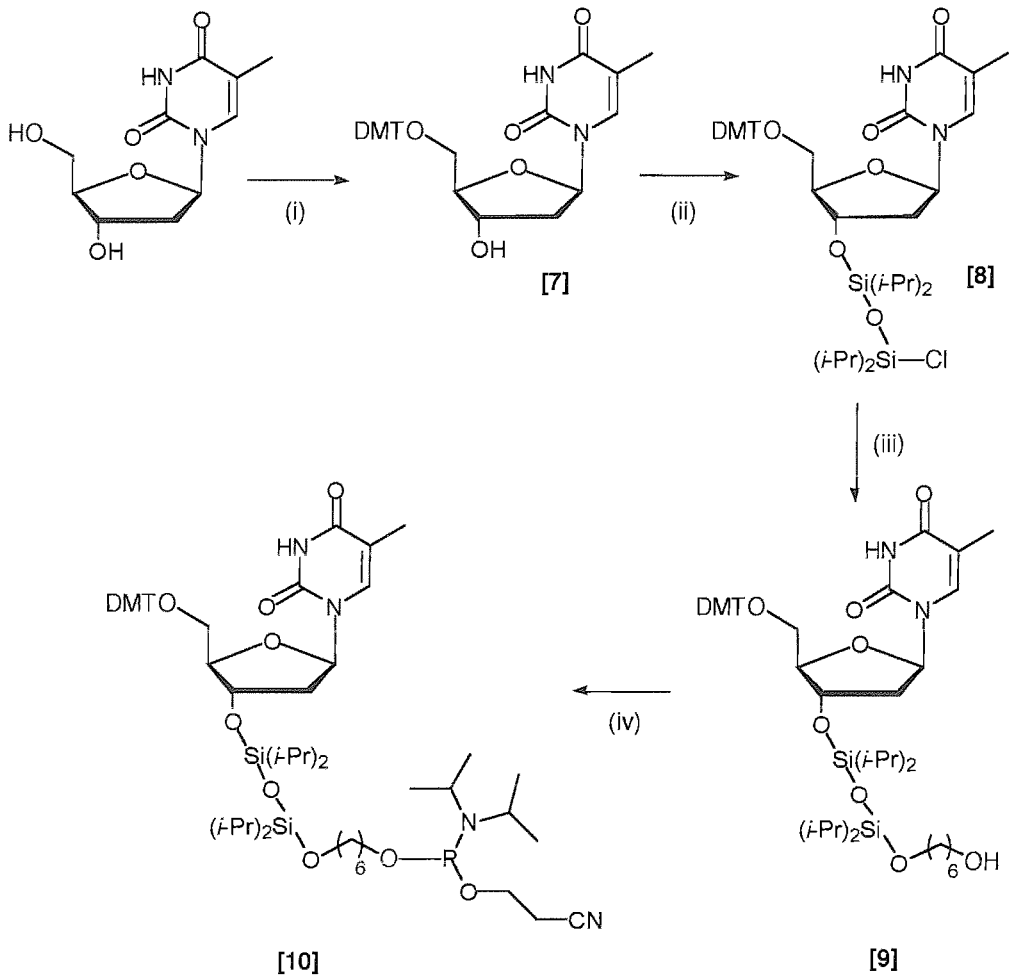
Kwiatkowski et al. reported the synthesis of a disiloxyl resin-oligonucleotide linker to replace the conventional succinyl linker to overcome the production of defective molecules, in particular depurinated molecules, during standard oligonucleotide synthesis[145].



(i) (ii)

Figure 2.5 - i, Conventional base labile succinyl linker, ii, fluoride labile disiloxyl linker

1,3-dichloro-1,1,3,3-tetraisopropyl disiloxane is coupled to the 3'hydroxyl of the first nucleoside to be attached to the resin, this is then coupled to an alcohol derivatised resin. The linker is stable to mild base but cleaved by fluoride ions. Fluorescein dipivalate is commercially available as its phosphoramidite monomer, utilising the monomer in conjunction with the disiloxyl linker would permit selective deprotection of fluorescein.



Reagents and conditions: i, 2'-deoxythymidine, 4-dimethylaminopyridine, 4,4'-dimethoxytrityl chloride, pyridine, rt, 24hrs, 82% ii, imidazole, 1,3-dichloro-1,1,3,3-tetraisopropyl disiloxane, pyridine, rt, 24hrs, iii, 1,6-hexanediol, pyridine imidazole, rt, 12hrs, 76% (last two steps), iv, diisopropylethylamine, 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite, CH_2Cl_2, rt, 1.5hrs, argon, 98%

Figure 2.6 - Preparation of disiloxyl phosphoramidite monomer

Synthesis of the disiloxyl nucleoside phosphoramidite monomer **[10]** was achieved. The 5' hydroxyl of 2'-deoxythymidine was protected as a 4,4'-dimethoxytrityl ether. 1,3-dichloro-1,1,3,3-tetraisopropyl disiloxane was coupled to the 3' hydroxyl and following conversion

of the starting material, hexan-1,6-diol added to the reaction mixture. Finally the primary alcohol of the hexyl spacer was transformed to the phosphoramidite (figure 2.6, **[10]**).

LCAA-CPG was derivatised with a poly alkyl linker (figure 2.7). The linker was assembled by protecting the primary hydroxyl group of 12-hydroxydodecanoic acid as 4,4' - dimethoxytrityl ether **[11]**. The following reaction coupled the carboxylic acid of this molecule with free amino moieties on the resin. Resin loading was calculated by acid-catalysed detritylation, DMT cation release was quantified at 495nm. The resin was capped with acetic anhydride prior to use in automated oligonucleotide synthesis. The first nucleoside coupled to the CPG (3') was the monomer **[10]** thus placing the fluoride scissile linkage between the oligonucleotide and the resin. Sequential addition of twelve standard thymidine monomers, followed by fluorescein dipivalate completed the synthesis of the resin-bound oligonucleotide **[13]**.

[11] **[12]**

Reagents and conditions: i, DMTCl, pyridine, rt, 2hrs; ii, LCAA-CPG, DIC, HOBT, DIPEA, CH_2Cl_2, rt, 1hr; iii, Ac_2O, pyridine, THF: 1-methylimaidazole, THF (1:1), rt, 1hr

Figure 2.7 - Derivatisation of CPG for oligonucleotide synthesis with monomer **[10]**

The stability of the disiloxyl linker to the conditions used for the deprotection of the fluorescein trimethylacetyl esters was established. Methanolic ammonia is the typical reagent used for such deprotections. However, the disiloxyl linker was labile when subjected to ammonia with significant loss of the oligonucleotide from the resin after 30 minutes, contrary to the published results[145]. Figure 2.7 shows the stability of the disiloxyl linker to methanol saturated with ammonia compared to the stability of the conventional succinyl linker, known to completely cleave in 30 minutes.

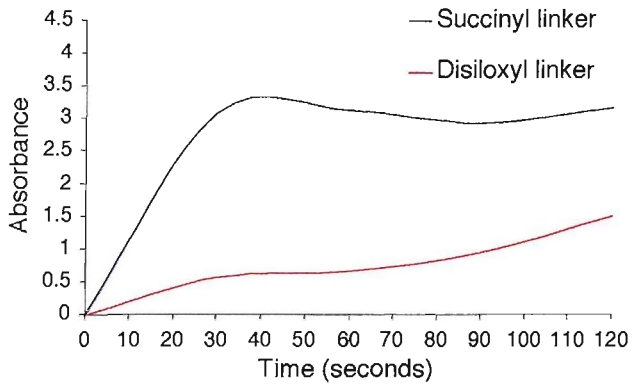


Figure 2.8 - Increase in absorbance at 260nm as oligonucleotide is released into solution from CPG derivatised with succinyl or disiloxyl linkers

High losses of oligonucleotide from the resin were impractical for on-resin fluorescein phosphorylation. Alternative deprotection systems (figure 2.8) were investigated in a series of model reactions on fluorescein dipivalate **[18]**. The results revealed DBU: MeOH (1: 20) as a suitable choice of deprotection reagent and subsequent experiments demonstrated the stability of the disiloxyl linker to this reagent over the 45 minutes reaction period.

Deprotection conditions	Deprotection time (Hrs)
Ammonia: MeOH (1: 20)	18
Triethylamine: MeOH (1:20)	18
Triethylamine: MeOH (1: 4)	18
Triethylamine: MeOH (1: 1)	12
$KHCO_3$ (0.1M): MeOH (1: 20)	18
$LiBH_4$ (2.0M): THF: MeOH (1: 2: 2)	6
DBU: MeOH (1: 20)	0.75

Figure 2.9 – Conditions and time periods from acetyl removal from fluorescein dipivalate

An oligonucleotide sequence FTTTTTTTTTTTT (**[14]**), F = fluorescein monomer, figure 2.10) was synthesised using the disiloxyl linker and resin. The fluorescein monomer was deprotected with DBU: MeOH (1: 20) producing the expected fluorescent resin. Initial phosphorylation of the fluorescein hydroxyl groups using the phosphate monomer shown in

figure 2.10 appeared successful (monitored by the disappearance of resin fluorescence). The oligonucleotide was removed from the resin using $3HF \bullet Et_3N$: NMP: Et_3N to give a colourless solution. (NB. using tetrabutylammonium fluoride gave a fluorescent solution indicating loss of phosphate groups) The cyanoethyl and ethylsulfonyl protecting groups on the phosphate monomer were removed with triethylamine: CH_2Cl_2 (1: 4). RPHPLC analysis of the oligonucleotide gave a major fluorescent peak corresponding to $FAMT_{12}$, and a minor peak that was fluorescent and therefore could not be the desired product. Repeated attempts at on-resin phosphorylation failed to yield the desired phosphorylated fluorescein labelled oligonucleotide.

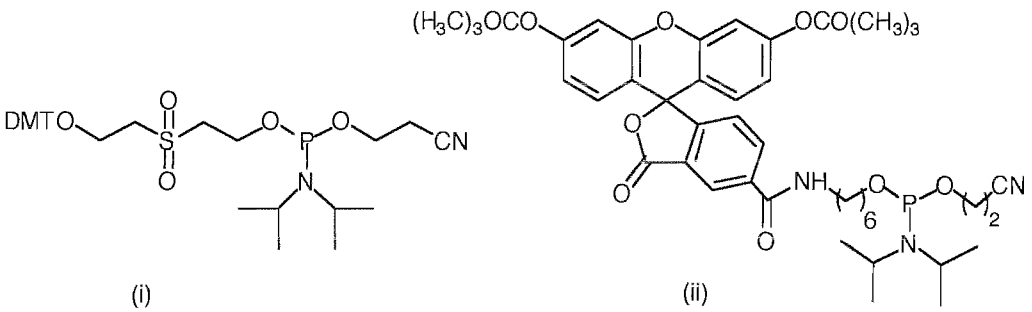
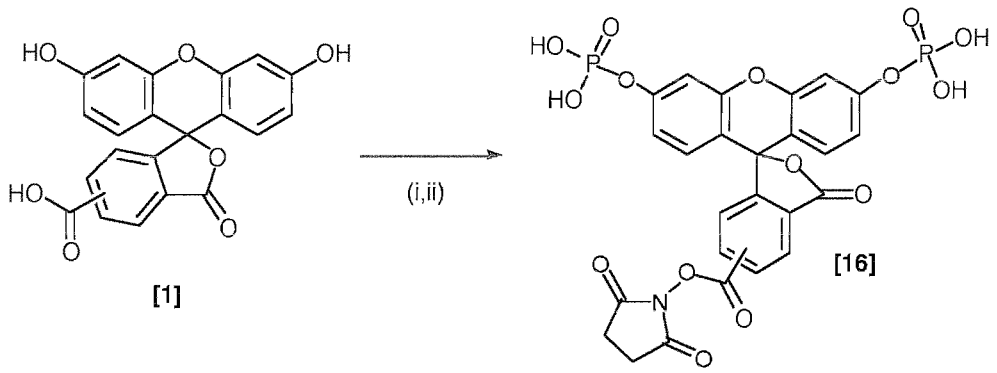


Figure 2.10 - i, phosphate phosphoramidite, ii, fluorescein phosphoramidite

2.2.3 Solution Phase Strategy

Synthetic oligonucleotides derivatised at the 3' or 5' terminus with an amino function (aminolink) react with *N*-hydroxysuccinimde esters of dyes in a simple labelling reaction. 5(6) Carboxyfluorescein *N*-hydroxysuccinimide ester **[15]** was treated with phosphorus oxychloride to give non-fluorescent crude solid. Reaction of the crude product with an aminolink oligonucleotide **[17]** in bicarbonate buffer, pH 9.75, gave one main peak by RPHPLC shown to be unreacted aminolink oligonucleotide. Two small peaks were observed; one fluorescent and one colourless. It was proposed that these corresponded to fluorescein and fluorescein diphosphate labelled products, however the amounts of product collected was insufficient for mass spectral analysis. The yield of the desired oligonucleotide was very low, and attempts to improve this were unsuccessful.



Reagents and conditions: i, *N*-hydroxysuccinimide, EDC, EtOAc, rt, 3hrs, 61%; ii, $POCl_3$, pyridine, 0°C, 2hrs

Figure 2.11 - Synthesis of phosphorylated fluorescein *N*-hydroxysuccinimide ester

2.2.4 Conclusions

Literature describes the use of fluorescein diphosphate and fluorescein monophosphate as a substrate to study alkaline phosphatase activity[146,147]. Rotman *et al*[148] encountered difficulties associated with diphosphate preparation, but successful synthesis has been described using fluorescein[146]. However, the literature methods do not subject the molecule to acidic or basic conditions. Phosphorylation of fluorescein derivatives has not been reported.

5(6) Carboxyfluorescein diphosphate derivatives appear to be relatively unstable to acid and base. Fluorescein is a good leaving group and its phosphates can be readily hydrolysed allowing fluorescein to tautomerise and regain fluorescence. Unfortunately, the inherent instability of this molecule deemed it unsuitable for the assay application described.

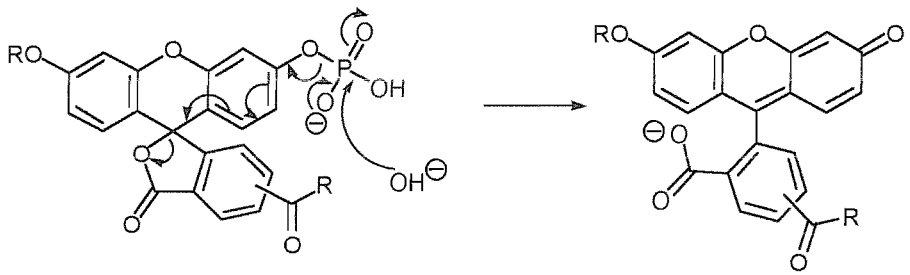


Figure 2.12 - Hydrolysis of fluorescein phosphate

2.3 β-D-Galactosidase Approach

2.3.1 Introduction

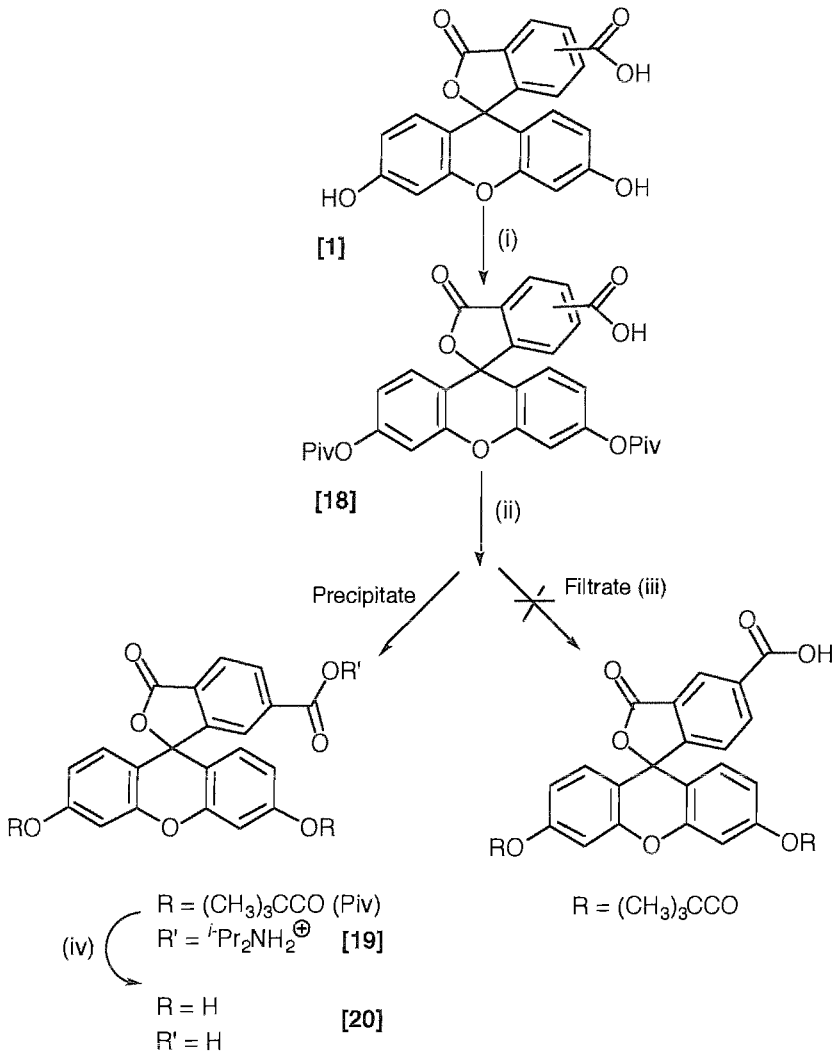
β-D-Galactosidase (EC 3.2.1.23) is oblate ellipsoid in shape and has a molecular weight of around 500,000. The native enzyme is tetrameric, comprising of four identical subunits, each with a molecular weight in the range of 130,000. The hydrolysis of β-D-galactosides by β-D-galactosidase may be followed by monitoring the liberation of either the glycone or aglycone products. The substrate fluorescein di-β-D-galactopyranoside (FDG) as been used to study the activity of the enzyme[149,150] which releases fluorescein in a two step hydrolysis process[151,152]. The mechanism involves the hydrolysis of fluorescein di-β-D-galactoside to the fluorescein mono-β-D-galactoside (FMG) intermediate which is in turn hydrolysed by one of two pathways: (i) *binding* - the FMG binds to enzyme in competition with FDG and is hydrolysed (ii) *channelling* - the FMG is retained in the binding site until completely hydrolysed into fluorescein and galactose before diffusing away. The synthesis of this substrate by the silver (I) catalysed glycosylation of fluorescein with 2,3,4,6-tetra-O-acetyl-α-D-galactopyransoyl bromide has been described[148] but reported yields are poor.

Synthesis of fluorescein-β-D-galactopyranoside phosphoramidite monomer is not found in the literature. This molecule covalently linked to the 5'end of an oligonucleotide creates the non-fluorescent signal probe. Linkage of the β-D-galactosidase to the 3'end of a second oligonucleotide creates the enzyme-probe, for signal probe detection.

2.3.2 Synthesis of the Signal Probe

Figure 2.15 outlines the final stepwise synthesis of the fluorescein-bis-β-D-galactopyranoside, non-fluorescent, phosphoramidite monomer. The reaction of two molecules of resorcinol with benzenetricarboxylic anhydride results in results in roughly equal mixture of the 5 and 6 regiosiomers of carboxyfluorescein. For the purposes of early synthetic exploration this starting material was used as an isomeric mixture. To reduce the complexity of NMR data and simplify each step of the synthesis, the isolation of the

isomers was investigated. The two isomers are inseparable by simple chromatographic and crystallisation purification methods, but acylation of the phenolic hydroxyls causes greater resolution between the isomers by TLC. Recrystallisation of the 5-isomer of the acetyl diester in isopropanol has been reported[153] but all endeavours to crystallise or isolate either isomer by column or radial chromatography failed. Derivatisation as a dipivalate ester allowed isolation of both isomers by column chromatography, however the quantity and purity of the individual compounds was insufficient to continue the stepwise monomer synthesis.



Reagents and conditions: i, pivaloyl chloride, pyridine, rt, 3hrs, 64%; ii, diisopropylamine, ethanol, boiling, 19%; iii, 1M HCl wash then cold nitromethane; iv, NaOH in MeOH,then 4M HCl, 92%

Figure 2.13 - Procedure for isolation of isomers of 5(6) carboxyfluorescein

Rossi *et al.* reported the separation of the isomers by derivatisation and precipitation (figure 2.13)[154]. Repetition of the method described by the authors failed to produce either isomer pure. In contrast, a slight modification of the method achieved acceptable yields of the pure 6-isomer. 5(6) carboxyfluorescein dipivalate was prepared by reaction with pivaloyl chloride instead of the anhydride described by Rossi, to give a cleaner product with greater conversion of starting material. The crude product was dissolved in a minimum volume of boiling ethanol and diisopropylamine was added using fewer equivalents than reported. Slow cooling to -20°C produced the diisopropylamine salt of the 6-isomer (90% by NMR) as a fine white solid, which was easily converted to 6-carboxyfluorescein by base catalysed removal of the pivaloyl groups. This provided sufficient material to complete the stepwise synthesis of the monomer **[25]**. The authors described the crystallisation of the 5-isomer from the acidified filtrate in nitromethane, yet all attempts to duplicate were unsuccessful (figure 2.13).

6-Aminohexanol was protected as a TBS ether, which was in turn coupled to 6-carboxyfluorescein to give the amide (**[21]**, figure 2.15). Glycosylation of fluorescein in low yields (14%) was originally performed by Rotman *et al*[148]. The reaction follows classic Koenigs-Knorr chemistry with inversion of the α-galactosyl halide to give exclusively the β-D-galactoside. In the presence of heavy-metal salts, a neighbouring group participation mechanism is favoured, dictating exclusive attack of the glycosyl acceptor from above the plane of the ring (figure 2.14). The correct configuration of the glycosidic linkage was essential for enzyme recognition and was confirmed by NMR and enzyme hydrolysis.

Rotman's reaction was reproduced using the amide **[21]**, 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide (prepared by the reaction of hydrogen bromide in acetic acid with penta-*O*-acetyl-β-D-galactopyranoside) and silver oxide. The reaction was slow, proceeding through a fluorescein mono-β-D-galactopyranoside intermediate, and gave a low yield. The reaction was hindered by the insolubility of the fluorescein amide in benzene and failed to produce enough material for stepwise synthesis.

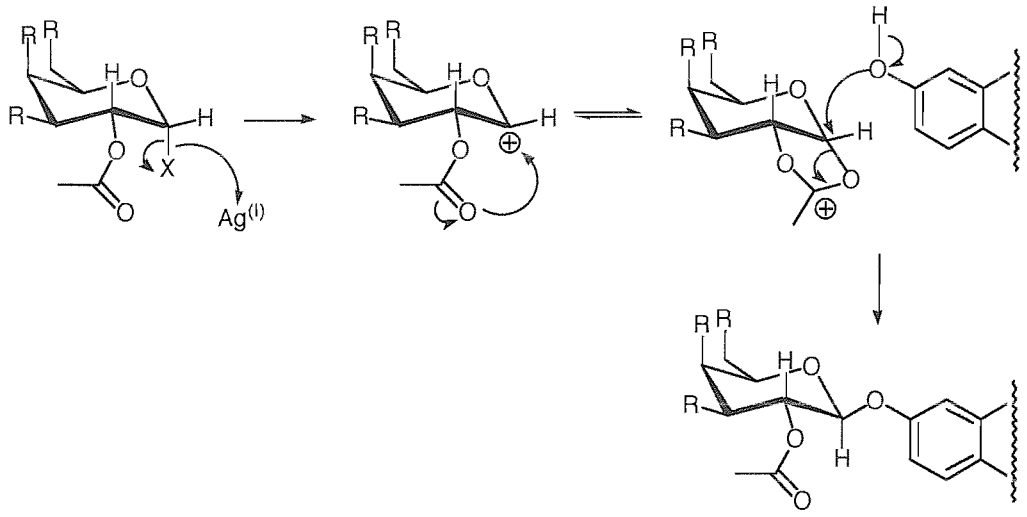
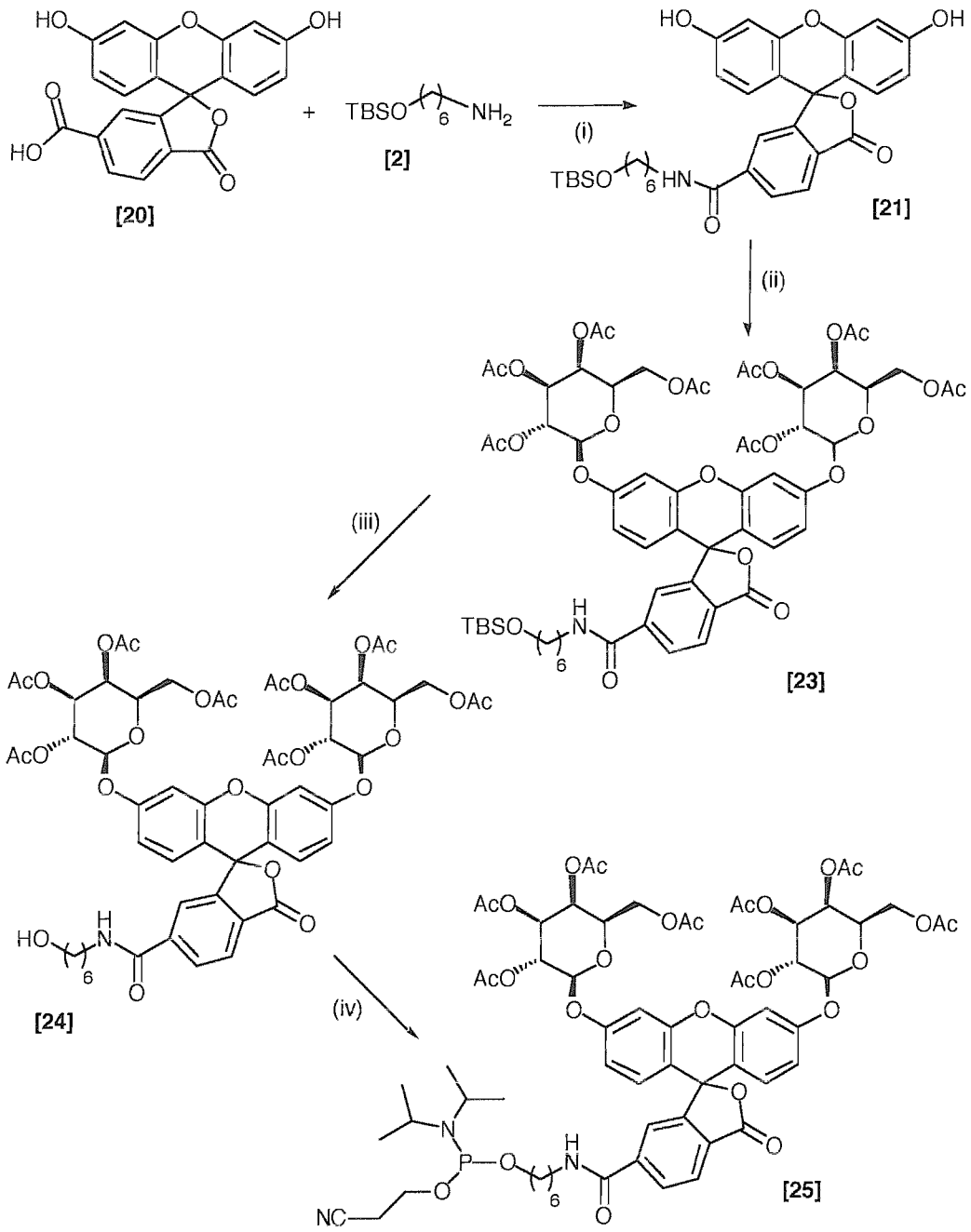


Figure 2.14 – Mechanism of inversion of configuration at anomeric centre ensuring β-D-galactoside formation

The use of trichloroacetimidates as glycosyl donors is reported to give high yields and anomeric selectivity in both solid phase and solution glycosylations[155,156]. Disappointingly, reactions of galactosyl trichloroacetimidate and fluorescein amide **[21]** in the presence of Lewis acids met with little success; changes in the Lewis acid catalyst or reaction solvents used did not give the desired product.

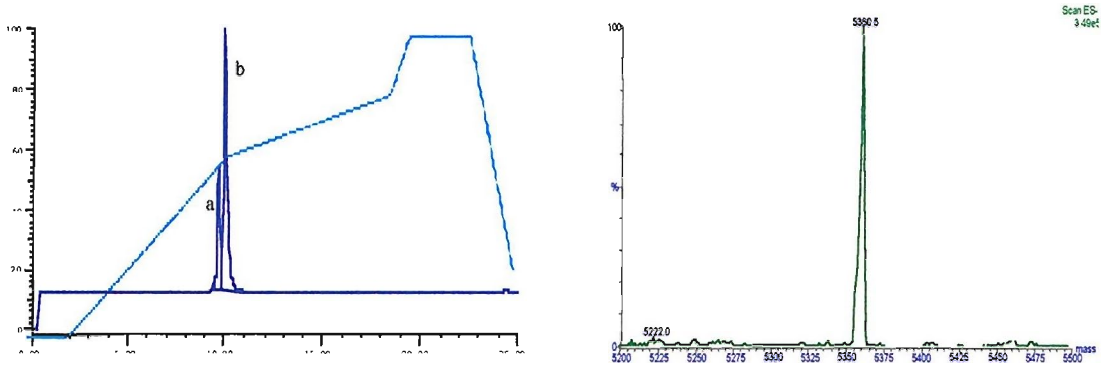
A silver silicate catalyst has been reported for glycosylations of glycosyl halides, giving improved yields and greater selectivity for the β-anomer[157,158]. This is due to the association of the sugar halide with the silver on the surface of the catalyst. Fortuitously, synthesis of the catalyst and its application in glycosylation of **[21]** was very successful with complete consumption of the starting material and a relatively high yield after repeated purification (60%). Removal of TBS protection by tetrabutylammonium fluoride caused the regeneration of fluorescence, however the deprotection proceeded cleanly with a hydrogen fluoride reagent ($3HF \bullet Et_3N$: NMP: Et_3N). The final phosphitylation step was completed yielding the non-fluorescent monomer (figure 2.15, **[25]**) for oligonucleotide synthesis.



Reagents and conditions: i, EDC, pyridine, rt, 24hrs, 66%; ii, α-D-tetraacetate galactosyl bromide **[22]**, silver silicate catalyst, CH_2Cl_2, dark, rt, 2 days, 55% or α-D-tetraacetate galactosyl bromide **[22]**, silver oxide, quinoline, benzene, dark, rt, 3 days, 19%; iii, 3HF•Et_3N:NMP:Et_3N (2: 3: 1.5), dark, 60°C, 1hr, 81%; iv, 2-cyanoethyl-*N',N*-diisopropyl-chlorophosphoramidite, DIPEA, THF, rt, 1hr, argon, 82%

Figure 2.15 - Synthesis of non-fluorescent fluorescein-bis-β-D-galactoside phosphoramidite monomer

Two oligonucleotides were prepared using standard solid phase DNA synthesis; T_{15} **[26]** control and BT_{15} (**[27]**, where indicates phosphoramidite **[25]** used). Two peaks eluted on RPHPLC of **[27]** (figure 2.16); the earlier peak (a) had the same retention time as **[26]** control indicating this peak was a T_{15} oligonucleotide, the later peak (b) was the desired non-fluorescent labelled oligonucleotide. The identity of both peaks was confirmed by electrospray mass spectrometry.

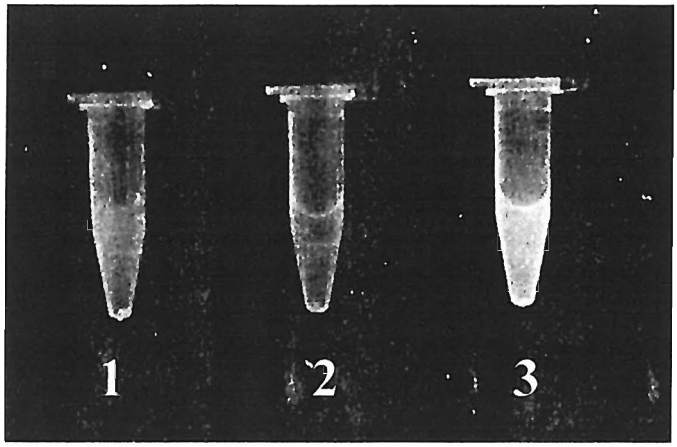


(i) (ii)

Figure 2.16 – i, RPHPLC of **[27]**, ii, ES^- **[27]**, calculated MW 5360.97

2.3.3 Evaluation of the Signal Probe **[27]**

A cleavage assay, in which the oligonucleotide labelled with the β-D-galactosyl protected fluorescein monomer **[25]** (the signal probe, **[27]**) was exposed to β-D-galactosidase, confirmed the galactosyl fluorescein ethers as effective substrates for the enzyme[159] (figure 2.17). A tube only containing signal probe emitted a very low level of background fluorescence (tube 1). A tube containing **[26]** and β-D-galactosidase emitted no fluorescence (tube 2). However, the tube comprising of signal probe **[27]** and β-D-galactosidase became intensely fluorescent over a 30minute period (tube 3). The contents of tube 1 and 3 were examined by UV spectroscopy, both samples absorbed at 260nm, but tube 3 displayed a characteristic fluorescein absorbance at 495nm.



tube 1 tube 2 tube 3

Figure 2.17 - Assay results (as viewed under transilluminator)

An identical assay performed in a UV spectrometer cell, monitoring at 495nm, demonstrated the increase in absorbance with time due to the enzymatic turnover of substrate (figure 2.18, i). A control experiment omitting the β-D-galactosidase showed insignificant change over the thirty minute period (figure 2.18, ii). .

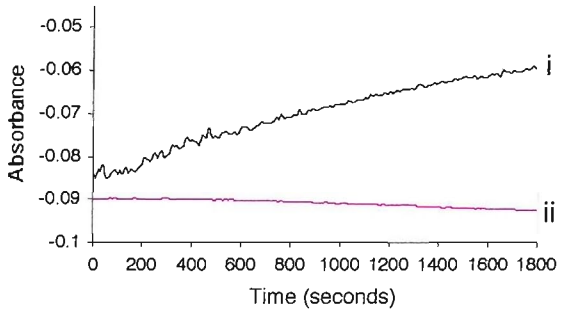


Figure 2.18 - UV monitored assay, i, assay of **[27]**, ii, control (no β-D-galactosidase)

2.3.4 β-D-Galactosidase-Oligonucleotide Enzyme-probe 1

Following the successful synthesis and enzymatic recognition of the signal probe **[27]**, the next step was to examine the conjugation of β-D-galactosidase to an oligonucleotide. Coupling of enzymes to proteins is a well-established technique[160], and the same principles have been applied in the preparation of oligonucleotide-enzyme conjugates[137]. Literature

searches produced no evidence of β-D-galactosidase coupled to oligonucleotides, however methods for attachment to various proteins have been described[161,162]. Attachment is achieved using a range of crosslinking reagents specific for any of the twenty free thiols on β-D-galactosidase and reactive groups on the protein. One example of conjugation between β-D-galactosidase and an antibody is shown in figure 2.19. Crosslinking reagents are homo- or heterobifunctional molecules containing two reactive functionality's that target reactive groups (amines, thiols, and carboxylic acids) on proteins or oligonucleotides[163]. The crosslinking reagent covalently links two biological molecules, providing a spacer that can reduce steric interactions. It is imperative that the crosslinking reagent or crosslinking reaction does not interfere with the performance of any biological functions.

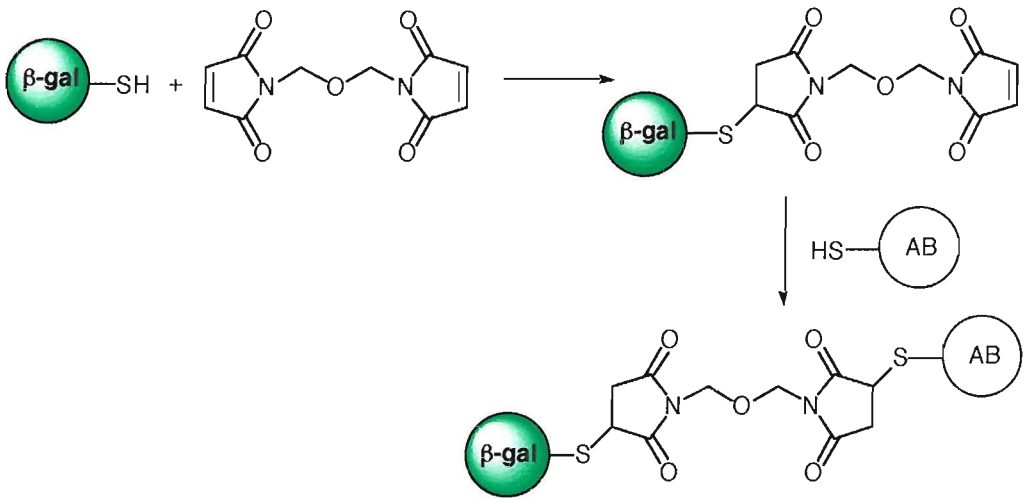
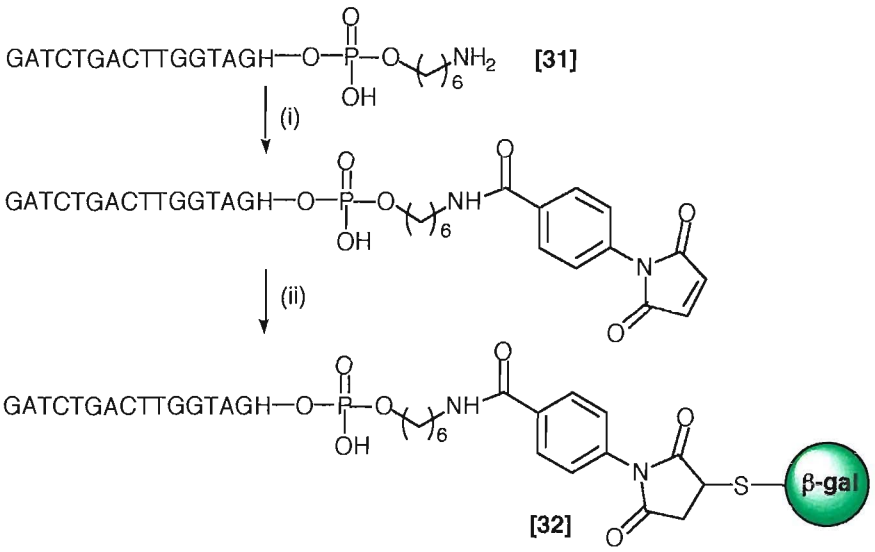


Figure 2.19 - Conjugation of β-D-galactosidase (β-GAL) to an antibody (AB) using dimaleimide, *N,N'*-oxydimethylenedimaleimide crosslinking reagent

By modifying reported methods a convenient strategy for conjugation of β-D-galactosidase to an oligonucleotide was proposed. Experiments were primarily performed on the 5' terminus of an oligonucleotide in order to establish the correct conditions for attachment; subsequent experiments employed 3' aminolink oligonucleotides (**[31]**, figure 2.20).



Reagents and conditions: i, maleimidobenzoyl-*N*--hydroxysuccinimde ester, Na_2CO_3 buffer, pH 6.0, rt, 3hrs; ii, β-D-galactosidase, Na_2HPO_4 buffer, pH 5.6, rt, 18hrs

Figure 2.20 – Conjugation of β-D-galactosidase to a 3' aminolink oligonucleotide

2.3.4.1 *Synthesis of MBS Oligonucleotide*

The preparation of aminolink oligonucleotides is carried out using commercial aminohexyl phosphoramidite monomers, the synthetic details have been well documented by Agrawal[50]. Initial attempts to couple maleimidobenzoyl-*N*-hydroxsuccinimde ester (MBS) at pH 7.0 to an amino-derivatised oligonucleotide **[28]** gave poor yields and demanded optimisation. Reactions of *N*-hydroxysuccimide esters of dye molecules with aminolink oligonucleotide are generally carried out at pH 8.75; therefore the pH of the reaction buffer was raised to this value. This change appeared to dramatically improve the yield (70%), however, the isolated product (RPHPLC) of the reaction failed to couple with β-D-galactosidase, as analysed by gel filtration and ion exchange chromatography of the products. Examination of the product revealed a molecular ion corresponding to a hydrated product formed by attack of hydroxide at the double bond (figure 2.21).

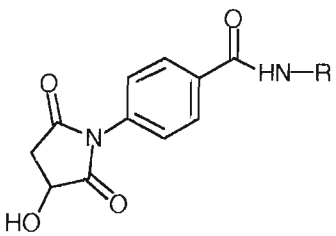


Figure 2.21 - Hydrated MBS product

This indicated a conjugate Michael addition of water had occurred at pH 8.75 rendering the derivative unreactive towards crosslinking with the enzyme. It was concluded that at pH 7.0 the major peak by RPHPLC (retention time 10.8min) was the desired product. At pH 8.75 a small peak of the same retention time is present (8%) but the major peak (52%, retention time of 10.1min) corresponds to the hydrated product (figure 2.22, i). When the reaction was repeated at pH 6.0 the undesirable hydrated peak contributed 3% of the total product and the desired alkene 34% of the total (54% unreacted starting material, figure 2.22, ii). It was concluded that the reaction of MBS with primary aminolink oligonucleotides should be carried out at pH 6.0 to prevent hydration of the desired unsaturated product, this observation has been made by others [164].

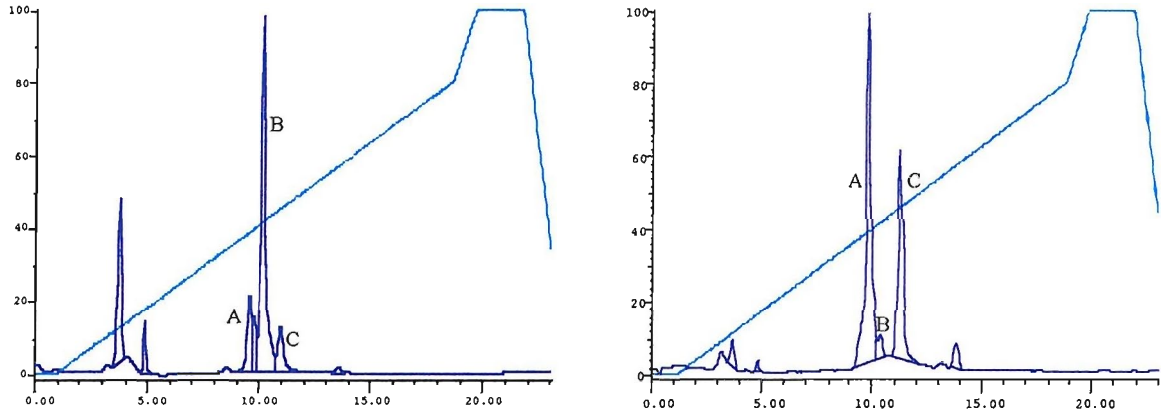


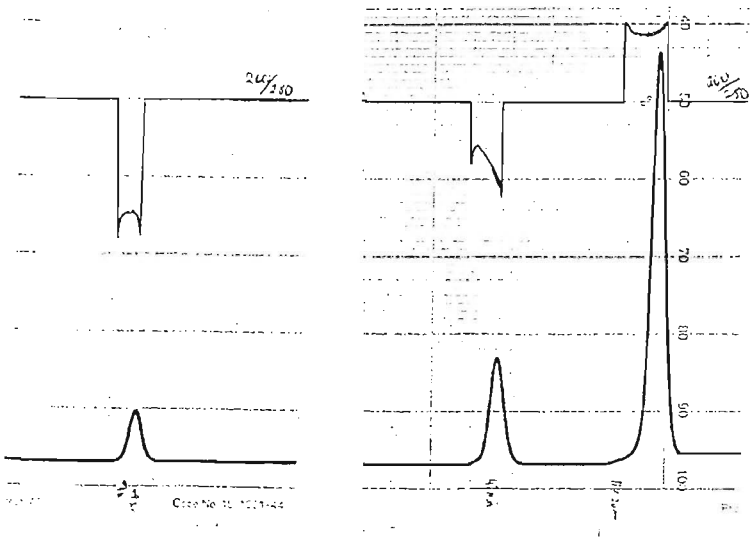
Figure 2.22 - RPHPLC i, pH 8.75; ii, pH 6.00; A: unreacted starting material; B: hydrated product; C: desired product

2.3.4.2 Conjugation of MBS derivatised oligonucleotide to β-D-Galactosidase

The purified MBS oligonucleotide was reacted with β-D-galactosidase in phosphate buffer at pH 5.6. Purification of the conjugate was achieved by gel filtration followed by ion exchange chromatography on a Pharmacia FPLC system. Peak elution was monitored by the ratio of absorption at 260/280nm; proteins giving large negative peaks, oligonucleotides strong positive peaks and conjugates weak negative peaks.

Gel filtration separates according to size, larger molecules are eluted first, as they can not enter the pores of the gel as easily as smaller molecules, and elution is achieved isocratically. The trace obtained from the native enzyme is a single peak (49min) with a

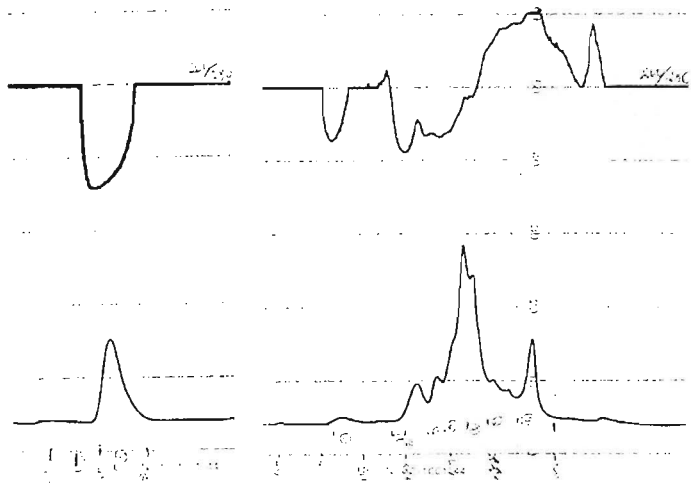
strong absorbance at 280nm (a, figure 2.23). The trace obtained from the conjugate produced two peaks, the first (45min) gave a shallower absorbance peak corresponding to the conjugate, the second (85min) was unreacted oligonucleotide absorbing strongly at 260nm (b, figure 2.23).



(a) (b)

Figure 2.23 - Gel filtration chromatography: a, β-D-galactosidase, b, conjugation reaction

Ion Exchange separates according to charge and gives greater resolution, separating the conjugate into several peaks of varying degrees of substitution of the enzyme by the oligonucleotide. The trace obtained from the native enzyme was a single peak (a, figure 2.24), whereas the conjugate gave approximately nine peaks (b, figure 2.24).



(a) (b)

Figure 2.24 - Ion exchange chromatography: a, β-D-galactosidase b, conjugation

With these results in hand a large-scale synthesis of the conjugate with a 3' amino derivatised oligonucleotide **[31]** was performed, coupling was again highly successful producing the enzyme probe 1 **[32]**.

2.3.5 Enzyme-probe 1 Activity

Enzyme activity of each fraction was verified by the turnover of the substrate *o*-nitrophenol-β-D-galactopyranoside. Hydrolysis of this substrate releases the yellow product *o*-nitrophenol which absorbs 405nm (figure 2.25).

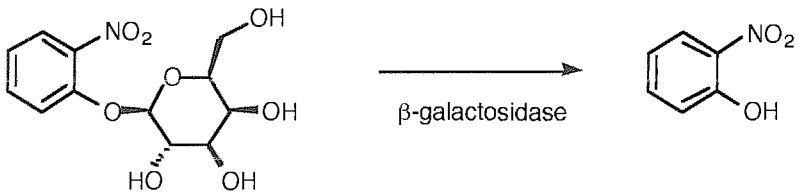
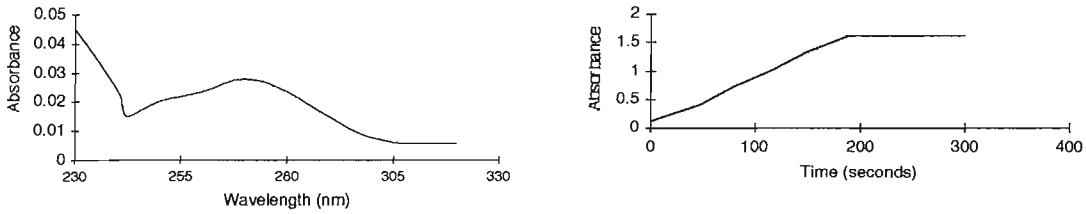


Figure 2.25 - Hydrolysis of *o*-nitrophenol-β-D-galactopyranoside by β-D-galactosidase

The conjugate fractions from ion exchange chromatography were also analysed for the necessary absorptions at 260nm for DNA, and 280nm for protein (figure 2.26, i). The peaks that displayed high rates of substrate turnover (figure 2.26, ii), and the correct absorption maxima for the protein (280nm) and the oligonucleotide (260nm), were combined **[32]**.

In order to establish the relative activity of the conjugate compared to that of the native enzyme, the turnover of one micromole of substrate by β-D-galactosidase was monitored at 405nm at 37°C over a five minute period. The experiment was repeated using the same quantity of substrate and 10μL of conjugate. From the gradient of the linear section of the two curves, and taking into account the relative amounts of native enzyme to conjugate used, calculations showed the conjugate exhibited a sixth of the activity of the free enzyme.

The approximate amount of DNA present in the conjugate was calculated to be $9.4\mu gmL^{-1}$ from the optical density reading at 260nm, the molecular weight of the oligonucleotide, and its molar extinction coefficient.



(i) (ii)

Figure 2.26 - UV traces i, typical spectrum of conjugate, ii, hydrolysis of *o*-nitrophenol-β-D-galactopyranoside by the β-D-galactosidase-oligonucleotide conjugate **[32]**

2.3.6 SDS Page Electrophoresis

To investigate further the nature of the β-D-galactosidase conjugate SDS-polyacrylamide gel electrophoresis was performed, and the bands were visualised by highly sensitive silver staining.

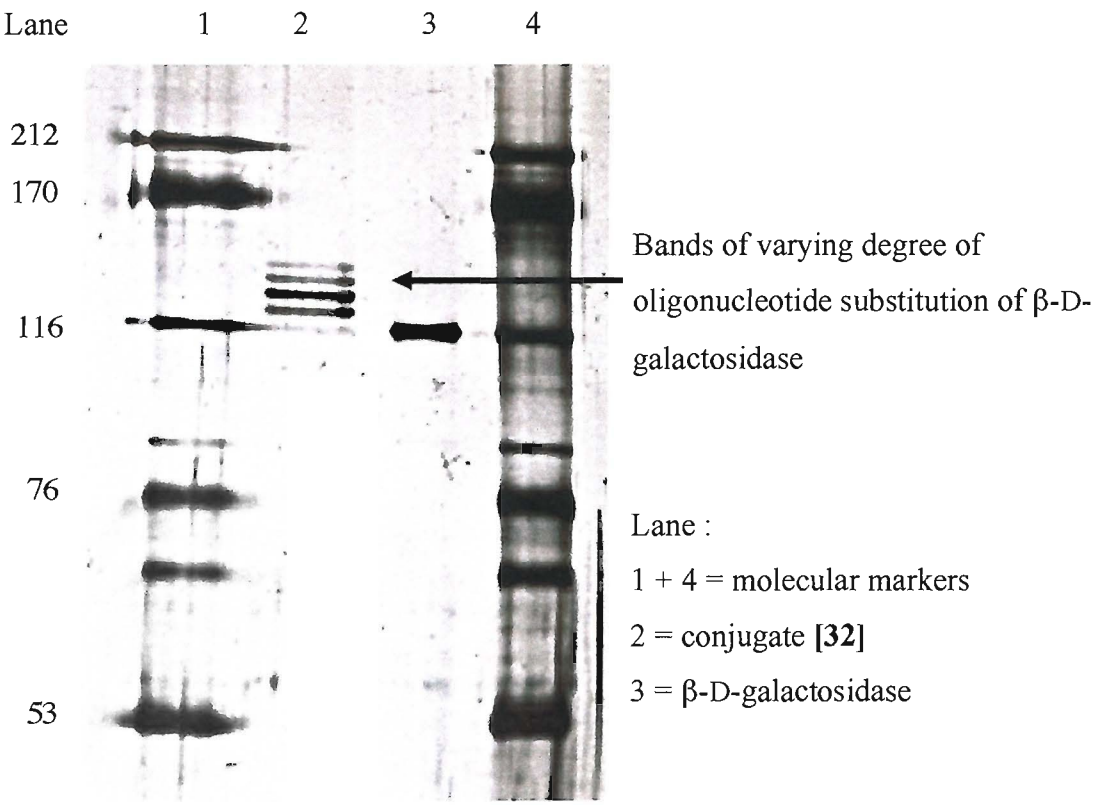


Figure 2.27 - Gel electrophoresis demonstrating the substitution of β-D-galactosidase with oligonucleotides

The gel obtained clearly displayed the conjugate with four bands of varying degrees of oligonucleotide substitution of the enzyme. The first two bands, corresponding to one and two oligonucleotides substituting the enzyme, are the most intense, therefore the most abundant. The molecular marker at 116 and lane 3 are native β-D-galactosidase proving there was little or no free enzyme present in the conjugate mixture **[32]**.

2.3.7 Assay Evaluation (as described section 2.1)

The β-D-galactosidase conjugate (enzyme probe 1, **[32]**) and signal probe had been assembled enabling the assay to be designed and evaluated. Oligonucleotides to act as target DNA and the signal probe were designed and synthesised. Figure 2.28 depicts the three-strand system (1) which brought the substrate and enzyme in head to head contact and system (2) which incorporated a short stem to keep the substrate and enzyme away from the target third strand. Enzyme and substrate were separated from the oligonucleotide strands by hexaethylene glycol spacers, this was to increase the degree of freedom to try and promote interactions between the pair.

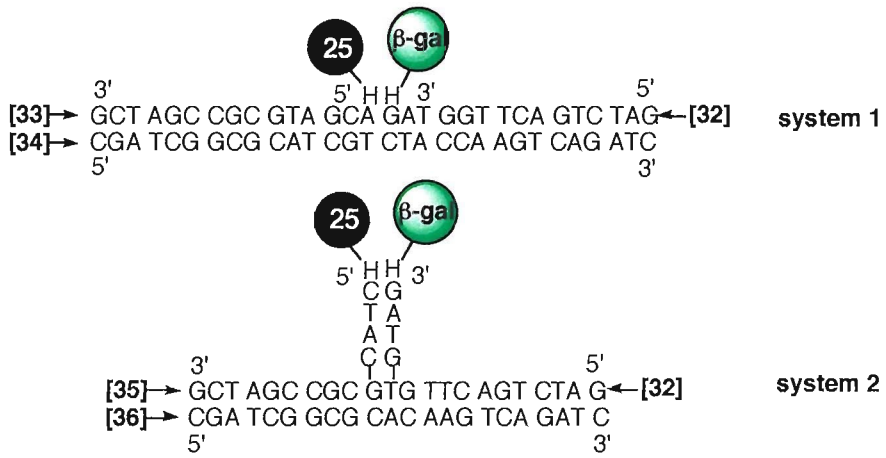
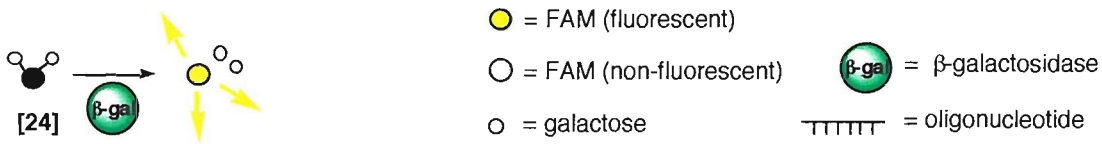


Figure 2.28 - Systems (1) and (2) designed to test the assay

To assess the performance of individual components of the assay experiments 1-5 were conducted in a fluorimeter cuvette and the increase in fluorescence absorption (495nm) was monitored in each case.



i) The substrate **[24]** was placed in buffer but was relatively insoluble even with the addition of DMSO, β-D-galactosidase was subsequently added. Fluorescence intensity increased from 125 to 632 over a 24 hour period indicating the slow hydrolysis of the substrate (figure 2.29).

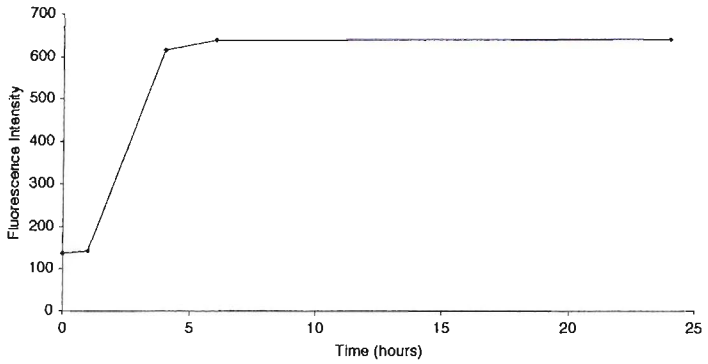
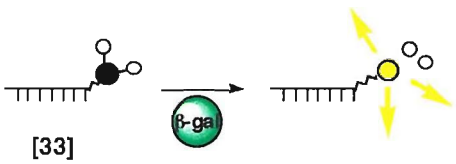


Figure 2.29 - Hydrolysis of β-galactose FAM molecule **[17]** by β-galactosidase over 24 hour, monitored by increase in fluorescence emission at 520nm.



ii) The oligonucleotide substrate (signal probe, **[33]**) was placed in buffer and β-D-galactosidase was added. Fluorescence intensity increased from 670 to over 1000 over 24 hours (figure 2.30). The background fluorescence of the substrate was higher than in the first experiment and the reaction proceeded at a much faster rate. These observations were explained by the increased solubility of the substrate in aqueous buffer due to the hydrophilic oligonucleotide.

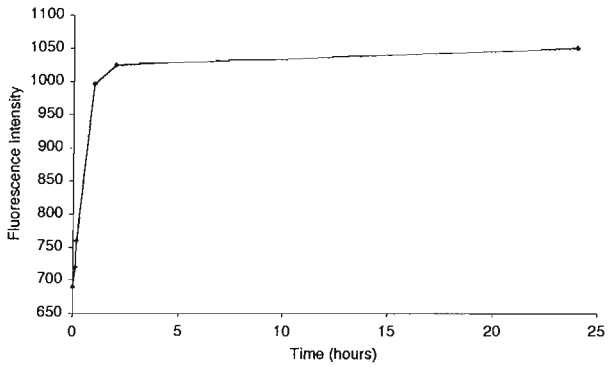
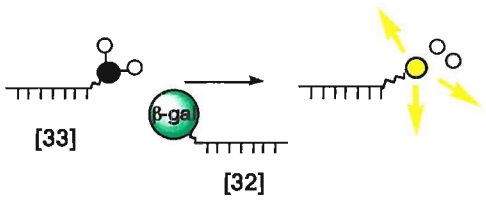


Figure 2.30 -Hydrolysis of β-galactose signal probe **[33]** by β-galactosidase over 24 hours, monitored by increase in fluorescence emission at 520nm.



iii) The signal probe **[33]** was placed in buffer and the β-D-galactosidase oligonucleotide conjugate (enzyme-probe, **[32]**) was added, no target strand was present therefore the two oligonucleotides remained free in solution. The fluorescence intensity reading increased from 360 to 462 over 24 hours (figure 2.31). The rate of hydrolysis of the signal probe by the enzyme-probe free in solution was slow and the change in fluorescence intensity was small. The slow hydrolysis was desirable if a significantly increased rate caused by hybridisation of the two probes adjacent to one another was to be observed.

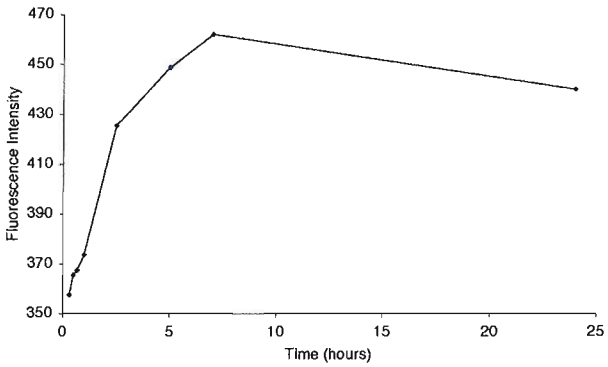
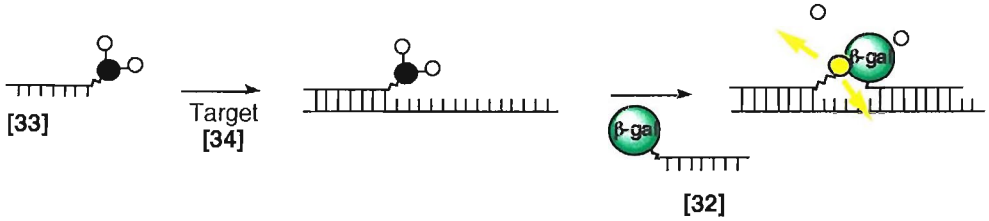


Figure 2.31 - Hydrolysis of β-galactose signal probe **[33]** by β-galactosidase-oligonucleotide conjugate (enzyme probe, **[32]**) in the absence of a target nucleic acid, over 24 hours. Monitored by increase in fluorescence emission at 520nm.



iv) The final experiment was designed to test the assay. In this case the signal probe **[33]** was hybridised to the target **[34]** first by heating to 80°C followed by slow cooling to room temperature. The enzyme-probe **[32]** was added and the change in fluorescence was monitored. The substrate was hydrolysed slowly, an increase in fluorescence intensity from 470 to 585 was observed with no significant change over 24 hours (figure 2.32). There was no notable increase in rate of hydrolysis or the change in fluorescence intensity in the presence of a target compared to the absence of a target (experiment iii). The results were consistent for both assay systems 1 and 2.

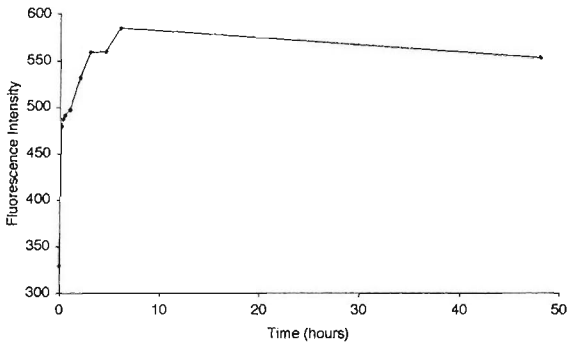


Figure 2.32 - Hydrolysis of β-galactose signal probe **[33]** by β-galactosidase-oligonucleotide conjugate (enzyme probe, **[32]**) in the presence of a target nucleic acid **[34]**, over 24 hours. Monitored by increase in fluorescence emission at 520nm.

2.3.8 Enzyme-probe 1 Hybridisation

The results of the initial assay evaluation suggested that hybridisation between the enzyme-probe and its solution complementary oligonucleotide was not occurring. Confirmation of the hypothesis was secured by the results of UV thermal melting studies and dot blot assays.

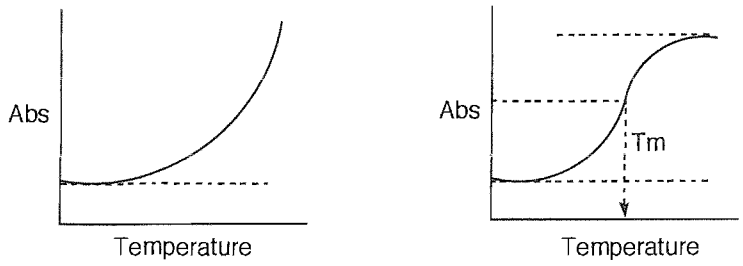
2.3.8.1 UV Thermal Melting

Slow heating of dsDNA causes the unwinding of the ordered helical structure into the two single-stranded constituents. This can be measured by a smooth transition of increasing UV absorption (260nm), the mid-point corresponding to the precise melting temperature of the duplex. UV melting experiments can be performed to determine the extent of annealing of two complementary strands by observing the shape of the curve produced on heating (section 1.5.3).

Equal samples of the target oligonucleotide **[34]** and the enzyme-probe **[32]** were mixed in hybridisation buffer. The sample was heated to 90°C and cooled slowly to room temperature, allowing efficient annealing of the two strands. The sample was then heated from 5°C to 85°C over 80 minutes following the change in absorbance at 260nm, this was repeated three times. Each melt showed the same gradual increase in absorbance from 0.231 to 0.291 (figure 2.33, i) but the transition expected due to the dissociation of the strands was not observed (figure 2.33, ii). A sample of the native β-D-galactosidase was melted under the same condition this produced an extremely similar curve to that of the hybridisation experiment.

Proteins also absorb UV light (λ_{max}=280nm) due to the bulky, aromatic, hydrophobic side chains of phenylalanine, tyrosine and tryptophan amino acids. At the wavelength of the experiment (260nm) there will be some absorbance by the protein. Elevated temperatures will denature proteins, causing the unwinding of the tertiary structure; this increase in disorder exposes a greater number of aromatic amino acid residues to UV irradiation and the absorbance will increase.

It was concluded that the observed increase in absorbance was due to conformational change of the enzyme with rising temperature, and not to the unwinding of the annealed oligonucleotides. The question of hybridisation of the target and enzyme-probe had not been resolved by this experiment, the effects of the protein masked the occurrence of a hybrid melting transition.



(i) (ii)

Figure 2.33 - UV thermal melting i, observed curve, ii, expected curve

2.3.8.2 Dot Blot Assay

The principle lies in the UV crosslinking of an oligonucleotide to a nitro-cellulose or nylon membranes (neutral or positively charged) via its heterocyclic bases[30]. Multiple samples of DNA are spotted next to each other on a single filter in dots of uniform diameter. For quantitative analysis known amounts of DNA are applied, usually by serial dilution of a standard to evaluate the extent of hybridisation[32]. As little as 1-3pg of DNA can be detected. The membrane is exposed to a solution of a complementary labelled oligonucleotide, which becomes immobilised on the membrane by annealing to the membrane-crosslinked oligonucleotide. Following stringent washing procedures, a label bound to the second oligonucleotide can be detected directly or, in the case of an enzyme label, by means of hydrolysis of a substrate releasing a coloured product.

In the case of the β-D-galactosidase conjugate (**[32]**, enzyme-probe), firstly the target oligonucleotide **[34]** was UV cross-linked to a nylon membrane. Secondly this membrane-bound oligonucleotide was exposed to an excess of β-D-galactosidase conjugate **[32]** for annealing. Unbound material was removed by membrane washing; the presence of bound conjugate was detected by the turnover of Xgal that produces an insoluble blue/green product on contact with β-D-galactosidase. After 3 days there was little evidence of the colourimetric product forming insinuating either the target had not bound to the membrane or more probably there had been no annealing between target and conjugate.

The conjugate **[32]** was directly spotted onto the nylon membrane and the oligonucleotide was covalently attached by UV crosslinking, but again colour failed to develop with Xgal substrate. The oligonucleotide of the conjugate was not able to crosslink to the membrane. From the UV melting and dot blot experiments it was concluded that the oligonucleotide linked to the β-D-galactosidase was not forming a duplex with its complementary target. This conjecture explained the slow hydrolysis observed in initial attempts to test the assay in the presence of a target oligonucleotide (experiment iv, section 2.3.7). Lack of, or poor annealing of the enzyme-probe to the target implied all enzymatic cleavage that occurred was due to random collision of the substrate and enzyme in solution, thus explaining the similarity in result between experiments iii and iv, section 2.3.7.

2.3.9 Factors Influencing Formation and Stability of Hybrids

The disappointing result from initial assay evaluation called for a re-examination of the conditions required for efficient enzyme-probe-target hybrid formation. Numerous factors effect the rate and stability of hybrid formation[40,165]. One factor is the relative concentrations of the probe or target, however all hybridisation experiments were performed with either a large excess of target, a large excess of enzyme-probe or equimolar quantities of each strand and in all cases hybridisation failed to occur. Monovalent cation concentration influences hybridisation and can alter the Tm of a duplex, yet results showed hybridisation was not occurring either at 0.1M or at 1.0M sodium chloride concentrations. Generally, as temperature increases the rate of hybridisation increases, provided the temperature is below the Tm of the duplex in question. The experiments performed gave no indication of a dsDNA to ssDNA transition; therefore further investigation of the effect of temperature was not necessary.

The three most important factors controlling the annealing of the enzyme-probe with a complementary target are length of the oligonucleotide, base composition of the oligonucleotide, and steric hindrance caused by the enzyme. The length of an oligonucleotide probe has a considerable effect on the rate and stability of the hybrid formed. Hybrids of a hundred or more base pairs are essentially stable at room temperature,

however, for shorter probes a decrease in length causes a decrease in Tm of the hybrid, some hybrids requiring temperatures below room temperature to form efficiently.

Altering the base composition of a duplex can raise or lower its Tm, as oligonucleotides with a higher (%G+C) form more stable hybrids. The effect has been determined empirically by measuring the dissociation temperature of hybrids relative to the length and (%G+C) content of oligonucleotides.

Bolton and McCarthy proposed an equation that is said to hold for oligonucleotides from 14 to 70 base pairs in length[166]

$$Tm = 81.5°C - 16.6 (\log_{10} M) + 0.41 (\%G + C) - 600 / L$$

Where M is the concentration of monovalent cation, L is the chain length of the hybrid in base pairs, Tm is the temperature at which 50% of the hybrids are dissociated and G and C are the number of corresponding nucleotides in the sequence.

Applying this equation to the 15mer β-D-galactosidase enzyme-probe it can be seen that the (%G+C) content is 46.6% and the predicted Tm is 77.2°C. In an attempt to improve hybridisation of the enzyme-probe to its target, an oligonucleotide for a new enzyme-probe was designed GAT CTG ACT TGG TAG ACT ATG CGC CGA TCG HHH (H = hexaethylene glycol. The (%G +C) content of the oligonucleotide was raised slightly to 53.3% but the length was doubled giving a new predicted Tm of 99.9°C (based on a constant salt concentration of 0.1M throughout). Therefore, the change in oligonucleotide length and content produces a more stable probe-target hybrid as the Tm of the duplex is elevated.

The final problem of steric interference by the β-D-galactosidase was a more difficult issue. Linking a longer oligonucleotide to the enzyme may increase the stability of a hybrid formed but if the oligonucleotide attached to the enzyme is inaccessible to its target then the hybrid will not form regardless of oligonucleotide length. Building a bridge of hexaethylene glycol spacers between the oligonucleotide and the enzyme allows both

partners enhanced flexibility. The original 15-mer enzyme-probe had one hexaethylene glycol spacer between β-D-galactosidase and the 3' of the oligonucleotide; the new probe was designed to incorporate three.

2.3.10 β-D-Galactosidase-Oligonucleotide Enzyme-Probe 2

The second longer enzyme-probe 2 **[37]** was prepared and analysed by the same methods as the first enzyme-probe 1 **[32]** (sections 2.3.4, 2.3.5, and 2.3.6). The 30-mer oligonucleotide derivatised at the 3' end with an amino moiety was reacted with MBS in a 31.1% yield and subsequently coupled to β-D-galactosidase and analysed by gel filtration, ion exchange chromatography and SDS page electrophoresis. The enzymatic activity of the conjugate 2 **[37]** was confirmed as before (section 2.3.5) and the amount of DNA present in the conjugate was calculated to be 19.6μgmL^{-1} from the optical density reading at 260nm (0.54mL^{-1}), the molecular weight of the oligonucleotide (10,480), and its molar extinction coefficient (287.28).

2.3.11 Enzyme-probe 2 Hybridisation

2.3.11.1 UV Thermal Melting

In a control experiment an oligonucleotide identical in sequence to the oligonucleotide of the enzyme-probe **[38]** was annealed to its perfectly complementary target **[39]**. UV thermal melting of the duplex gave a Tm in excess of 85°C in agreement with a computer generated prediction of 92°C. A second control experiment heated the enzyme probe 2 **[37]** in buffer alone from 5.3°C to 83.3°C. This displayed a gradual rise in absorbance, without any significant transition, as the protein unfolds. UV melting the conjugate annealed to its complementary target from 6°C to 84°C displayed a weak transition at 51.3°C. Unfortunately a control UV melt of the target **[39]** on its own produced a similar transition at 50°C, the nature of this transition was investigated by computer simulation but still remains unknown.

2.3.11.2 Dot Blot Assay

The target **[34]** was UV cross-linked to a nylon membrane, and then incubated with excess of enzyme-probe 2 **[37]** for annealing. Unbound material was removed by membrane washing; the presence of bound conjugate was investigated by the turnover of the substrate Xgal, no blue/green product appeared.

The enzyme-probe 2 **[37]** was directly spotted onto the nylon membrane and the oligonucleotide was bound by UV crosslinking, but again after washing, the blue colour failed to develop with Xgal substrate. However, repetition of the experiment with very concentrated samples of the probe produced very faint spots.

Finally native β-D-galactosidase control and the enzyme-probe 2 **[37]** were spotted onto nitro-cellulose membrane (binds proteins directly). β-D-galactosidase developed the expected strong blue/green spot, the probe gave a weak coloured spot.

Hybridisation of two oligonucleotides in solution is sterically favoured over membrane-bound hybridisation. It can be concluded from the dot blots that the enzyme-probe can not hybridise to a membrane-bound target or bind via the oligonucleotide to a membrane. However, target-probe hybrids may be forming in solution. UV thermal melting results would normally confirm hybridisation but in this case the results are complicated and inconclusive

2.3.11.3 Molecular Beacon Approach

Molecular beacons are single-stranded oligonucleotides probes designed to detect specific nucleic acids in homogeneous solution by the generation of fluorescence upon hybridisation[114] (see section 1.6.2). A molecular beacon was synthesised with a loop sequence complementary to the oligonucleotide portion of the enzyme-probe 2 FCGCACGCGATCGGCGCATAGTCTACCAAGTCAGATCCGTGCGM **[40]**

(i)The molecular beacon (**[40]**, 0.04OD) was heated to 90°C and allowed to cool slowly to room temperature, this achieves efficient annealing of the stems. The sample was heated and cooled between 20°C to 80°C in the absence of a target strand (figure 2.34). The curve displays the expected result, increasing temperature causes increasing fluorescence due to

stem dissociation and the molecular beacon adopting random conformations. Decreasing temperature allows re-annealing of the stem and a drop in fluorescence to original background signal. The melting temperature of the hairpin was 60°C.

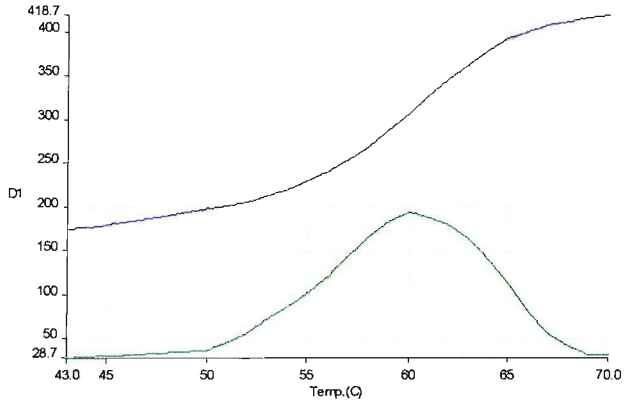


Figure 2.34 - Molecular beacon **[40]** heated and cooled between 20°C to 80°C in the absence of a target strand, monitored at 520 nm.
Derivative of fluorescent melting curve displaying a hairpin melting temperature of 60°C

(ii) Addition of an excess of target (**[38]**, 0.198OD, 4.4eq) to the molecular beacon sample, followed by heating to 80°C produced the same stem-dissociation curve as observed in (i). However, no drop in fluorescence intensity was observed when cooled to 20°C or reheated to 80°C, the entire molecular beacon was annealed to the target (figure 2.35).

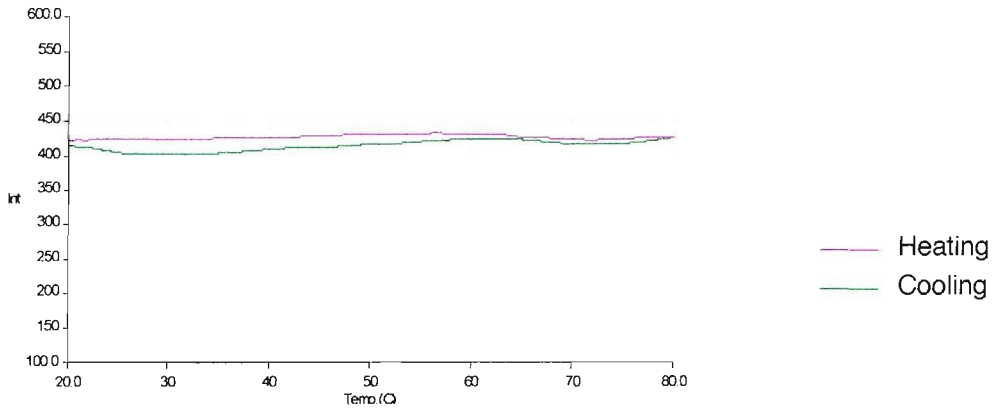


Figure 2.35 - Molecular beacon **[40]** heating and cooling between 20°C and 80°C after annealing to a target in excess (**[38]**, 4.4eq), monitored at 520nm

(iii) The molecular beacon was heated with an excess of enzyme-probe 2 (**[37]**, 0.108OD, 2.4eq) to 80°C giving stem dissociation curve initially observed. Cooling to 20°C caused a fall in fluorescence intensity yet the final intensity reached was a third of its original value (figure 2.36).

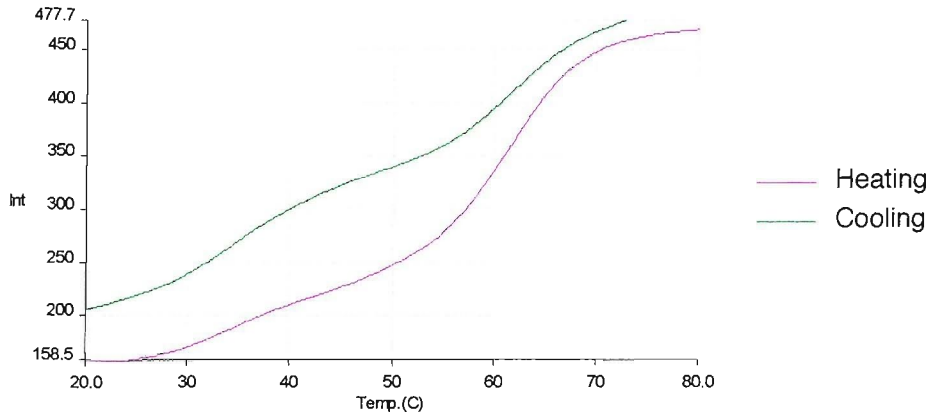


Figure 2.36 - Molecular beacon **[40]** heating from 20°C to 80°C with the enzyme-probe 2 (2.4eq), and subsequent cooling from 80°C and 20°C, monitored at 520 nm

It was concluded a proportion of the oligonucleotides attached to β-D-galactosidase were sterically accessible to the molecular beacon and hybridisation of these oligonucleotides maintained a level of fluorescence. The remaining molecular beacon re-adopted the hairpin conformation at lower temperatures and fluorescence intensity was reduced. The result demonstrated that hybridisation of the molecular beacon to the enzyme-probe was taking place, but the extent of hybridisation was not known.

2.3.12 Assay Evaluation (as described in section 1.2)

Evidence of enzyme-probe hybridisation prompted the re-evaluation of the assay. Figure 2.37 depicts assay systems (1) and (2) that required four new oligonucleotides, two targets (**[42]**, **[44]**) and two signal probes (**[41]**, **[43]**). System (1) brought the substrate and enzyme in head to head contact and system (2) incorporated a short stem to keep the substrate and enzyme away from the target third strand. Enzyme and substrate were separated from the oligonucleotide strands by hexaethylene glycol spacers to increase their flexibility encouraging enzymatic scission.

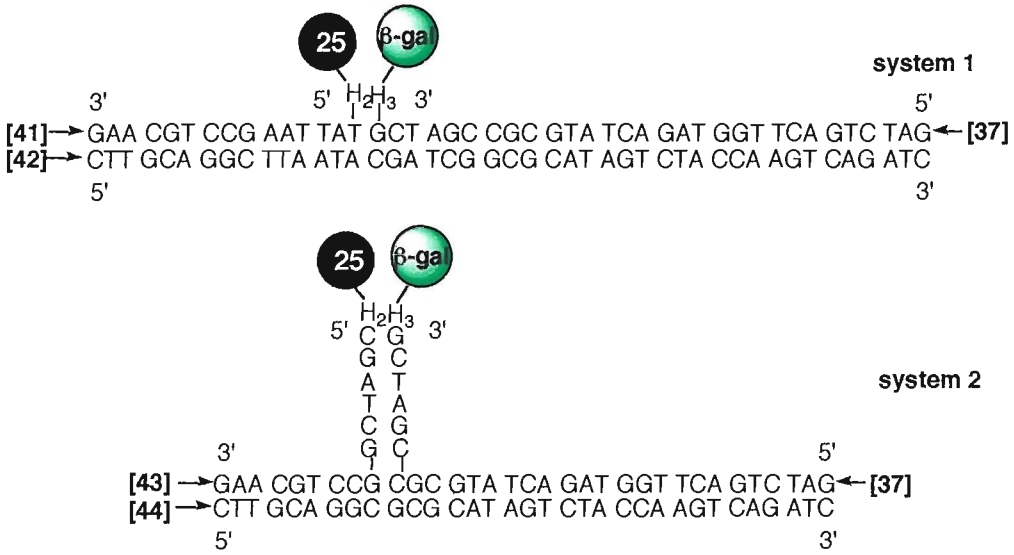
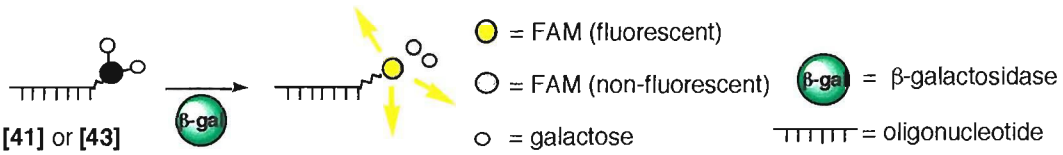
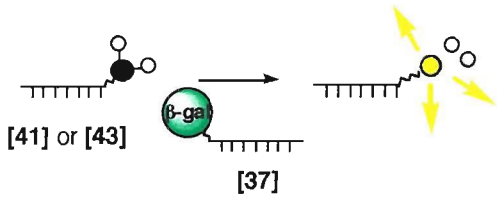


Figure 2.37 - Systems (1) and (2) designed to re-test assay

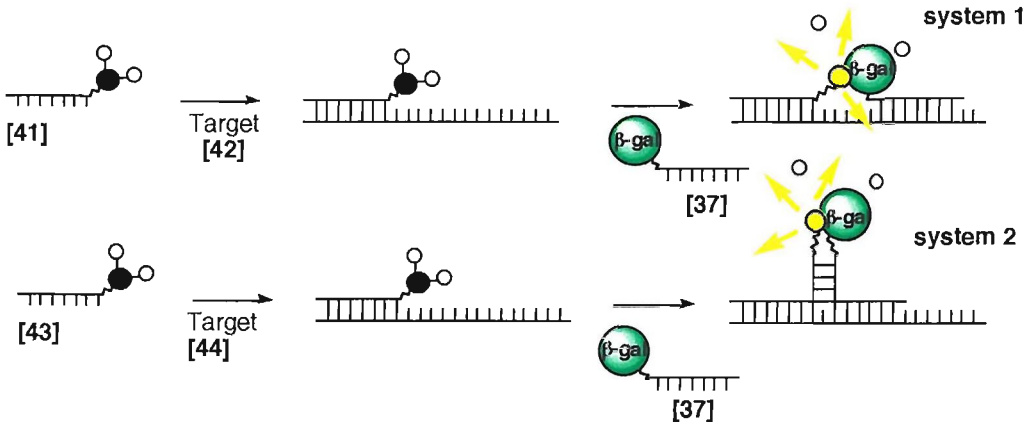
Each assay was conducted in stages in a fluorescence spectrometer and the change in fluorescence was monitored over time.



i) The signal probe ([**41**], [**43**], 0.19nmol) was placed in buffer, excess β-D-galactosidase was added and the change in fluorescence intensity was monitored over 1 hour. The signal probes from system 1 and 2 gave similar curves of increasing intensity with the final reading a three fold increase of the background reading (figure 2.38, i).



ii) The signal probe ([**41**], [**43**], 0.19nmol) was placed in buffer and an equimolar amount of enzyme-probe ([**37**], 0.19nmol) was added, no target strand was present and the two oligonucleotides remained free in solution. Over the course of one hour both systems 1 and 2 showed no significant change in fluorescent intensity (figure 2.38, ii).



iii) The assay was tested with both systems, in each case the signal probe (**[41]**, **[43]**, 0.19nmol) was hybridised to the target (**[42]**, **[44]**) by heating followed by slow cooling. The enzyme-probe (**[37]**, 0.19nmol) was added and the change in fluorescence was monitored over 1 hour. Again, both systems expressed no notable change in fluorescent intensity (figure 2.38, iii).

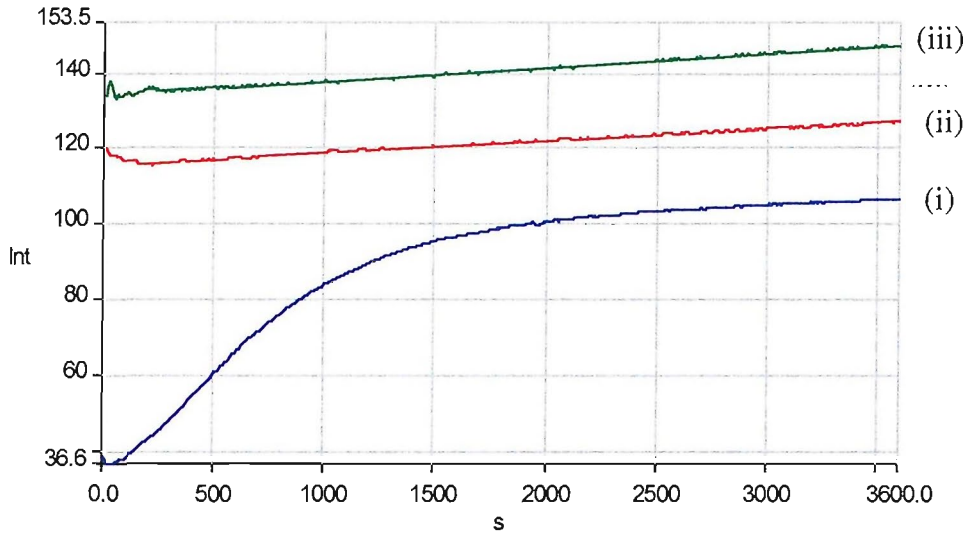


Figure 2.38 -

——— Signal probe **[41]** with excess β-galactosidase, fluorescence intensity at 520nm over 1 hour.

——— Signal probe **[41]** with an equimolar amount of enzyme-probe 2 (**[37]**), no target strand, fluorescence intensity at 520nm over one hour.

——— Signal probe **[41]** with an equimolar amount of enzyme-probe 2 (**[37]**), with target strand **[42]**, fluorescence intensity at 520nm over one hour.

2.3.13 Conclusions

The synthesis of a non-fluorescent phosphoramidite monomer **[25]** was accomplished, and the monomer was utilised in the assembly of a range of oligonucleotide signal probes. The signal probe was an efficient substrate for native β-D-galactosidase, demonstrated by the visualisation of fluorescence and the curve generated upon enzymatic hydrolysis of the substrate. Conjugation between β-D-galactosidase and a 15mer oligonucleotide was achieved creating an enzyme probe 1 for the intended assay. However, results established that the enzyme probe would not hybridise to a complementary synthetic DNA target. In the absence of annealing, the rate of signal probe hydrolysis and the intensity of fluorescence released were shown to be similar with or without a target nucleic acid.

On the basis of these results a second enzyme probe 2 **[37]** was designed which was longer (30mer) and slightly more GC rich. Initial investigation suggested although the oligonucleotide of the enzyme probe would not anneal to a membrane, hybridisation may occur in homogeneous solution. Disappointingly, attempts to validate the proposed assay in two different systems did not produce significantly different changes in fluorescent intensity in the presence or absence of the target.

Previous successful enzyme probes have used much smaller enzymes such as alkaline phosphatase (AP, 89kDa) and horseradish peroxidase (40kDa). In these cases the conjugation to oligonucleotides has little affect on the hybridisation kinetics, selectivity or efficiency of the oligonucleotide itself (HRP conjugation; no change in Tm, AP conjugation; approximately 10°C fall in Tm[167]). β-D-Galactosidase is a relatively large enzyme with a molecular weight of approximately 500kDa[168] and has twenty free thiols for conjugation. However, the location of the thiols may mean that not all are involved in the conjugation reaction and the number of oligonucleotides per enzyme molecule may vary. Both enzyme probes prepared possessed oligonucleotides relatively small (15mer: 5kDa, 30mer: 10kDa) in comparison with β-D-galactosidase and it is conceivable that hybridisation is prevented for steric reasons.

The assay was carried out at the optimum pH for β-D-galactosidase activity (pH 7.5), at this pH the overall charge of the tetrameric enzyme is -158.78. This large negative charge excludes the possibility of electrostatic attraction between the protein and the conjugated oligonucleotides which bear an overall negative charge due to their phosphate backbones. However, poor annealing between the oligonucleotides linked to the protein and target nucleic acids may be explained by electrostatic repulsion of the incoming target oligonucleotides by the protein. This is an inherent problem as the pI of the enzyme is 5.28[169] it requires a pH of 5.28 or lower to possess a positive charge, however at this pH the enzyme is no longer active.

To study the assay further requires an enzyme probe capable of hybridisation to its complementary target. This might be achieved using a longer oligonucleotide for conjugation and a longer spacer between the oligonucleotide and enzyme. However, an intrinsic problem lies in the size of β-D-galactosidase which may always sterically prevent annealing. Another consideration for future development is signal amplification. Thermal cycling the annealing and enzymatic cleavage steps would enhance the fluorescent signal, yet this would require a thermostable β-D-galactosidase.

Chapter 3

Heterogeneous Assay

3.0 Heterogeneous Assay

This chapter explains the basis of heterogeneous assay and the development of new novel heterogeneous assays, which use support-bound probes to detect nucleic acid sequences in solution.

3.1 Introduction

Heterogeneous or separation assay is probably the most commonly used format for binding assays, and involves immobilisation of single-stranded DNA or RNA onto an inert solid phase[135]. The immobilised nucleic acid is capable of forming a hybrid with a single stranded probe added in solution, when incubated under conditions that favour duplex formation. The remainder of unbound probe can simply be washed away. The probing species can be either labelled directly or visualised after additional binding steps with secondary labelled species. The sensitivity of this assay format can be greater that its homogeneous rival as background signals and interference are reduced by repeated washing procedures removing excess unbound label.

3.2 Molecular beacons on beads

3.2.1 Introduction

Tyagi and Kramer recently reported a novel probe technology for the detection of specific nucleic acids in homogeneous solution[114]. The probes named 'Molecular Beacons' produce sensitive, real-time fluorescent signals that indicate hybridisation of a probe to a target nucleic acid[115,118]. They have a stem and loop structure, the loop portion probing for complementary target nucleic acids in solution and the stem consisting of two short arms one terminally labelled with a fluorophore and the other with a quencher. Annealing of the arms causes intramolecular energy transfer from the fluorophore to the quencher and the probe is non-fluorescent. Hybridisation of the loop to its target causes the stem arms to

dissociate, the fluorophore and quencher are no longer in close proximity and fluorescence is emitted (see section 1.6.2). Molecular beacons are designed so that the arm sequences are unrelated to the target and hybridisation of the arm sequences in the hairpin loop must produce a weaker interaction than target-probe annealing. There is only one recent example of molecular beacons supported on a solid phase. This involves labelling the beacon with biotin, the biotin then binds to surface-immobilised avidin linking the molecular beacon to the silica support[121].

The aim of the project was to adapt this technology to heterogeneous assay by supporting molecular beacons on solid phase. The intention was to show that resin-bound beacons exhibit similar properties to their homogeneous predecessors, non-fluorescent in the absence of a target nucleic acid and fluorescent in the presence of a complementary target. This technology could be harnessed in nucleic acid screening as hybridisation of resin-bound beacons to target DNA or RNA would generate fluorescent beads that could be isolated and analysed either directly or by cleavage of the oligonucleotide from the resin. The oligonucleotides could be examined by mass spectrometry[170,171].

The principle behind the molecular beacon beads is shown in figure 3.1. A molecular beacon is synthesised by standard automated DNA synthesis and deprotected. Initially the resin is dry, therefore the conditions are unsuitable for hairpin formation and the beads are fluorescent. Placing the resin in hybridisation buffer allows the stems to anneal and the fluorescence is quenched. A probe-target hybrid is formed upon exposure to a loop complementary nucleic acid, the arms are separated and the resin regains its fluorescence.

Development of molecular beacon heterogeneous assay required a suitable solid support that was (i) suitable for automated oligonucleotide synthesis giving satisfactory coupling efficiencies (>98%) at each step, (ii) allow annealing between the immobilised DNA and the solution target nucleic acid, (iii) easily manipulated for bead isolation and characterisation.

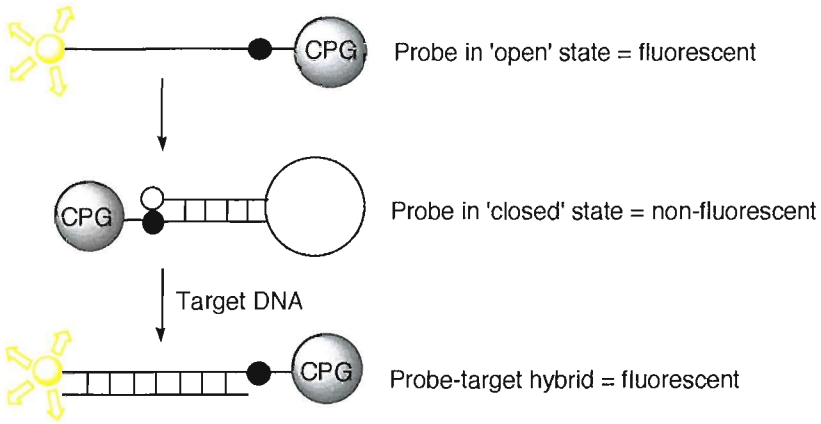
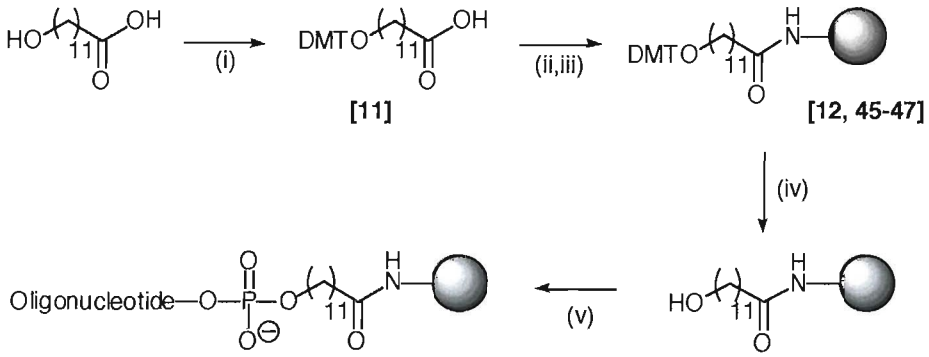


Figure 3.1 - Mechanism of action of molecular beacon on beads

3.2.2 Resin Derivatisation

The purpose of the initial experiments was to find a suitable resin for supporting the synthesis of an oligonucleotide, and to establish suitable conditions for the hybridisation of the support bound oligonucleotide to complementary oligonucleotides in solution. All resins investigated displayed different swelling, shrinking and sinking properties, which would influence the efficiency of annealing. Four resins were examined: long chain aminoalkyl-controlled pore glass (LCAA-CPG, Link Technologies), aminoalkylpolystyrene (Link Technologies), Novasyn TentaGel™ amino resin (NovaBiochem) and TentaGel™ N amino resin (Rapp Polymere).

Each resin was derivatised with a poly-alkyl linker as shown in figure 3.2. Firstly the primary hydroxyl of 12-hydroxydodecanoic acid was protected as a 4,4'-dimethoxytrityl ether. Secondly, the carboxylic acid moiety was coupled to free amino groups on the resin using DIC/HOBT in 1% DIPEA/CH_2CH_2 creating a stable amide bond between the linker and resin. The loading of the linker was determined by acid-catalysed detritylation followed by quantitation of the DMT cation release from the resin at 495nm.The appropriate amount of each resin for 0.2 micromole scale oligonucleotide synthesis was utilised on an ABI 394 DNA synthesiser.



Reagents and conditions: i, DMTCl, pyridine, rt, 2hrs, 87%; ii, resin, DIC, HOBT, DIPEA, CH_2Cl_2, rt, 1-2hrs, loading = 10 to 67μmolg^{-1}; iii, Ac_2O, pyridine, THF: 1-methylimidazole, THF (1: 1), rt, 1hr; iv, 5% TCA, CH_2Cl_2, rt, 5mins; v, standard automated DNA synthesis

Figure 3.2 - Generic scheme for resin derivatisation

In order to establish suitable conditions for the hybridisation of resin-bound oligonucleotides to solution nucleic acids, oligonucleotides **[49-52]** were assembled on each of the resins, each oligonucleotide preceded by a hexaethylene glycol spacer. The resins were heated (60°C) in conc. aq. ammonia: ethanol (4:1) for four hours and washed thoroughly with anhydrous CH_2Cl_2 and Et_2O, to remove the heterocyclic base and phosphate protecting groups.

3.2.3 Heterogeneous Hybridisation

Oligonucleotides of complementary sequence to the resin-bound oligonucleotide were synthesised and purified using standard methods **[53-54]**. The solution oligonucleotide (0.25 OD) was mixed with the resin-bound oligonucleotide in buffer such that the resin-bound oligonucleotide was in approximately four times excess. The heterogeneous mixture was allowed to stand at room temperature for 3 hours, after which the absorbance (260nm) of the resin solution was measured. The resins were also assessed for uniform shape and good sinking and swelling properties

The Novasyn TentaGel™ (TG) amino resin gave substandard synthesis giving low coupling efficiencies (coupling efficiency of TG = 91.5%, compared to CPG >98.5%)

leading to a low overall yield. The resin was designed for peptide synthesis[172]; the beads swell considerably, the large size rendered the resin unsuitable for oligonucleotide synthesis.

The Rapp TentaGel™ amino resin coupled with efficiencies >96.4% which was just acceptable for short sequences. The beads were uniform and medium in size but were sticky and difficult to dry, making manipulation difficult. Heterogeneous annealing was partially successful with a drop in UV absorbance of 0.06OD corresponding to annealing 24% of the solution oligonucleotide.

The aminopolystyrene beads were relatively small and uniform and gave high coupling efficiencies of 99.1%, but were slow sinking in UV experiments. Hybridisation of the immobilised oligonucleotide to the solution oligonucleotide was very effective, with a fall in UV absorbance of 0.21 corresponding to a reduction of 84% of oligonucleotide in solution. UV melting of the resin solution gave a smooth transition as the oligonucleotide was released from the resin (Tm = 83.6°C, figure 3.3).

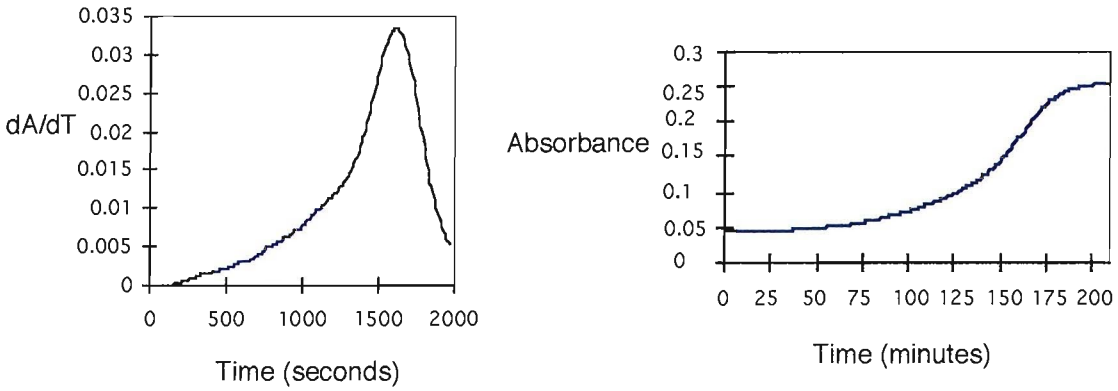


Figure 3.3 - UV thermal melting of immobilised hybrid

Controlled-Pore Glass, a rigid, porous borosilicate glass support has the advantage of being a well-known support for DNA synthesis. CPG is hydrophilic and can be dried or frozen allowing long-term storage of immobilised biomolecules, it is also heat stable and therefore amenable to temperature cycling. Oligonucleotide assembly on LCAA-CPG proceeded with >98.5% coupling efficiencies, hybridisation was successful and the particles were easy

to handle, sinking quickly in aqueous buffer, which is an important factor for immediate UV readings. The only drawback was the random structure of the glass particles, but despite this controlled pore glass was chosen for initial resin-bound molecular beacon studies.

3.2.4 Molecular Beacons on Controlled Pore Glass

Two molecular beacons were assembled by solid phase DNA synthesis on the polyalkylamide CPG previously described **[12]**. Both oligonucleotides were labelled at the 5' position with a fluorescein fluorophore and at 3' end with a methyl red quencher (figure 3.4); CGCACGCTTAAAGTCACTTCATTTTCGTGCG **[49]** and CGCACGATGTAGCACATCAGAAGCGTGCG **[55]**. Three hexaethylene glycol spacers were coupled to the resin preceeding the quencher to minimise steric interactions between the resin and the oligonucleotide during annealing (figure 3.4, 3.5).

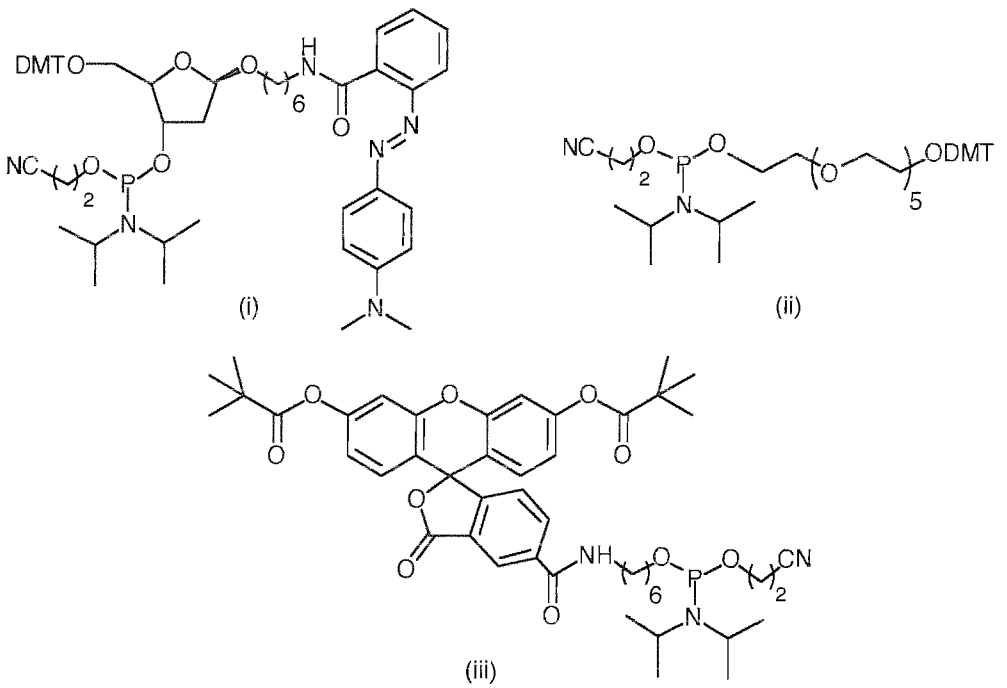


Figure 3.4 - i Methyl red phosphoramidite, ii, hexaethylene glycol phosphoramidite, iii, fluorescein phosphoramidite

The molecular beacon-functionalised CPG's were heated (60°C) in concentrated aqueous ammonia: ethanol (4:1) for four hours, to deprotect the heterocyclic bases and phosphodiesters. Both solutions became fluorescent during this period implying loss of oligonucleotide from the resin. However, subsequent thorough washing of the resin afforded glass beads that remained fluorescent, indicating that there was oligonucleotide still present on the beads (approximately 70 % loss of the oligonucleotide from the resin). Cleavage of a proportion of the silicon-oxygen bonds within the glass was inevitable under these strongly basic conditions. Alternative deprotection conditions or resin materials may be beneficial for future applications.

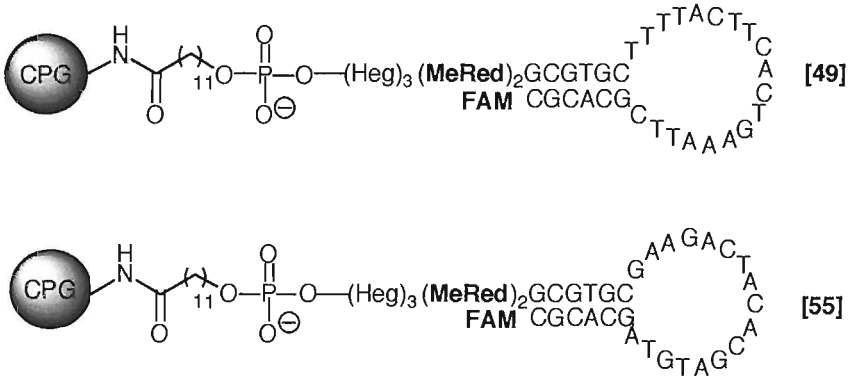


Figure 3.5 - Sequences of CPG bound molecular beacons

Two complementary solution oligonucleotides were synthesised as targets for molecular beacon probes; AAAATGAAGTGACTTTAAG **[53]**, CTTCTGATGTGCTACAT **[56]**.

The heterogeneous beacon assay was carried out in two stages. Firstly, the resins (**[49]**, and **[55]**, 2mgs) were placed in hybridisation buffer to allow stem annealing. Loss of fluorescence was monitored under a fluorescence microscope and photographed. Secondly, the complementary oligonucleotides (**[53]** and **[56]**) were added and after two hours at room temperature any increase in fluorescence due to hybridisation (stem dissociation) was observed and photographed (figure 3.6).

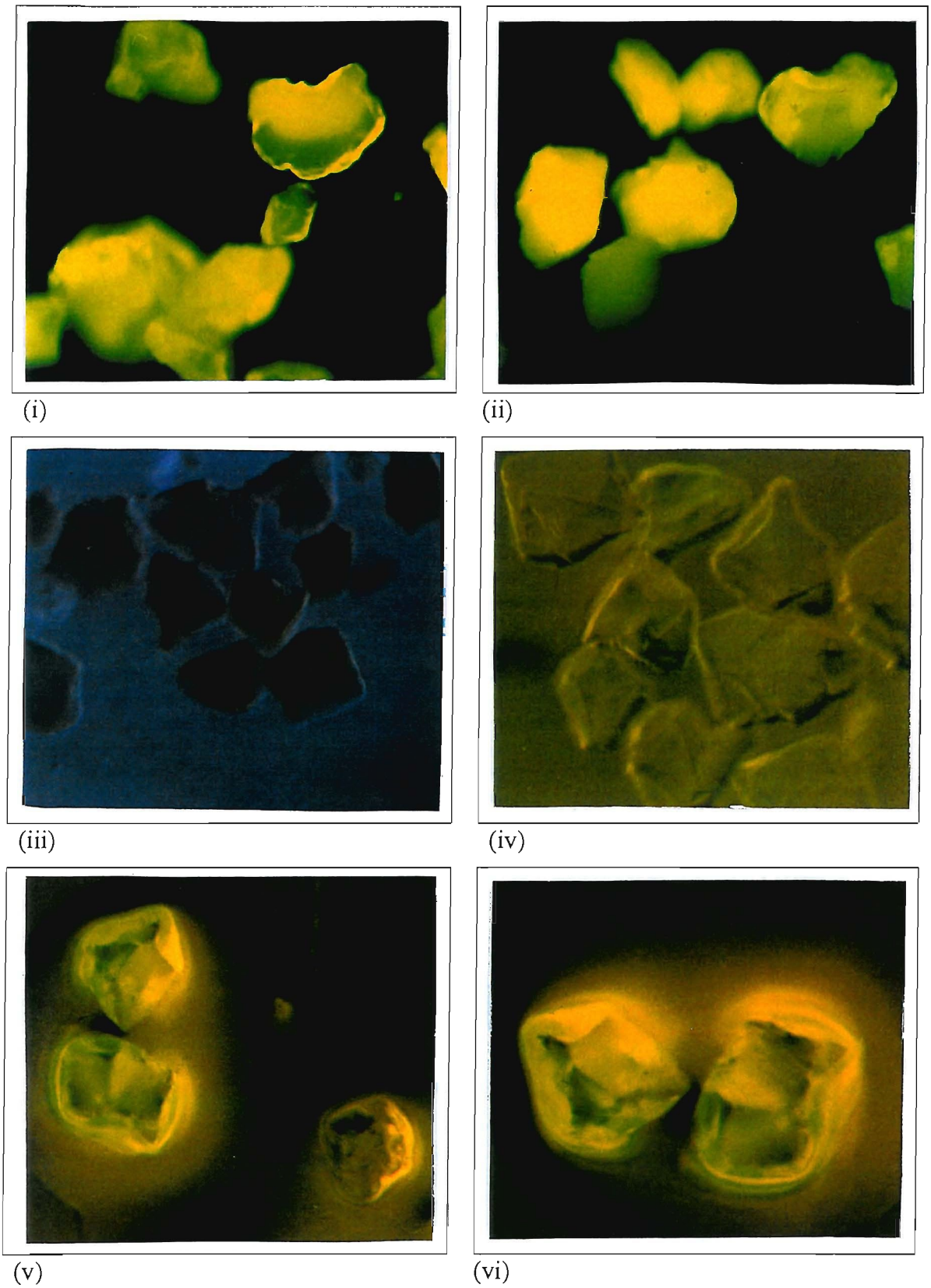


Figure 3.6 - i, ii dry fluorescent CPG; iii, iv, buffered non-fluorescent CPG; v, vi, buffered fluorescent CPG and target

Figure 3.7 shows the approximate percentages of the total number of non-fluorescent or fluorescent beads / total number of beads viewed. Lower percentages than expected were observed due to loss of oligonucleotide during ammonia deprotection.

	Dry Resin	Resin in buffer	Resin in buffer with target
[49]	fluorescent beads (≈ 70%)	non-fluorescent beads (≈ 90 %)	fluorescent beads (≈ 50%)
[55]	fluorescent beads (≈ 70%)	non-fluorescent beads (≈ 90 %)	fluorescent beads (≈ 50%)

Figure 3.7 - Approximate percentages of bead types under different experimental conditions

The results showed that when the resin was dry the glass beads possessing probes were fluorescent, therefore the fluorophore and quencher must be separated (stems are dissociated). Addition of a suitable annealing buffer caused the beads to become non-fluorescent, indicating closure of the probes by stem annealing. Finally, introduction of a complementary solution oligonucleotide caused a significant increase in fluorescence on the beads; the loop portion of the probe has hybridised to its target causing the solid-supported molecular beacons to exist in the fluorescent 'open' state. Exposure of the CPG-beacons to a non-complementary oligonucleotide in buffer did not increase the level of fluorescence on the beads.

3.2.5 Molecular Beacons Immobilised on Alternative Supports

Initial experiments proved molecular beacons bound to glass particles would anneal to their targets, and display similar behaviour to homogenous molecular beacons. However a number of weaknesses were uncovered during the experiments using glass particles that may be overcome by other solid phase materials: i, deprotection in aqueous base caused significant loss of the probe from the resin reducing the intensity of fluorescence observed, this might be avoided by milder cleavage conditions or using resins chemically inert to base, ii, isolation of individual particles for analysis would be tricky, as the glass chips

were irregular in shape and size. These disadvantages led to a search for alternative resin types.

3.2.5.1 Polystyrene Beads

Polystyrene beads have the advantage of being spherical and uniform in shape allowing easy isolation. Previous results with aminopolystrene resin (Link Technologies) recorded good coupling efficiencies and excellent heterogeneous hybridisation properties. However molecular beacons synthesised on polystyrene beads [57] persistently displayed low levels of fluorescence caused by a number of factors.

(i) The beads were not as suited to oligonucleotide synthesis as CPG, displaying average coupling efficiencies of around 96%. This causes high loading of the quencher at the 3' terminus at the beginning of the synthesis but low loading of the fluorescent dye at the 5' terminus at the end of the synthesis. The consequence of an excess of quencher to fluorophore is that the beads are always quenched either intra or intermolecularly. However, couplings were improved by using a RNA coupling cycle which prolongs the coupling time of each monomer to 20 minutes and this increased the coupling efficiency to 98.5% [58].

(ii) There was a small amount of quenching of the fluorescent dyes by the polystyrene itself rather than the quencher molecules. This leads to reduced fluorescence of the beads even when the probes are in the open state (i.e. when hybridised to a target). In an attempt to overcome this problem hexaethylene spacers were coupled to the resin prior to the molecular beacon [59], [60].

(iii) Polyaromatic polystyrene resin is highly hydrophobic being made up of mainly 1% divinylbenzene (DVB) cross-linked polystyrene and does not swell well in aqueous environments. If the beads are not swollen there may be a large degree of inter- and intramolecular quenching and access of target oligonucleotides may be prevented. In polystyrene resin the spacer separating the bound species and the hydrophobic resin matrix is generally short, this can disfavour reactions involving incoming polar or charged species such as target oligonucleotides.

Due to the problems described, it was concluded that polystyrene was an unsuitable solid phase for the heterogeneous assay. The resin cannot afford to be hydrophobic nature, as annealing of the immobilised molecular beacon to target oligonucleotides requires aqueous environments. Hexaethyleneglycol spacers attached primarily to the resin can increase the hydrophilicty and swelling of polystyrene but not to a satisfactory level for the assay. It was concluded that the physico-chemical properties of a resin required careful consideration for an aqueous assay.

3.2.5.2 TentaGel™ Beads

The architecture of TentaGel™ resin is based on a very small proportion of cross-linked 1% DVB-polystyrene backbone that is extensively grafted with long PEG spacers making up 70% of the total resin weight[173]. The reactive sites are therefore situated at the end of long, flexible spacers and are well-separated form the polystyrene backbone; this reduces problems caused by the hydrophobic nature of the polystyrene matrix and steric hindrance of incoming molecules. TentaGel™ appeared an appropriate resin for hybridisation experiments swelling in polar aqueous solvents due to its increased hydrophilicity. Previous experiments with Rapp amino TentaGel™ gave disappointing stepwise coupling efficiencies, however improved yields (>96.9%) were achieved using an RNA cycle extending the coupling time to 10 minutes [61]. All attempts to repeat the molecular beacon assay gave results similar to those using polystyrene in that the beads were consistently orange and non-fluorescent.

3.2.5.3 Macroporous Beads

This type of resin has a permanent well-developed porous structure even when completely dry, and because of the unchanging structure the beads do not need to swell to allow access to the interior. Provided the surfaces of the pores are wetted with solvent the pores can be entered, essentially all solvents can be used, even water[174]. Amino Argopore™ is a macroporous resin with a high internal surface area that provides low and predictable swelling[175]. The resin was derivatised and used in automated synthesis, but unfortunately the average stepwise yield was >85.5 giving an overall yield of 7% for a 35mer molecular beacon oligonucleotide **[62]**. Attempts to increase the coupling by extending the reaction times did not significantly improve the yield.

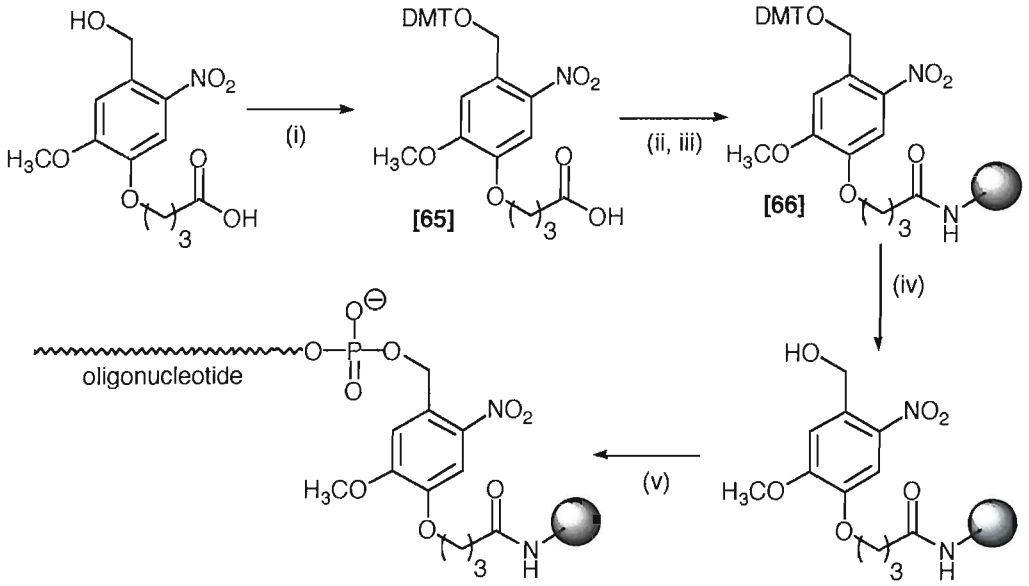
3.2.6 Conclusions and Future Work

Molecular beacons attached to glass beads have been synthesised. The glass-bound beacons in the absence of a complementary DNA sequence are 'closed', quenching occurs and the glass particles are non-fluorescent. The clear glass beads become brightly fluorescent when exposed to a complementary target nucleic acid, indicating that the probes are in their 'open' state. Ranges of structurally dissimilar resins have been investigated for the same purpose, however each resin proved unsuitable for the different reasons described.

This technology could be harnessed in nucleic acid screening by hybridisation of molecular beacons to target DNA or RNA, followed by isolation and analysis of the fluorescent beads. If the assay were to be carried out in a multiplex format it would require a characteristic dye for each molecular beacon sequence. However, this is currently limited by the availability of fluorophores with suitable emission characteristics.

A few tentative experiments exploring the possibility of using only one fluorophore and one quencher, to screen any number of targets were performed. A linker that was stable to DNA synthesis and deprotection but cleaved by UV irradiation[176] was placed between the resin and the molecular beacon (figure 3.8). Synthesis of a polythymidine oligonucleotide showed coupling to be successful **[63]**, however, mixed sequence oligonucleotide **[64]** required ammonia deprotection that caused high losses of oligonucleotide from the resin (≈60%). Continuation of this work employing fast-deprotecting phosphoramidite monomers or alternative deprotection conditions may lead to interesting applications.

A range of different molecular beacons could be synthesised and combined into a single vessel and exposed to target nucleic acids. Hybridisation of target to the probes would be indicated by the generation of fluorescence on the complementary beads that could be isolated. UV irradiation of the beads would release the oligonucleotides into solution, subsequent mass spectral analysis would elucidate the molecular beacon and therefore its complementary target nucleic acid (figure 3.9).



Reagents and conditions: i, 4,4'-dimethoxytrityl chloride, DMAP, pyridine, 60°C, 2hrs, 86%; ii, LCAA-CPG, HOBT, DIC, DIPEA, CH_2Cl_2, rt, 2hrs, loading = 22μmolg^{-1}; iii, acetic anhydride, pyridine, THF : 1-methylimidazole, THF (1:1), rt, 0.5hr; iv, 3% TCA in CH_2Cl_2; v, automated DNA synthesis

Figure 3.8 - Derivatisation of controlled pore glass with UV linker

Other linkers that could be applied to the same or similar uses with optimisation are described in chapter 4. The linkers are cleaved by enzymatic hydrolysis and contain amide bonds. Therefore they are completely orthogonal to automated DNA synthesis and deprotection. Advances in MALDI-TOF mass spectrometry allow molecular ions of samples in the types of aqueous buffers required for enzyme scission to be detected[177,178].

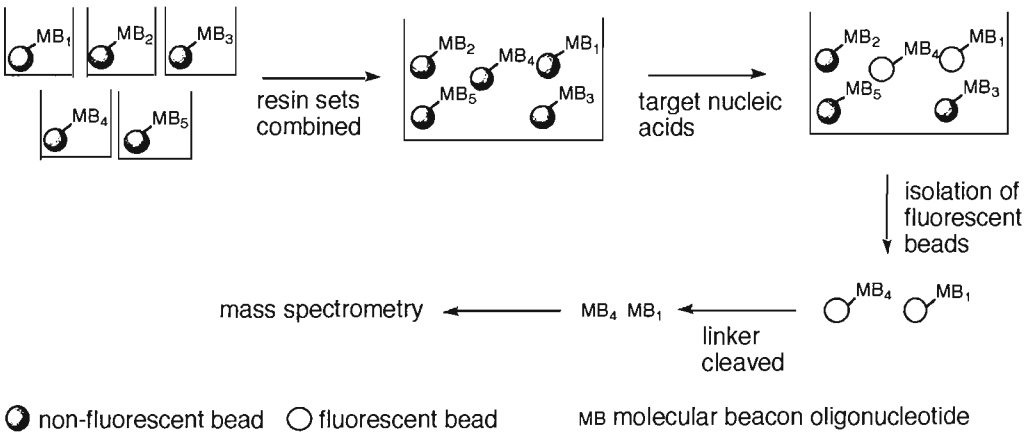


Figure 3.9 - Representation of multiplex molecular beacon assay

3.3 Heterogeneous Capture Assay

A new strategy for the use of solid supported probes was investigated. The idea involved the capture and release of oligonucleotide probes on and off a solid support

3.3.1 Principle

Single-stranded oligonucleotides, assembled by solid phase synthesis, that are attached to the solid support by inert linkers will remain bound to the side-chain after deprotection. Synthesis of resin-bound oligonucleotides labelled at the 3' end with a quencher can be used to capture complementary oligonucleotides (denoted as capture oligonucleotides). The complementary solution oligonucleotides can be labelled (5') with fluorescent molecules (denoted as probe oligonucleotides) and are longer than the resin-bound capture oligonucleotides.

The principle lies in the hybridisation of the probe oligonucleotide to the capture oligonucleotide on the resin (figure 3.10, i). In this state the fluorophore and quencher are adjacent and fluorescence will be quenched. As the probe oligonucleotide is longer than the capture oligonucleotide it will have a 3' tail. This is important, as heating the resin in the presence of a probe complementary target will cause dissociation of the fluorescent probe from the resin (figure 3.10, iv). Upon cooling the probe will preferentially hybridise to the longer target. Target-probe annealing is signalled by a gain in fluorescence of the resin solution, in the presence of non-complementary targets the probe oligonucleotide will simply re-anneal to the resin and fluorescence will be lost by quenching (figure 3.10, v).

3.3.2 Results

Polystyrene beads were derivatised with inert linkers [45] and used to assemble single stranded oligonucleotides, labelled at the 3' end with a quencher, that remained bound to the resin after synthesis and deprotection (capture oligonucleotide, [67]). A second 5' fluorescently labelled solution oligonucleotide was synthesised, deprotected and purified

[68]. This longer oligonucleotide was complementary to the capture oligonucleotide on the resin.

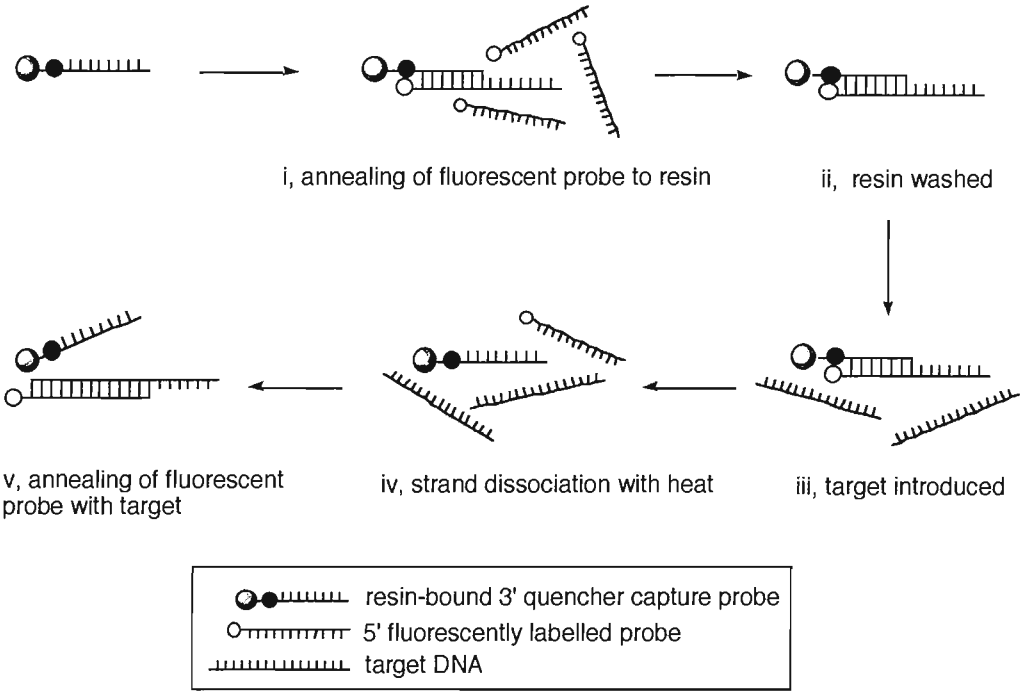
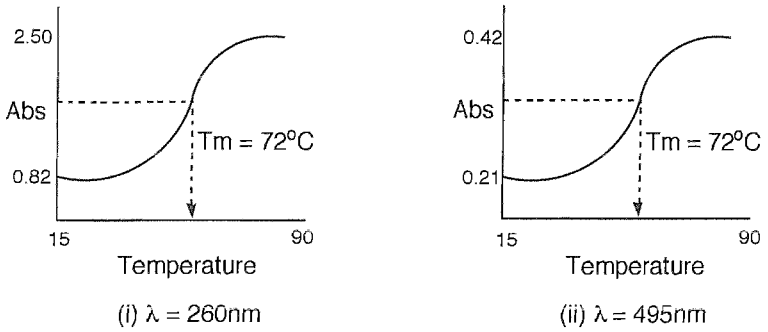


Figure 3.10 - Principle of capture assay

The resin was heated to 90°C in a solution of the fluorescent oligonucleotide **[68]** and cooled to room temperature to allow annealing between the fluorescent oligonucleotide and the capture oligonucleotide **[67]** (i, figure 3.10). The resin was then washed to remove any unbound fluorescent oligonucleotide (ii, figure 3.10). To ensure that the fluorescent oligonucleotide was being held on the resin, the resin was heated from 15 to 90°C over a period of 75 minutes and the increase in absorbance at 495nm and 260nm followed. Both melts showed characteristic S shaped curves for the release of the fluorescent oligonucleotide into solution as the two strands dissociated, Tm = 72°C (figure 3.11). The beads were allowed to cool for re-annealing, this process was slower than the dissociation due to the dilution of the resin required for UV measurements.



3.11 - Results of resin heating at i, 260nm, ii, 495nm

A sample of the re-annealed resin was placed in a cuvette with hybridisation buffer and an excess of target solution oligonucleotide **[69]** was added (iii, figure 3.10). The sample was heated to 90°C (iv, figure 3.10) and the increase in absorbance at 495nm and 260nm was recorded. At 495nm the absorbance rose from 0.048 to 0.15, and at 260nm from 3.49 to 3.65 indicating release of the fluorescent oligonucleotide from the resin. The sample was cooled causing an initial drop in absorbance to 0.125 but remained constant at this value for 48 hours as the fluorescent oligonucleotide was bound to its target (v, figure 3.10).

A control experiment (buffer only, no target) showed the an increase in absorption at 495nm of 0.057 to 0.455 at 90°C, cooling caused a drop to 0.252, but after 48 hours the reading was back down to 0.08, as the fluorescent oligonucleotide was re-annealed to the resin.

3.3.3 Conclusions

The initial experiment performed confirmed the principles of the assay. The re-annealing of the probe oligonucleotide to the resin-bound capture oligonucleotide was slow and would require optimisation to allow the assay to be thermal cycled in a PCR format, which requires numerous heating and cooling cycles. Saturation of the solution with beads may enhance annealing, however this may make fluorescent reading difficult. Another approach could involve coating the walls of a cuvette or capillary with the capture oligonucleotide this would simplify fluorescent readings by removing any obstructions.

Chapter 4

Enzyme Labile Linkers

4.0 Enzyme Labile Linkers

This chapter explains the need for chemically stable linkers between an oligonucleotide and a resin, or to separate different sections of an oligonucleotide. The synthesis of four linkers is described and successful enzymatic cleavage of two tripeptide linkers.

4.1 Introduction

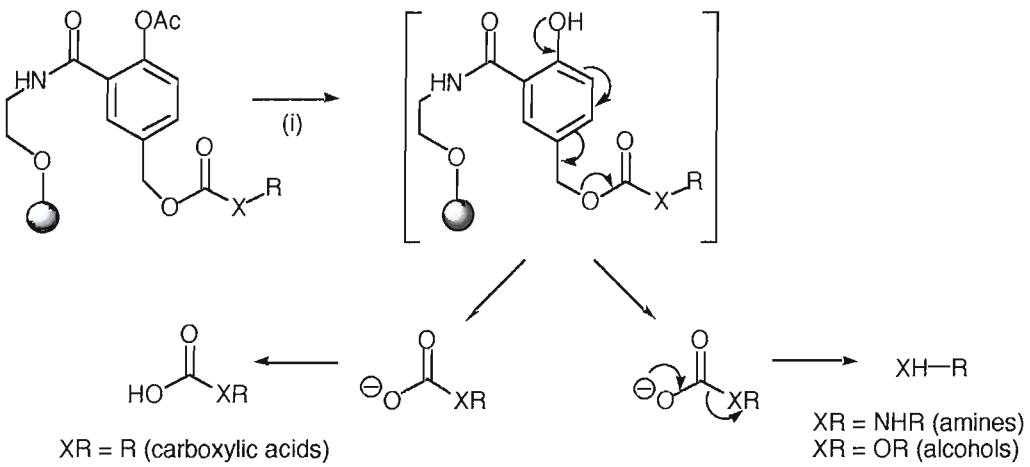
Solid phase chemistry of oligonucleotides employs linkers that are cleaved chemically, for example the previously described succinyl and disiloxyl linkers[145] (section 2.2.2.5), or by UV irradiation[179]. A linker is described as a bifunctional protecting group that can be easily attached to the molecule to be synthesised by a bond labile to the cleavage conditions, but is held on the solid support via a more stable bond or a bond with completely orthogonal reactivity. Ideally linkers allow the attachment of the starting molecule in high yields and are stable to the chemistry required for synthesis of the supported molecule. It is also important that the conditions for linker cleavage are sufficiently mild, so not to destroy the product. A recent comprehensive review of linker strategy in solid-phase synthesis has been published[141].

The most commonly used linker in oligonucleotide assembly, the succinyl linker, is base labile. This means manipulation of the oligonucleotide on-resin under basic conditions (e.g. selective removal of protecting groups) is impractical. Therefore manipulations of this kind are generally performed after the oligonucleotide has been released from the resin into solution. However, performing chemical manipulations on the oligonucleotide in solution is non-selective due to the numerous reactive sites and can require laborious purifications. The need for linkers exhibiting a higher degree of orthogonality to the conditions of automated DNA synthesis has therefore increased[180].

Developments in probe technology have increased the demand for modified oligonucleotides. Assembly of modified molecules whilst still attached to a solid support simplifies both synthesis and purification. Linkers are generally employed to retain a

molecule on a resin during synthesis, however, another important application arises when a labile linker is placed internally in an oligonucleotide sequence. Labile linkers inserted within solution oligonucleotides have implications in fluorescence resonance energy transfer and quenching experiments. A fluorophore and quencher positioned either side of a linker may be non-fluorescent but linker scission would cause increased in fluorescence intensity. Similarly a reduction in fluorescence may be observed upon linker hydrolysis when two fluorophores are placed either side of a linker.

Enzyme labile linkers are of interest, as the cleavage conditions are mild, neutral, generally aqueous and proceed with chemo-, regio- or stereo- selectivities. A few enzymes have been used to cleave linkers for solid phase chemistry including phosphodiesterase[181], chymotrypsin[182] and penicillin amidase[183]. Sauerbrei *et al.* describe a linker that releases carboxylic acids, alcohols or amides (after decarboxylation) by variation of X, figure 4.1[184]. Enzymatic hydrolysis of the acetate by lipases or esterases is followed by elimination of the desired molecule without any undesirable cleavage products being retained on the molecule.



Reagents and Conditions: i, lipase RB001-05, MES buffer (50mmol): MeOH (4: 1), $NaHSO_3$ (0.2M), pH 6.5, 30°C

Figure 4.1 - Enzyme-labile linker described by Sauerbrei *et al.*

To design a linker for oligonucleotide synthesis a number of factors require careful consideration: (i) the linker requires two points of diversity; (ii) it must exhibit stability to

the conditions of automated oligonucleotide synthesis; (iii) the linker should be easily incorporated during DNA synthesis. For enzyme labile linkers the choice of enzyme is important, the linker must be a recognisable substrate, and the enzyme must not harm the oligonucleotide.

The linkers designed were terminated at one end with a protected hydroxyl and at the other with a phosphoramidite. This was to allow the linker to be coupled using standard DNA chemistry. Automated oligonucleotide synthesis would then be continued following detritylation of the hydroxyl group. This phosphoramidite method permitted incorporation of the linker not only directly to the resin, but also internally within an oligonucleotide for solution hydrolysis. Synthesis of a phosphoramidite also ensured linker incorporation maintained high coupling yields, which are essential for efficient DNA synthesis. Enzyme labile linkers of the types shown in figure 4.2 were initially proposed.

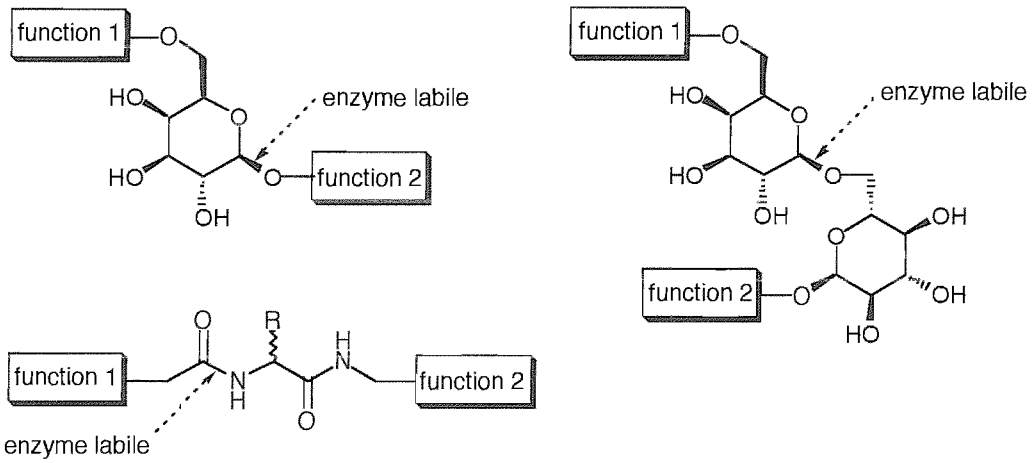


Figure 4.2 - Proposed enzyme labile linkers based on monosaccharides, disaccaharides or peptoids.

4.2 β-Galactose Linker

4.2.1 Principle and Preparation

It was envisaged that this linker would be cleaved by exposure to a glycosylase enzyme severing the glycosidic linkage and releasing an alcohol.

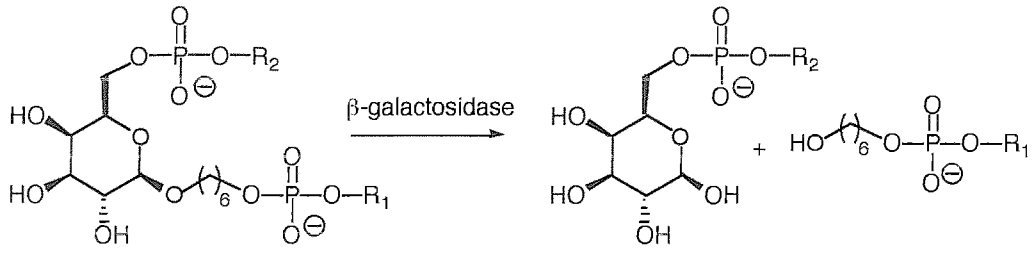


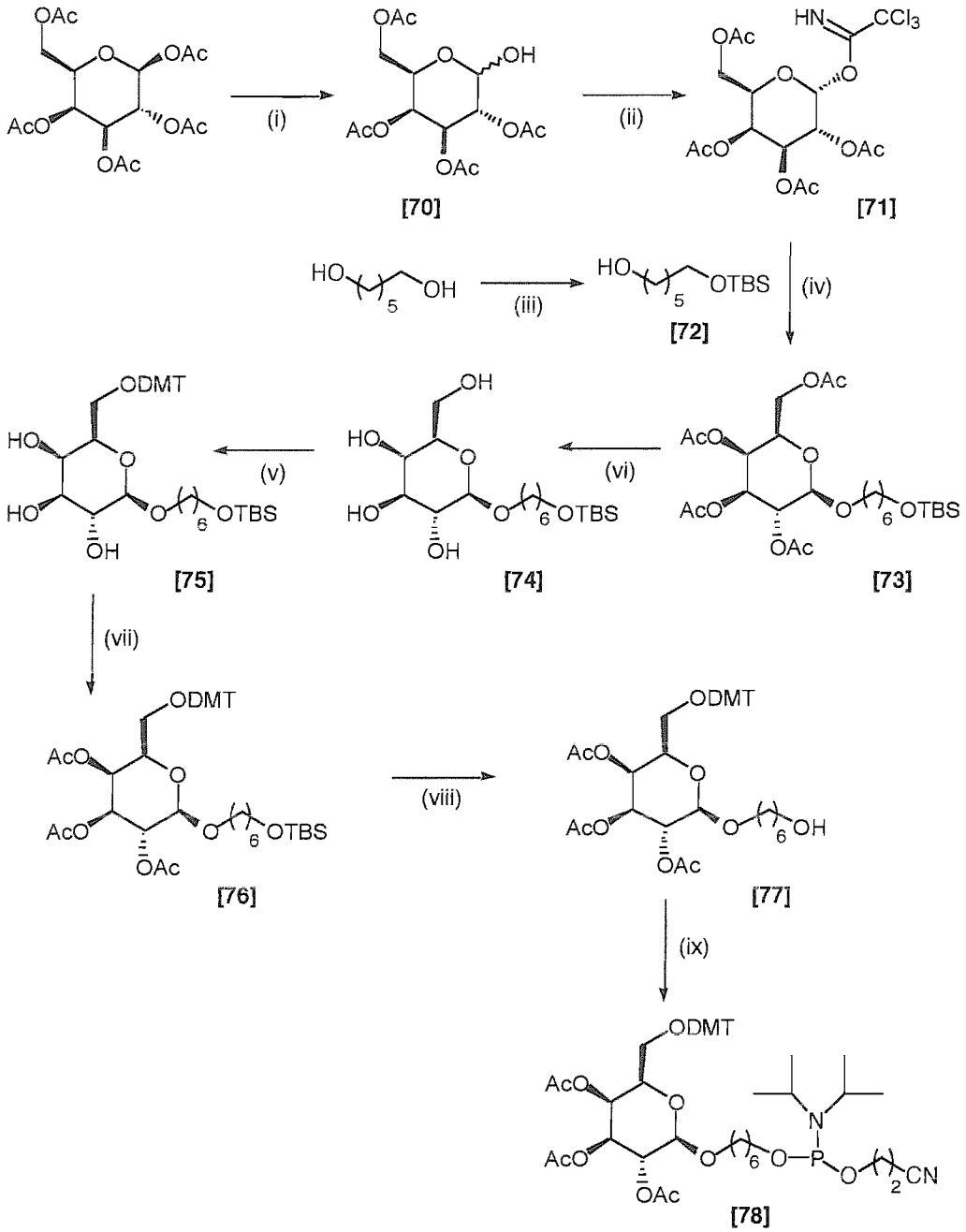
Figure 4.3 - β-galactosyl linker cleaves to release alcohols from galactose-containing residue

The commercially available β-galactose pentaacetate was selectively deprotected at the anomeric centre to give a mixture of β and α anomers (α: β, 1:3)[185]. Subsequent conversion to the crystalline trichloroacetimidate **[71]** was first attempted using a simple biphasic reaction between trichloroacetonitrile and 50% aqueous potassium hydroxide, containing a catalytic amount of tetrabutylammonium hydrogen sulfate[186]. The method was successful in producing the trichloroacetimidate, yet resulted in cleavage of the acetyl protecting groups. An alternative method using sodium hydride, gave the desired α-anomer selectively in a satisfactory yield, however, the yield was improved using 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) as the base for the reaction[187,188].

Monosilylation of the symmetric hexan-1,6-diol was achieved in reasonable yield by generating the monosodium alkoxide salt and reacting it with *tert*-butyldimethylsilyl chloride[189]. The glycosylation step between the trichloroacetimidate glycosyl donor and the alcohol acceptor was catalysed by Lewis acid[190,191]. Results showed trimethylsilyl trifluoromethanesulphonate proved to be a superior catalyst than boron trifluoride etherate giving only the β-anomer in high yield. It was important to ensure that the β-anomer was retained, as the molecule needed to be a recognisable substrate for β-galactosidase. NMR analysis confirmed the configuration as the β-anomer, an axial-axial configuration of the hydrogens at positions C1 and C2 gave rise to a large coupling constant.

Removal of the acetyl groups by base-catalysed transesterification proceeded as expected. The primary hydroxyl was protected as the 4,4'-dimethoxytrityl ether with only a small amount of bis- and tris- tritylation observed. The three remaining hydroxyl functions were

peracetylated with acetic anhydride. Slow desilylation was observed with TBAF, however, a hydrogen fluoride reagent (3HF•Et_3N: NMP: Et_3N) gave a clean and rapid reaction. The final phosphitylation yielded the desired monomer ([78] figure 4.4).



Reagents and conditions: i, NH_3, MeOH, 0°C, 1hr, 73%; ii, 1,8-diazabicyclo(5.4.0)undec-7-ene, Cl_3CCN, CH_2Cl_2, rt, 0.5hr, 70%; iii, TBS-Cl, NaH, THF, rt, 2hrs, 62%; iv, TMSOTf, CH_2Cl_2, -15°C, 15mins, 57%; v, K_2CO_3, MeOH, 0°C, 2hrs, 96%; vi, DMT-Cl, DMAP, pyridine, rt, 20mins, 45%; vii, Et_3N, Ac_2O, DMAP, CH_2Cl_2, rt, 0.5hr, 89%; viii, Et_3N•3HF: NMP: Et_3N, 60°C, 1hr, 97%; ix, 2-cyanoethyldiisopropylchlorophosphoramidite, DIPEA, CH_2Cl_2, rt, 20mins, 96%

Figure 4.4 - Synthesis of the β-galactose linker phosphoramidite

4.2.2 Oligonucleotide synthesis and linker cleavage

Two oligonucleotides were assembled placing the linker between a fluorescent dye and a polythymidine oligonucleotide. The coupling efficiency of the monomer was > 99%. FST_{15} **[79]**, $FSST_{15}$ **[80]** where **S** indicates incorporation of monomer **[78]** and F = fluorescein.

The oligonucleotides were designed such that cleavage of the linker by β-galactosidase would separate the fluorescent dye and the colourless polyT oligonucleotides. The cleavage reaction was performed and the products analysed by gel filtration. Successful scission would be characterised by elution of a colourless polyT oligonucleotide, with the fluorescent dye fragment retained on the gel. However, when the reaction was passed through the gel the labelled fluorescent oligonucleotide was eluted as a single entity indicating cleavage was unsuccessful. Mass spectrometry confirmed the observation; no cleavage products were detected, the only molecular ion being that of the starting oligonucleotide.

4.2.3 Conclusions

Results showed the linker failed to hydrolyse in solution, this implied cleavage from a resin was extremely unlikely. The disappointing result could be caused by poor or no recognition of the linker as a substrate for the enzyme or that the enzyme could not access the linker. In favour of the former hypothesis, there is some evidence to suggest β-D-galactosidase may not tolerate substitution in the C-6 position[168]. Future work could utilise the precursor to the phosphoramidite **[77]** to synthesise a disaccharide linker. For example linking galactose to glucose would form a lactose-based linker that could be a substrate for β-galactosidase or other endoglycosylases. However, due to the large size of the β-galactosidase causing potential steric problems in solid supported applications, attention switched to alternative linkers and enzymes.

4.3 Peptoid Linkers

4.3.1 Phenylalanine Linker 1

4.3.1.1 Principle and preparation

Chymotrypsin is a monomeric, serine protease that hydrolyses peptide bonds in the middle of polypeptide chains (endopeptidase). It has a large, hydrophobic binding pocket that accommodates the side chains of amino acids such as phenylalanine, tyrosine and tryptophan[192]. Cleavage occurs at the *C*-terminus of the L-isomers of any of these residues. Coloured and luminescent substrates based of these three amino acids have traditionally been used to assay the activity of chymotrypsin. A linker was designed that incorporated alkyl spacers either side of a central phenylalanine residue.

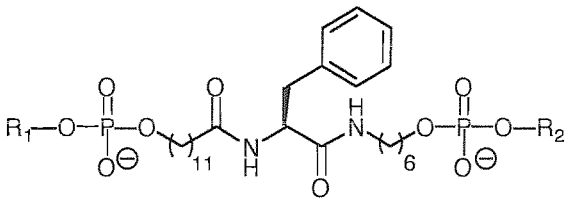
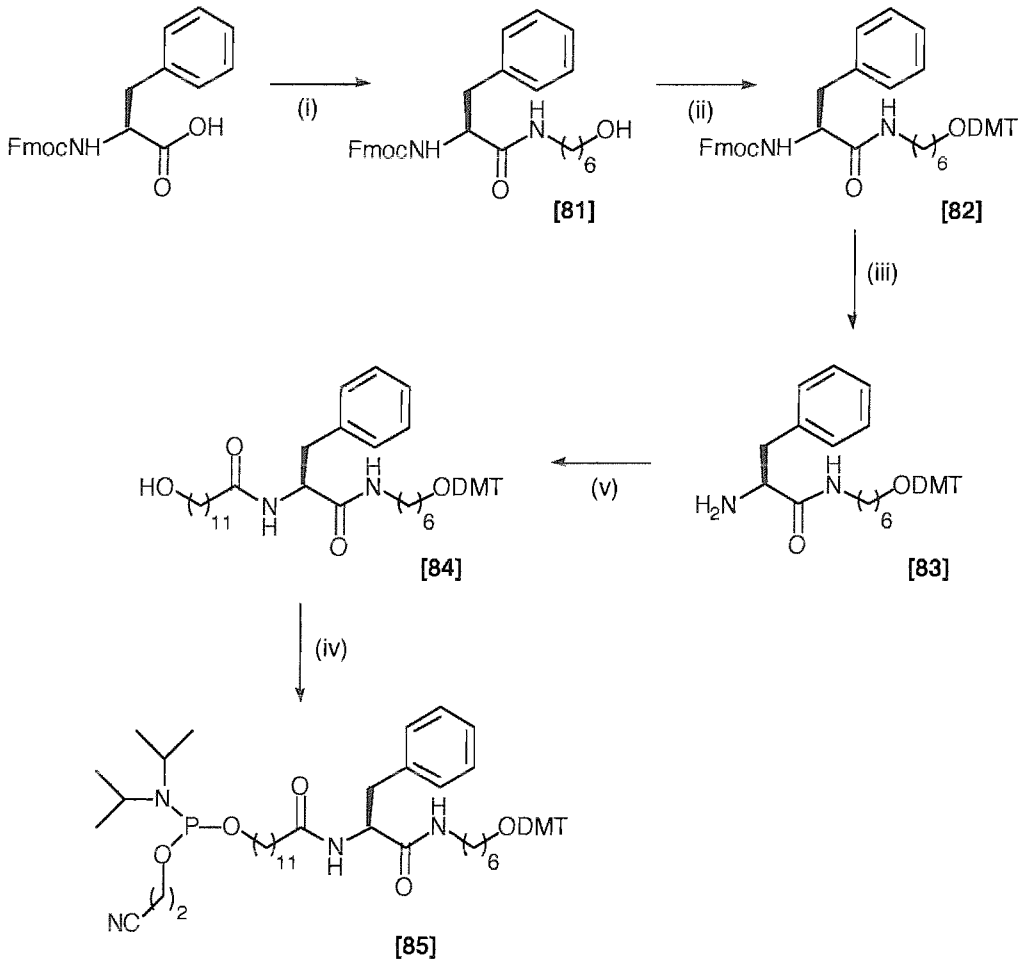


Figure 4.5 - Phenylalanine Linker 1

Synthesis of the linker began with the commercially available Fmoc-phenylalanine, which was coupled to the bifunctional 6-aminohexan-1-ol to give the desired amide with no significant ester formation. Tritylation proceeded in high yield to give the white crystalline product **[82]**, subsequent Fmoc deprotection afforded the free amine **[83]**. In order to generate an alcohol at the *N*-terminus, the amine was refluxed with butyrolactone in an attempt to achieve ring-opening amide formation[193]. However, this resulted in slow detritylation and gave none of the desired amide. An alternative reaction coupled the amine with 12-hydroxydodecanoic acid in good yield, producing a more flexible, long chain, primary alcohol **[84]**. Subsequent conversion to the phosphoramidite gave the desired monomer **[85]** (figure 4.6) that was incorporated into an oligonucleotide by solid phase synthesis with 99.5% coupling efficiency.



Reagents and conditions: i, 6-aminohexan-1-ol, EDC, DMF, rt, 18hrs, 62%; ii, 4,4'-dimethoxytrityl chloride, DMAP, pyridine, rt, 18hrs, 92%; iii, 20% piperidine in DMF, rt, 2hrs, 96%; iv, 12-hydroxydodecanoic acid, EDC, CH_2Cl_2, rt, 18hrs, 76%; v, 2-cyanoethyldiisopropylchlorophosphoramidite, DIPEA, CH_2Cl_2, rt, 20 mins, 92%

Figure 4.6 - Synthesis of phenylalanine linker 1 phosphoramidite

4.3.1.2 Oligonucleotide assembly and linker testing

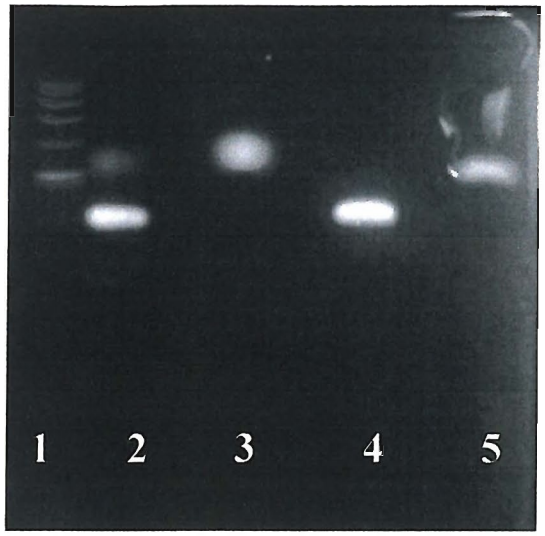
Two oligonucleotides were prepared $X\mathbf{P^1}T_{15}$ **[86]**, $XH\mathbf{P^1}HT15$ **[87]** (where X = hexachlorinated fluorescein, H = hexaethylene glycol and $\mathbf{P^1}$ indicates incorporation of the phenylalanine linker 1 monomer **[85]**). The oligonucleotides were designed such that after hydrolysis of the amide bond at the C-terminus of the phenylalanine residue by chymotrypsin, the dye would be released as a small molecule fragment from the oligonucleotide chain.

The oligonucleotide was placed in buffer with an excess of chymotrypsin for 24 hours and the reaction products were examined by gel filtration (size-exclusion chromatography). If

cleavage had occurred the small fluorescent dye fragment would be retained in the pores of the gel and the large colourless oligonucleotide would be eluted. After prolonged exposure to chymotrypsin both oligonucleotides were eluted though the gel as fluorescent solutions, suggesting no cleavage had occurred.

Further analysis of the assay products was carried out by gel electrophoresis, lanes containing unreacted oligonucleotides should appear as single bands, whereas lanes containing cleavage products should display a band for the released oligonucleotide and a fluorescent band of the dye. Staining was achieved with SYBR Green II™ which stains single-stranded DNA.

Figure 4.7 shows the result of the agarose gel electrophoresis, lane 1 contained PCR markers to ensure staining was accomplished, lanes 2 and 4 contained **[86]** and **[87]** respectively and displayed single fluorescent bands only, as expected. Lanes 3 and 5 contained the products of the chymotrypsin cleavage assay of **[86]** and **[87]** respectively. There was an obvious change in migration through the gel in both cases, however, the expected result in which there would be two bands, one from the oligonucleotide and another from the fluorescent dye was not observed. Nucleic acid staining ensured that any oligonucleotide fragments would be detected, removing the possibility that the band visualised was a dye fragment and not the labelled oligonucleotide. The shift in migration suggested the dye-labelled oligonucleotides were forming a complex with the enzyme, the increase in size causing the oligonucleotides to move more slowly through the gel. This complex formation is feasible as the fluorescein based dye possesses aromatic functionalities that could interact with the hydrophobic pocket at the active site of chymotrypsin.



Lane **1** PCR markers (50 -1000bp)
2 [86]
3 [86] chymotrypsin cleavage assay
4 [87]
5 [87] chymotrypsin cleavage assay

Figure 4.7 - 3% Agarose gel electrophoresis of chymotrypsin cleavage assay

To examine the enzyme cleavage further, the linker was placed between the resin and the oligonucleotide. Successful cleavage of the peptide linker by chymotrypsin would be indicated by the release of the oligonucleotide from the resin into solution. The resin bound oligonucleotide was assembled and deprotected using chemistry described in section 2.2.2.5, CGTGACATCTGCAGTC$\mathbf{P^1}$DR$_1$ **[88]** where D = disiloxyl thymidine monomer **[10]**, $\mathbf{P^1}$ = peptide linker and R$_1$= CPG **[12]**. Unfortunately, monitoring a suspension of the resin in the presence of the chymotrypsin at 260nm for 3 hours showed no significant increase in absorbance, implying all oligonucleotide remained on the resin as the linker had failed to hydrolyse.

One further experiment involved an oligonucleotide FCAATGTT$\mathbf{P^1}$TTCATTGM **[89]** (where F = fluorescein fluorophore, M = methyl red quencher (figure 3.5) and $\mathbf{P^1}$ indicates incorporation of the phenylalanine linker 1 monomer **[85]**). At neutral pH the oligonucleotide exists as a hairpin loop, in this configuration energy transfer between the fluorophore and quencher occurs and the oligonucleotide is non-fluorescent. When the linker is cleaved the single-stranded hairpin oligonucleotide is separated into two strands, these are sufficiently short that they will rapidly dissociate and this can be observed by an increase in fluorescence due to separation of fluorophore and quencher. The hairpin oligonucleotide was exposed to chymotrypsin but no change in fluorescence was observed,

indicating no cleavage. Conformation that the single stranded hairpin loop oligonucleotide remained intact was achieved by UV thermal melting which gave the same transition (Tm = 61°C), corresponding to stem dissociation, before and after exposure to chymotrypsin.

4.3.1.3 Conclusions

The results showed the peptide linker was not able to act as a substrate for chymotrypsin, there was either no recognition of the linker by the enzyme or in the case of a dye-labelled oligonucleotide, a complex between the dye and the enzyme may be forming. To overcome these difficulties design of a new linker required greater rigidity of the recognition site by a more peptidic linker structure in attempt to gain substrate recognition.

4.3.2 Phenylalanine Linker 2

4.3.2.1 Principle and Preparation

Design focused around a tripeptide substrate for chymotrypsin, phenylalanine was sandwiched between two glycine residues and alkyl spacers incorporated each side. Successful cleavage of the linker would occur at the *C*-terminal amide of the phenylalanine residue (figure 4.8).

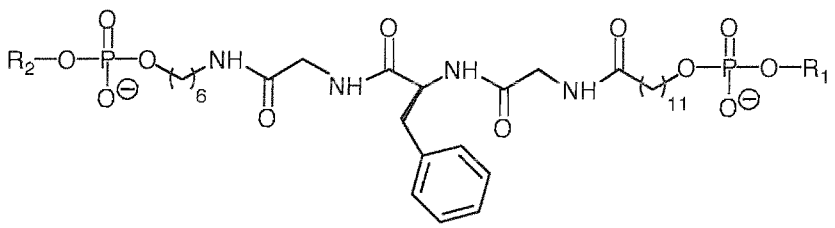
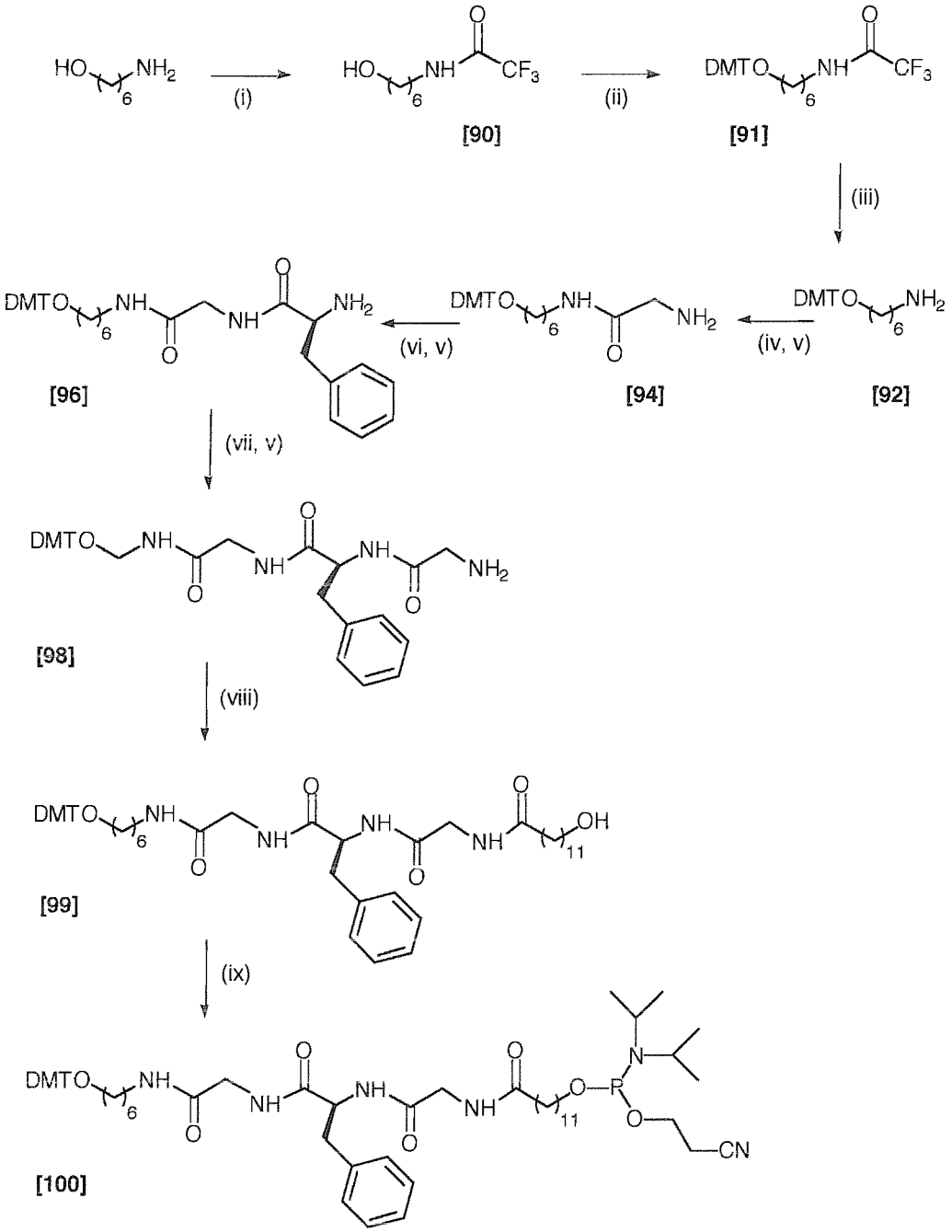


Figure 4.8 - Phenylalanine Linker 2

The tripeptide section of the linker was assembled by solution phase peptide synthesis beginning with the protected hydroxyamine spacer **[92]**. A series of carbodiimide coupling and piperidine deprotection reactions generated a tripeptide amine onto which a twelve-carbon spacer was added to provide the final alcohol for phosphitylation (figure 4.9).

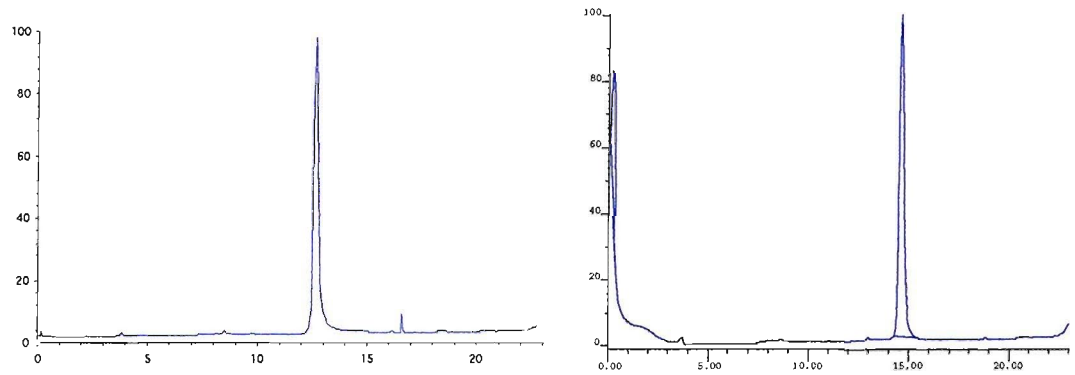


Reagents and conditions: i, $EtOCOCF_3$, Et_3N, CH_2Cl_2, rt,12hrs, 71%; ii, DMTCl, DMAP, pyridine, rt, 15hrs, 81%; iii, NH_3 (g), CH_3OH, rt, 18hrs, 88%; iv, Fmoc-Gly-OH, EDC, CH_2Cl_2, DMF, 67%; v, 20% piperidine, DMF, rt, 1-24hrs, **[94]** = 53%, **[96]** = 83%, **[98]** = 92%; vi, Fmoc-Phe-OH, EDC, CH_2Cl_2, DMF, rt, 14hrs, 91%; vii, Fmoc-Gly-OH, EDC, CH_2Cl_2, DMF, rt, 1hr, 83%; viii, 12-hydroxydodecanoic acid, EDC, CH_2Cl_2, rt, 12hrs, 83%;
xi, 2-cyanoethyldiisopropylchlorophosphoramidite, DIPEA, CH_2Cl_2, rt, 1hr, 92%

Figure 4.9 - Synthesis of phenylalanine linker 2 phosphoramidite

4.3.2.2 Linker cleavage

Initial experiments to examine the hydrolysis of the linker were performed in solution. Two oligonucleotides were synthesised and purified FTTTTTTT**P²**TTTTTT **[101]**, F**P²**TTTTTT **[102]** (where F = fluorescein and **P²** indicates incorporation of the phenylalanine liner 2 monomer **[100]**, figure 4.10)



(i) (ii)

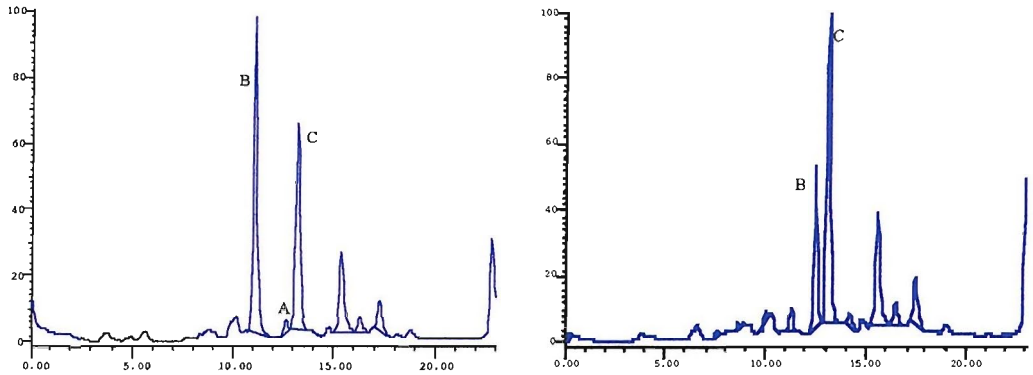
Figure 4.10 - Phenylalanine linker 2 oligonucleotide prior to hydrolysis (t = 0 min) (i) **[101]**, (ii) **[102]**

Initial experiments incubated the oligonucleotides in buffer with a large excess of chymotrypsin at 37°C for 3 days. The enzyme was removed by centrifugal ultrafiltration and the reaction products analysed by RP.HPLC, eluting under identical conditions to initial oligonucleotide purification.

The experiment was performed on both oligonucleotides and showed in each case a complete disappearance of starting material with the formation of new product peaks corresponding to complete hydrolysis of the linker. To assess the rate of cleavage, a time-controlled study was performed with both oligonucleotides **[101]** and **[102]** using an excess of enzyme.

Six samples of the modified oligonucleotides in separate tubes were incubated at 37°C, in buffer, five tubes containing chymotrypsin. After time periods of 10mins, 30mins, 1hr, 3hrs, and 24hrs a tube was removed, denaturation was achieved by rapid heating to 95°C and the enzyme removed by centrifugal filtration. RP.HPLC analysis of the reaction

products showed almost complete conversion of starting oligonucleotide to hydrolysed product after 10mins with very little change after longer time periods (figure 4.11).



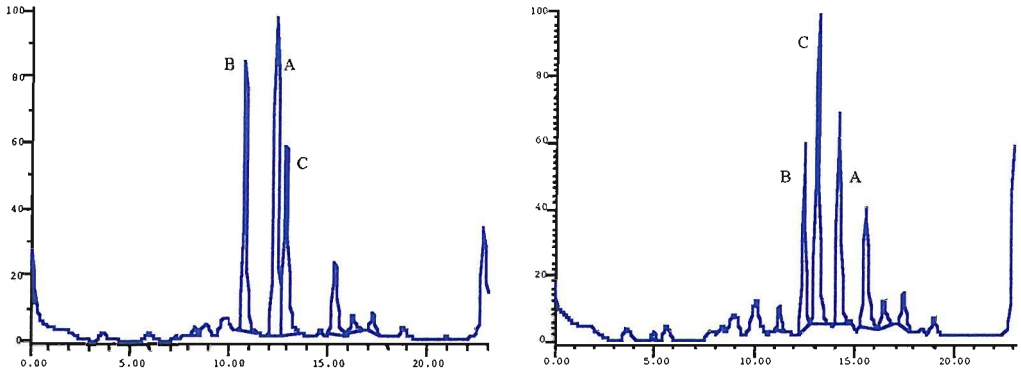
(i) (ii)

Figure 4.11 - (i) **[101]**, (ii) **[102]** almost complete hydrolysis of starting oligonucleotide (A) by chymotrypsin, generating cleavage fragments (fluorescent product B and non-fluorescent product C). All other peaks are enzyme fragments (t = 10 min).

The results were consistent for both oligonucleotides and collection of the individual cleavage peaks revealed one fluorescent product and one colourless product as expected. Mixed injection of starting oligonucleotide with the reaction products confirmed that the oligonucleotide had been completely hydrolysed (figure 4.12). Repetition of the experiment using one equivalent of chymotrypsin showed the formation of the products on a much slower timescale, the reaction was almost complete after 24 hrs.

To examine the hydrolysis of the linker placed between a resin and an oligonucleotide, the following sequences were assembled and left attached to the resin F**P**^{2}TTTTTTR$_2$ **[103]**, F**P**^{2}HH-CPG **[104]** (where H= hexaethleneglycol, **P**^{2} indicates incorporation of the phenylalanine linker 2 monomer **[100]** and R_2 = polystyrene). The compound **[103]** was designed to release dye from the resin into solution. A sample of the resin in buffer was incubated with excess chymotrypsin and monitored for increase in absorbance at 495nm over time. The reading increased from 0.059 to 0.275 over 21 hours, however, the experiment showed only qualitative cleavage of the linker by detachment of the dye.

The second oligonucleotide **[104]** was assembled on standard controlled pore glass using a trityl-on programme, this ensured the thymidine added possessed trityl protection at the 5' position. A sample of the resin was used to determine the loading by acid catalysed detritylation and quantification at 495nm. The remainder of the resin was incubated with chymotrypsin for 4hrs, washed and the loading recalculated. Unfortunately only 10% loss of the nucleoside from the resin was observed.



(i) (ii)

Figure 4.12 - Mixed injection of oligonucleotide containg phenylalanine linker 2 (i, A = **[101]**, ii, A = **[102]**) starting material and cleavage fragments (B and C) from chymotrypsin hydrolysis.

4.3.2.3 Conclusions

The solution studies clearly display the rapid hydrolysis of the linker with excess enzyme, and a more controlled hydrolysis is achieved using one or a few equivalents of chymotrypsin. Cleavage of the linker adjacent to a resin was less successful. This may be due to steric factors, the resin preventing the enzyme accessing the substrate. Resins made of polystyrene are hydrophobic and therefore do not swell in aqueous environments that may hinder hydrolysis. It is also possible that the resin may interact with chymotrypsin due to its aromatic nature. To improve the cleavage alternative hydrophilic resins suitable for enzyme-catalysed reactions such as polyethyleneglycol grafted polystyrene[173] could be examined. However, it is important that the resin chosen displays high coupling yields during automated oligonucleotide assembly.

4.3.3 Lysine Linker

4.3.3.1 Principle and preparation

A previous problem encountered had been the formation of complexes because of the similarities between the aromatic rings of dye labels or the aromatic heterocyclic bases of nucleotides and chymotrypsin substrates. A second tripeptide linker was designed that incorporated lysine as the central amino acid. This linker was proposed as a substrate for trypsin, which requires an amino acid with a basic side chain, such as lysine or arginine, for catalysis.

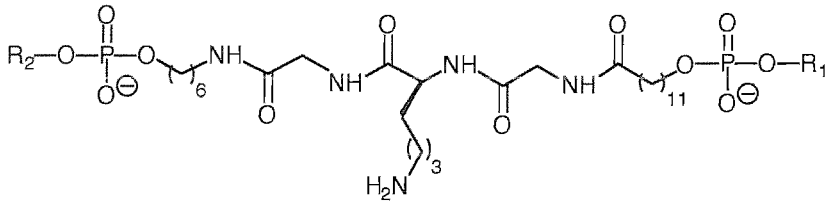
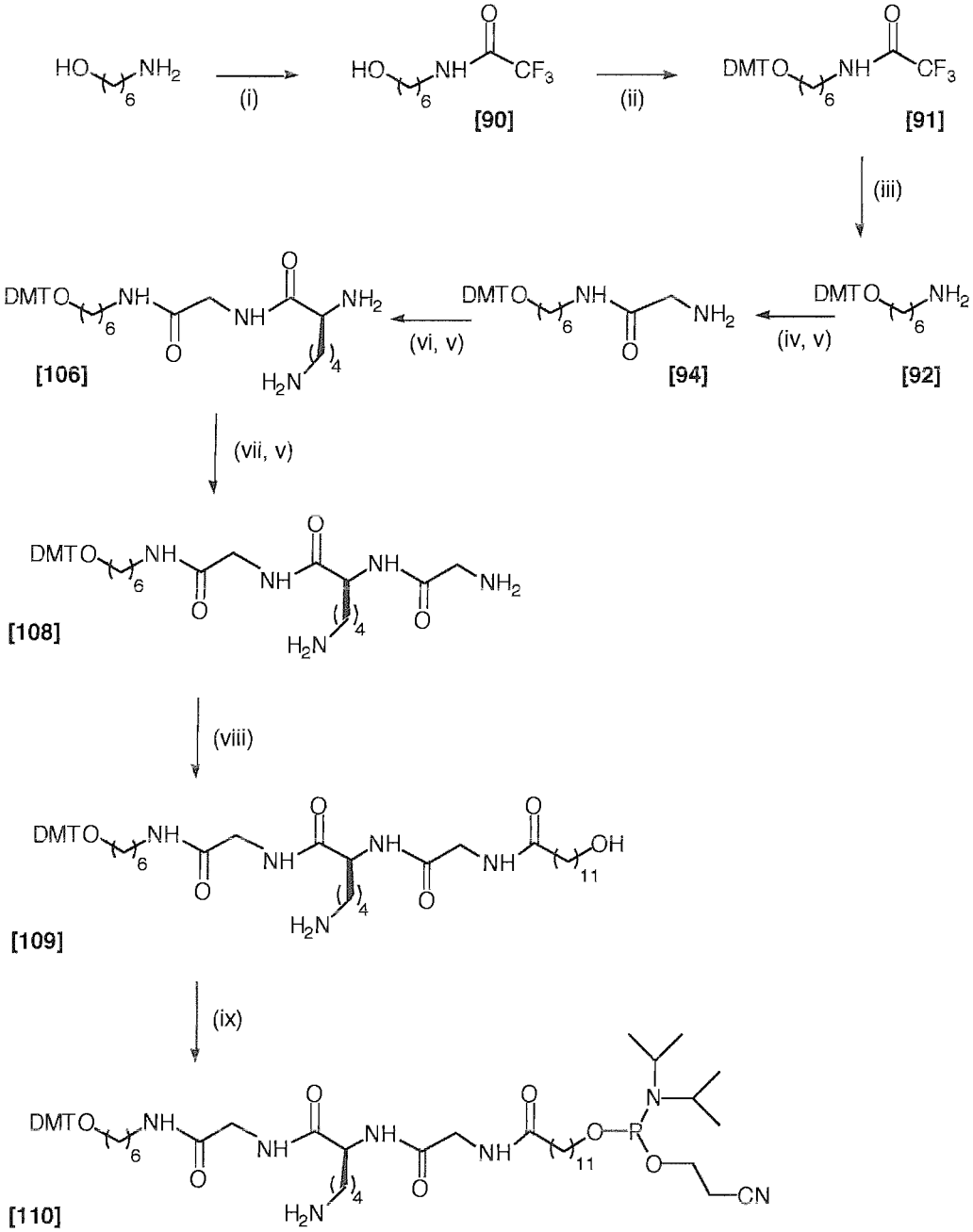


Figure 4.13 - Lysine Linker

The ε-amino moiety of lysine was protected with 4,4'-dimethyl-2,6-dioxocyclohex-1-y (idene)ethane (Dde), as the conditions for removal of this group (hydrazine in DMF) are orthogonal to the conditions of DNA synthesis. This choice of protecting group allowed the molecule to be assembled by solution phase peptide chemistry and deprotected after oligonucleotide assembly. The tripeptide section of the linker was assembled from the with the protected hydroxyamine spacer **[92]**. A series of carbodiimide coupling and piperidine deprotection reactions generated a tripeptide amine, onto which a twelve-carbon spacer was added to provide the final alcohol for phosphitylation (figure 4.14).

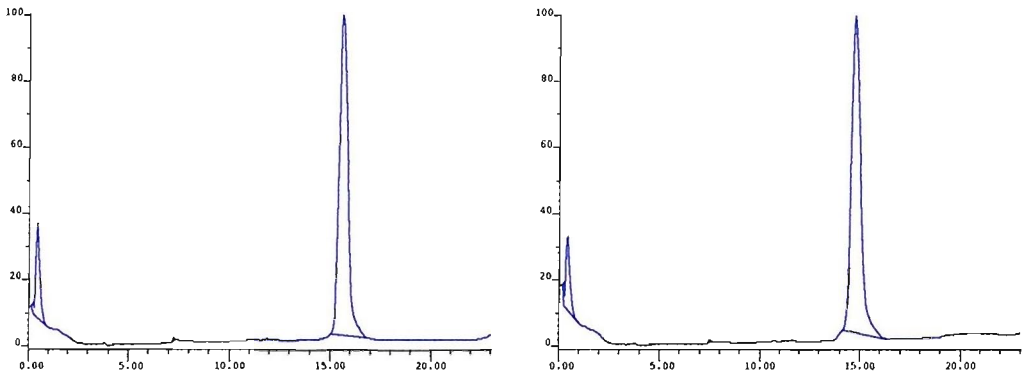


Reagents and conditions: i, $EtOCOCF_3$, Et_3N, CH_2Cl_2, rt,12hrs, 71%; ii, DMTCl, DMAP, pyridine, rt, 15hrs, 81%; iii, NH_3 (g), CH_3OH, rt, 18hrs, 88%; iv, Fmoc-Gly-OH, EDC, CH_2Cl_2, DMF, 53%; v, 20% piperidine, DMF, rt, 1-24hrs, **[94]** = 67%, **[106]** = 69%, **[108]** = 82%; vi, Fmoc-Lys(Dde)-OH, EDC, CH_2Cl_2, rt, 1hr, 88%; vii, Fmoc-Gly-OH, EDC, CH_2Cl_2, DMF, rt, 2.5hrs, 67%; viii, 12-hydroxydodecanoic acid, EDC, CH_2Cl_2, rt, 2.5hrs, 82%; xi, 2-cyanoethyldiisopropylchlorophosphoramidite, DIPEA, CH_2Cl_2, rt, 2.5hrs, 91%

Figure 4.14 - Synthesis of lysine linker phosphoramidite

4.3.3.2 Linker cleavage

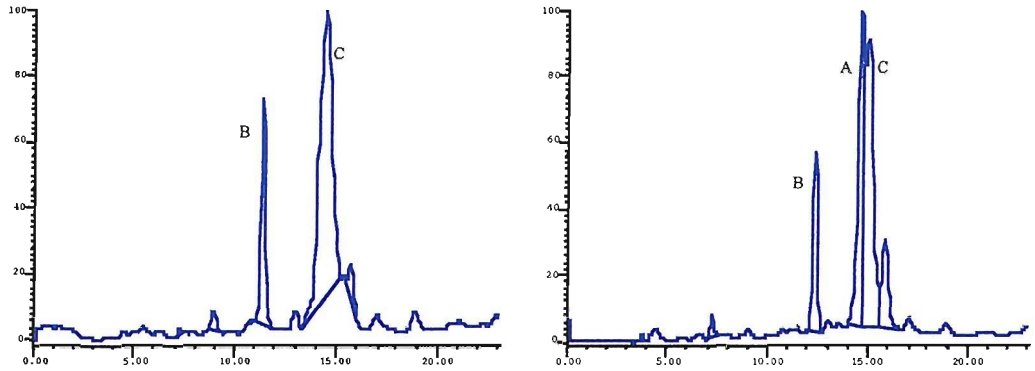
Initial experiments to examine the hydrolysis of the linker were performed in solution. Two oligonucleotides were synthesised and purified by RPHPLC TTTTTT**L**TTTTTTTTTTTTTTT **[111]**, TTCTTCTT**L**TCCTCTTCTTCTCCTTT **[112]** (where **L** indicates the incorporation of the lysine linker monomer **[110]**, figure 4.15).



(i) (ii)

Figure 4.15 - Lysine linker oligonucleotide prior to hydrolysis (t = 0 min) i, **[111]** ii, **[112]**

Deprotection of the oligonucleotides with concentrated aqueous ammonia was shown to remove Dde protection from the lysine side chain by RPHPLC and mass spectral analysis. The fully deprotected oligonucleotides were incubated, in buffer, with a large excess of trypsin at 37°C for 24 hours. Trypsin was removed by centrifugal ultrafiltration and the reaction products were analysed by RPHPLC eluting under identical conditions to initial oligonucleotide purification. Hydrolysis of the lysine linker was shown by almost total conversion of starting material to two product peaks (figure 4.16, i). Mixed injection of starting oligonucleotide with the reaction products confirmed that the oligonucleotide had been completely hydrolysed (figure 4.16, ii).

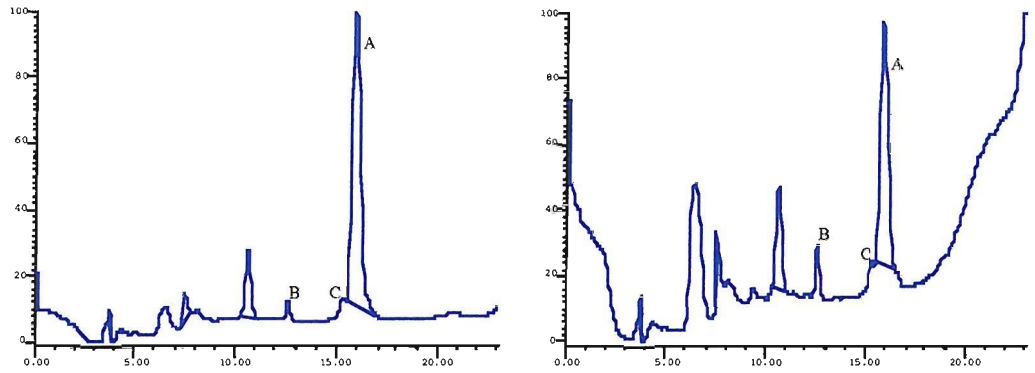


(i) (ii)

Figure 4.16 - i, Complete hydrolysis of starting oligonucleotide **[112]** by trypsin generating cleavage fragments (fluorescent product B and non-fluorescent product C). All other peaks are enzyme fragments (t = 24hrs), ii, mixed injection of oligonucleotide **[112]** (A) and cleavage fragments (B and C) from trypsin hydrolysis

To assess the rate of cleavage of the oligonucleotides **[111]** and **[112]** six samples of the modified oligonucleotides were placed in separate tubes and incubated at 37°C, in buffer, five tubes contained excess trypsin, one omitting trypsin as a control. At 10mins, 30mins, 1hr, 3hrs, and 24hrs intervals one tube was removed, denatured (95°C) and the trypsin was removed. Results of RPHPLC analysis for both oligonucleotides, inferred complete conversion of starting oligonucleotide to hydrolysed product after 10mins (or less) with no further significant change with time. The experiment was repeated using **[111]** and one equivalent of trypsin, the starting material was hydrolysed more slowly and the reaction was approximately 80% complete after 24 hrs (figure 4.17).

The hydrolysis of the linker located between a resin and an oligonucleotide was investigated. T**L**HH-CPG **[113]** where H = hexaethyleneglycol and L = lysine linker was assembled using trityl-on programme ensuring the thymidine possessed trityl protection at the 5' position, and remained attached to the glass. The resin was exposed to hydrazine in DMF for 15 minutes to remove the Dde group from the lysine of the linker. Acid catalysed detritylation and quantification at 495nm was used to ascertain the loading of the resin. The remainder of the resin was incubated with trypsin for 48hrs, washed and the loading recalculated in an identical manner. A 40% loss of the nucleoside from the resin was observed.



(i) (ii)

Figure 4.17 - Lysine linker oligonucleotide **[111]** (A) hydrolysis using one equivalent of trypsin generating cleavage fragments (B and C). All other peaks are enzyme fragments i, t = 10 mins, ii, t = 1 hour

4.3.3.3 Conclusions

Results of solution studies demonstrate that hydrolysis of the lysine linker is very fast with excess enzyme. Retardation of the rate is achieved when only one equivalent of trypsin is available. Cleavage of the linker adjacent to a resin was more successful than cleavage of the phenylalanine tripeptide linker. However, the exposure time was extended which may explain the result. Incomplete cleavage may be due to steric factors between resin and enzyme; improved yield might be achieved with more hydrophilic resins, or inserting additional spacer molecules between resin and linker.

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Chapter 5
Experimental

5.0 Experimental

5.1 Preparation of Compounds

5.1.1 General Methods

All reactions requiring anhydrous conditions were performed in dry glassware under an atmosphere of nitrogen or argon. Methylene chloride, pyridine and triethylamine were distilled from calcium hydride. Tetrahydrofuran was freshly distilled from sodium wire and benzophenone. Methanol was distilled from magnesium and iodine. Quinoline was distilled from 3Å molecular sieves. Anhydrous benzene, dimethylformamide and diisopropylethylamine were purchased from Aldrich. Other reagents were purchased from Aldrich/Sigma or Lancaster.

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

A: CH_2Cl_2: MeOH 4:1 + 1% AcOH
B: CH_2Cl_2: MeOH: Et_3N 8.75:0.25:1.00
C: CH_2Cl_2: MeOH: Et_3N 8.5:0.5:1.0
D: CH_2Cl_2: MeOH 9:1
E: EtOAc
F: $CHCl_3$: MeOH 19:1
G: EtOAc: Et_3N 9:1
H: CH_2Cl_2: MeOH: Et_3N 8.75:1.00:0.25
I: CH_2Cl_2: MeOH: Et_3N 9:1:1
J: EtOAc: hexane 9:1

K: EtOAc: hexane 1:9

L: EtOAc: hexane 3:7

M: EtOAc: hexane 1:1

O: CH_2Cl_2: MeOH 4:1

P: CH_2Cl_2: MeOH 19:1

Q: EtOAc: MeOH 19:1

Infrared spectra were measured on a Perkin-Elmer 1600 series FTIR machine using samples supported on sodium chloride cells, or on a BIORAD FT-IR instrument using a Golden Gate adapter and BIORAD WIN-IR software. Absorbtions are described as strong (s), medium (m), weak, or broad (br).

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

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

Proton NMR was recorded at 300MHz and carbon NMR at 75.5MHz on a Brucker AM 300 spectrometer in either deuterated chloroform or D_6-DMSO. The multiplicites of carbon-13 signals were determined using distortionless enhancement by phase transfer (DEPT) spectral editing technique.

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

Microanalytical data was obtained from University College, London.

5.1.2 List of Compounds

[1]	5(6) Carboxyfluorescein	117
[2]	6-(*t*-Butyldimethylsilyloxy)-1-hexylamine	118
[7]	5'-O-(4,4'-Dimethoxytrityl)-2'-deoxythymidine	119
[9]	5'-O-(4,4'-Dimethoxy)trityl-2'-deoxythymidyl-3'-O-(1,1,3,3-tetra-isopropyl-disiloxyl-3-) hexanol	120
[10]	5'-O-(4,4'-Dimethoxytrityl)-2'-deoxythymidyl-3'-O-(1,1,3,3-tetra-iso propyl-disiloxyl -3'O-cyanoethoxy)-*N,N*-diisopropylamino) phosphine	121
[11]	12-O'-(4,4'-Dimethoxytrityloxy)dodecanoic acid	122
[15]	5(6)-Carboxyfluorescein-*N*-hydroxysuccinimide ester	123
[18]	5(6)-Carboxyfluorescein Dipivalate	124
[19]	6-Carboxyfluorescein dipivalate diisopropylamine salt	125
[20]	6-Carboxyfluorescein	126
[21]	6-(*t*-Butyldimethylsilyloxy)-1-hexyl-6-carboxyfluoresceinamide	127
[22]	α-D-Tetraacetate galactosyl bromide	128
[23]	6-(*t*-Butyldimethylsilyloxy)-1-hexyl-6-carboxyfluoresceinamide-(3,6)-*bis*-β-D-galactose tetraacetate	129
[24]	1-*O*-Hexyl-6-carboxyfluoresceinamide -(3,6)-*bis*-β-galactose tetraacetate	131
[25]	1-*O*-(cyanoethoxy-*N,N*-diisopropylamino phosphine) hexyl-6-carboxyfluoresceinamide(3,6)-*bis*-β-Galactose tetraacetate	132
[65]	4-(4-(O-4, 4'-dimethoxytrityl)-hydroxmethyl-2-methoxy-5-nitro-phenoxy)-butyric acid	133
[70]	2,3,4,6-Tetra-*O*-acetyl-D-galactose	134
[71]	*O*-(2,3,4,6-Tetra-*O*-acetyl)-α-D-galactosyl trichloroacetimidate	135
[72]	6-(*t*-Butyldimethylsilyl)oxy-1-hexanol	136
[73]	*O*-β-D-[(2,3,4,6-Tetra-*O*-acetyl)-galactosyl-[6-(*t*-butyldimethylsilyl) oxy hexane]	136
[74]	*O*-β-D-[6-(*t*-Butyldimethylsilyl)oxy hexane]-galactose	137
[75]	*O*-β-D-(6-*O*-4,4'-Dimethyloxytrityl)-galactosyl-[6-(*t*-butyldimethylsilyl) oxy hexane]	138

[76]	*O*-β-D-[(2,3,4-Tri-*O*-acetyl)-6-*O*-(4,4'-dimethyloxytrityl)]-galactosyl -[6-(*t*-butyldimethylsilyl)oxy] hexane	139
[77]	*O*-β-D-[(2,3,4-Tri-*O*-acetyl)-6-*O*-(4,4'-dimethyloxytrityl)]-galactosyl hexan-6-ol	141
[78]	*O*-β-D-[(2,3,4-Tri-*O*-acetyl)-6-*O*-(4,4'-dimethyloxytrityl)]-galactosyl-[6-(2-cyanoethyl diisopropylphosphoramidyl)oxy hexane]	142
[81]	Fmoc-phenylalanine-6-hydroxyhexylamide	143
[82]	Fmoc-phenylalanine-6-hydroxy(-*O*-4,4'dimethoxytrityl-)hexylamide	144
[83]	Phenylalanine-6-hydroxy(-*O*-4,4'dimethoxytrityl-)hexylamide	145
[84]	12-Hydroxy-dodecanamido-phenylalanine-6-hydroxy (-*O*-4,4'dimethoxytrityl-) hexylamide	146
[85]	12-(2-Cyanoethyl-diisopropylphosphoramidyl)oxy-dodecanamido-phenylalanine-6-hydroxy(-*O*-4,4'dimethoxytrityl-)hexylamide	147
[90]	1-Hexanol-6-trifluoroacetamide	147
[91]	1- O'-(4,4'-Dimethoxytrityloxy) hexyl-6-trifluoroacetamide	148
[92]	1-*O*-(4,4'-Dimethoxytrityloxy)-6-hexylamine	149
[93]	Fmoc-glycine-6-hydroxy(-*O*-4,4'-dimethoxytrityl)hexylamide	150
[94]	Glycine-6-hydroxy(-*O*-4,4'-dimethoxytrityl)hexylamide	151
[95]	Fmoc-phenylalanine-glycine-6-hydroxy(-*O*-4,4'-dimethoxytrityl) hexylamide	152
[96]	Phenylalanine-glycine-6-hydroxy(-*O*-4,4'-dimethoxytrityl)hexylamide	153
[97]	Fmoc-glycine-phenylalanine-glycine-6-hydroxy(-*O*-4,4'-dimethoxytrityl)hexylamide	154
[98]	Glycine-phenylalanine-glycine-6-hydroxy(-*O*-4,4'-dimethoxytrityl) hexylamide	155
[99]	12-Hydroxydodecanamido-glycine-phenylalanine-glycine-6-hydroxy (-*O*-4,4'-dimethoxytrityl)hexylamide	156
[100]	12-(2-Cyanoethyl diisopropylphosphoramidyl)oxy-dodecanamido-glycine-phenylalanine-glycine-6-hydroxy (-*O*-4,4'-dimethoxytrityl)hexylamide	157
[105]	Fmoc-lysine(Dde)-glycine-6-hydroxy(-*O*-4,4'-dimethoxytrityl) hexylamide	158
[106]	Lysine(Dde)-glycine-6-hydroxy(-*O*-4,4'-dimethoxytrityl)hexylamide	159

[107] Fmoc-glycine-lysine(Dde)-glycine-6-hydroxy(-*O*-4,4'-dimethoxytrityl) hexylamide 161

[108] Glycine-lysine-(Dde) -glycine-6-hydroxy (-*O*-4,4'-dimethoxytrityl) hexylamide 162

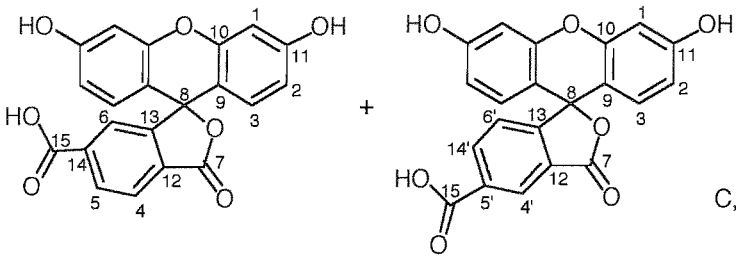
[109] 12-Hydroxydodecanamido-glycine-lysine-(Dde)-glycine-6-hydroxy (-*O*-4,4'-dimethoxytrityl)hexylamide 163

[110] 12-(2-Cyanoethyl-diisopropylphosphoramidyl)oxy-dodecanamido-glycine-lysine(Dde)-glycine-6-hydroxy (-O-4,4'-dimethoxytrityl)hexylamide 164

Silver oxide catalyst 165

Silver silicate catalyst 165

5.1.3 Experimental



$C_{21}H_{12}O_7$
Mol. Wt.: 376.32
C, 67.02; H, 3.21; O, 29.76

5(6) Carboxyfluorescein [1][194]

Method 1

4-Carboxyphthalic anhydride (4.80g, 25.0mmol) was ground to a fine powder and placed in an open metal pot. Resorcinol (5.45g, 49.5mmol) was added, and the mixture heated to 190°C for 1 hr with manual stirring. During this period anhydrous zinc chloride (1.75g, 12.5mmol) was added portionwise, and the reaction stirred until too viscous to continue. The red solid was cooled to room temperature and then allowed to stand for 1hr, water (50mL) and aqueous hydrochloric acid (concentrated, 2.5mL) were added. The mixture was heated to boiling, filtered and the residue dissolved in aqueous sodium hydroxide solution (2M, 40mL). This was dropped into rapidly stirring aqueous hydrochloric acid (4M, 50mL) affording an orange precipitate, which was removed by filtration. The orange suspension was taken up in MeOH (50mL) to aid handling and then the solvent removed *in vacuo*. The residue was triturated with Et_2O (30mL), filtered and evaporated to dryness. The product was dried over P_2O_5, *in vacuo*, for 24 hr to give orange flakes that were ground to a fine powder (5.32g, 14.1mmol, 57%, improved to 70%).

Method 2

4-Carboxyphthalic anhydride (38.40g, 0.2mol) was ground to a fine powder and place in an open metal pot. Resorcinol (43.60g, 0.4mol) was added, and the mixture heated to 190°C for 1 hr with manual stirring. The resulting red solid was cooled to room temperature and allowed to stand for 1hr, dissolved in aqueous sodium hydroxide solution (2M, 300mL) and dropped into rapidly stirring aqueous hydrochloric acid (4M, 300mL). This gave an orange precipitate that was filtered, dissolved in MeOH (200mL) and the solvent removed *in*

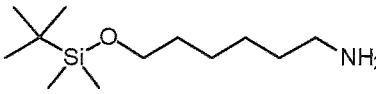
vacuo. The product was dried over P_2O_5, *in vacuo* for 24 hrs to give orange flakes that were ground to a fine powder (64.00g, 170.2mmol, 85%).

R_f (A): 0.78; LRMS (ES^- mode): *m/z* 375.2 (M^-), 489.2 ($MTFA^-$); HRMS: $C_{21}H_{13}O_7$ requires 377.06613, found 377.06613; λ_{max} (MeOH): 483nm; mp: 350-355°C (lit.mp: 356-358°C)

IR (CH_2Cl_2): ν = 2922.9 (s), 2842.7 (s), 1629.4 (m), 1459.2 (m), 1374.0 (m), 722.5 (w) cm^{-1}

^1H (d_6-DMSO): δ = 8.40 (1H, s, $H_{4'}$), 8.32 (1H, d, J = 9.5Hz, H_4), 8.20 (1H, d, J = 8.1Hz, $H_{14'}$), 8.12 (1H, d, J = 8.1Hz, H_5), 7.65 (1H, s, H_6), 7.40 (1H, d, J = 8.1Hz, $H_{6'}$), 6.75 (4H, s, H_1), 6.58-6.61 (8H, m, $H_{2,3}$), 3.50 (2H, br, OH)

^{13}C (d_6-DMSO): δ = 167.9, 166.2 ($C_{7, 15}$), 159.7 (C_{10}), 156.1 (C_{11}), 151.9 (C_{13}), 136.2 (C_{12}), 133.2 (C_{14}), 129.3, 126.9, 125.6, 124.6 ($C_{3\text{-}6, 9}$), 112.8 (C_2), 109.0 (C_8), 102.4 (C_1)



$C_{12}H_{29}NOSi$
Mol. Wt.: 231.45
C, 62.27; H, 12.63; N, 6.05; O, 6.91; Si, 12.13

6-(*t*-Butyldimethylsilyloxy)-1-hexylamine [2][195]

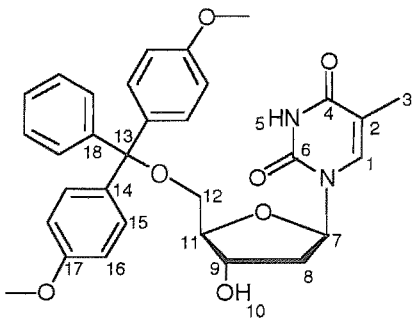
To a solution of 6-amino-1-hexanol (6.21g, 53.1mmol) in anhydrous pyridine (50mL) was added *t*-butyldimethylsilyl chloride (9.52g, 57.2mmol). The yellow solution was stirred at room temperature, under argon, for 20hrs. The solvent was removed *in vacuo*, and the resulting viscous brown oil was dissolved in CH_2Cl_2 (100mL), washed with saturated sodium bicarbonate solution (2x100mL) and dried (Na_2SO_4). Removal of the solvents *in vacuo* afforded an opaque yellow oil, which was purified by silica gel column chromatography eluting with CH_2Cl_2: MeOH (9:1). The title compound was obtained as a pale yellow oil, which was dried over P_2O_5 *in vacuo* for 24 hrs (9.63g, 41.6mmol, 79%).

R_f (H): 0.40; LRMS (ES^+ mode): *m/z* 232 (MH^+); HRMS: $C_{12}H_{30}NOSi$ requires 232.20967, found 232.20967; λ_{max} (MeOH): 254nm

IR (CH_2Cl_2): ν = 3350.0 (w), 2829.7 (s), 2374.6 (m), 1473.0 (m), 1254.9 (s), 1095.3 (s), 834.6 (s) cm^{-1}

^{1}H ($CDCl_3$): δ = 3.55 (2H, t, J = 6.6 Hz, CH_2O), 2.67 (2H, t, J = 6.6 Hz, $C\underline{H}_2NH_2$), 1.70 (2H, br s, NH_2), 1.52-1.42 (4H, m, $C\underline{H}_2CH_2O$, $C\underline{H}_2CH_2NH_2$), 1.38-1.25 (4H, m, CH_2), 0.90 (9H, s, $(CH_3)_3CSi$), 0.00 (6H, s, $(CH_3)_2Si$)

^{13}C ($CDCl_3$): δ = 63.3 (CH_2OSi), 42.2 (CH_2NH_2), 33.7, 32.9(CH_2), 26.8 (CH_2), 26.1 ($(\underline{C}H_3)_3C$), 25.9 (CH_2), 18.5 ($(CH_3)_3\underline{C}$), -3.3 (CH_3Si)



$C_{31}H_{32}N_2O_7$
Mol. Wt.: 544.60
C, 68.37; H, 5.92; N, 5.14; O, 20.56

5'-O-(4,4'-Dimethoxytrityl)-2'-deoxythymidine [7]

Anhydrous pyridine (2 x 20mL) was coevaporated from thymidine (6.06g, 25.0mmol), 4-dimethylaminopyridine (0.36g, 2.9mmol) was added, and pyridine (20mL) was coevaporated from the mixture a second time. The resulting mixture was dissolved in pyridine (30mL) and a solution of 4,4'-dimethoxytrityl chloride (9.32g, 27.5mmol) in pyridine (10mL) was added portionwise over 1.5 hrs. After stirring under argon for 24hrs, the reaction was quenched with MeOH (20mL), and the solvents removed *in vacuo*. The resulting solid was dissolved in EtOAc (150mL), washed with saturated sodium bicarbonate solution (3 x 100mL), dried ($MgSO_4$), and the solvents removed *in vacuo* to give a yellow foam. Recrystallisation from benzene afforded the title compound as a white solid, which was dried over P_2O_5 *in vacuo* for 24 hrs (11.10g, 20.4mmol, 82%).

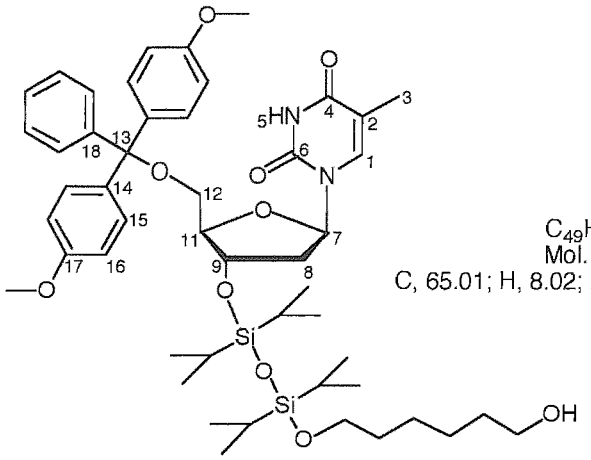
R_f (D): 0.63; LRMS (ES^+ mode): *m/z* 303.2 (DMT^+), 567.2 (MNa^+); HRMS: $C_{31}H_{32}N_2O_7$ requires 544.22095, found 544.22095; λ_{max} (MeOH): 269, 234nm; mp: 124-127°C

IR:ν = 3036.8 (w), 2834.16 (w), 1677.6 (s), 1606.3 (m), 1252.6 (s), 1175.8 (s), cm^{-1}

^{1}H ($CDCl_3$): δ = 11.40 (1H, s, H_5), 7.50 (1H, s, H_1), 7.40-7.15 (9H, m, Ph$\underline{H}$, H_{15}), 6.90 (4H, d, J = 8.8 Hz, H_{16}), 6.23 (1H, t, J = 6.6 Hz, H_7), 5.35 (1H, d, J = 4.4 Hz, H_9), 4.33 (1H, s,

H_{10}), 3.92-3.88 (1H, m, H_{11}), 3.75 (6H, s, OCH_3), 3.35 (3H, s, H_3), 3.24-3.16 (2H, m, H_{12}), 2.35-2.10 (2H, m, H_8)

^{13}C ($CDCl_3$): δ = 163.8 (C_4), 158.3 (C_{17}), 150.5 (C_6), 144.8 (C_{18}), 135.5 (C_{14}), 129.8 (C_1), 128.4, 128.0, 127.8, 126.9 ($C_{Ph,\ 15}$), 113.3 (C_{16}), 109.7 (C_2), 86.0 (C_{13}), 85.6 (C_7), 83.9 (C_9), 70.7 (C_{11}), 63.9 (C_{12}), 55.1 (OCH_3), 39.6 (C_8), 11.8 (C_3)



$C_{49}H_{72}N_2O_{10}Si_2$
Mol. Wt.: 905.27
C, 65.01; H, 8.02; N, 3.09; O, 17.67; Si, 6.20

5'-O-(4,4'-Dimethoxy)trityl 2'-deoxythymidyl-3'-O-(1,1,3,3-tetra-isopropyl-disiloxyl-3-) hexanol [9][145]

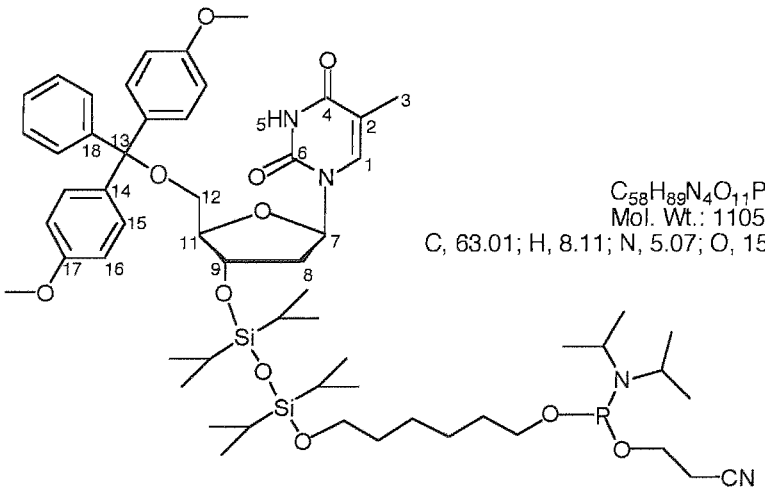
Freshly distilled pyridine (2 x 10mL) was coevaporated from the alcohol **[7]** (2.60g, 4.8mmol), imidazole was added (0.64g, 9.6mmol) and pyridine (10mL) was coevaporated from the mixture. To a stirring suspension of the mixture in pyridine (40mL), under argon, was added 1,3-dichloro-1,1,3,3-tetraisopropyl disiloxane (1.50g, 4.8mmol). The reaction was stirred at room temperature for 24 hrs. This afforded 5'-O-(4,4'-Dimethoxytrityl)-2'-deoxythymidyl 3'-O-(1,1,3,3-tetra-isopropyl-disiloxyl) chloride **[8]**, which was used directly reacted in the following reaction. Hexane-1,6-diol (5.00g, 42.4mmol) was added and the reaction stirred for a further 12 hrs. The solvent was removed *in vacuo* and the resulting cloudy oil was dissolved in CH_2Cl_2 (150mL), washed with saturated sodium bicarbonate solution (3 x 100mL), then dried ($MgSO_4$). Removal of the solvent *in vacuo* yielded a cloudy oil that was purified by silica gel column chromatography eluting with MeOH: CH_2Cl_2 (0:100 then 2:98 then 1:19). Drying over P_2O_5 for 12 hrs *in vacuo* afforded the title compound as a clear oil (3.15g, 3.5mmol, 76%).

R_f (F): 0.65; LRMS (ES^+ mode): *m/z* 303.1 (DMT^+), 926.9 (MNa^+); λ_{max} (MeOH): 268, 233nm

IR: ν = 2936.06 (m), 2864.85 (m), 1687.4 (s), 1248.6 (m), 1607.27 (m), 1032.7 (s), cm^{-1}

^1H (DMSO): δ = 11.40 (1H, s, H_5), 7.50 (1H, s, H_1), 7.40-7.20 (9H, m, PhH, H_{15}), 6.90 (4H, d, J = 8.6 Hz, H_{16}), 6.23 (1H, t, J = 6.0 Hz, H_7), 4.60-4.58 (1H, m, H_9), 4.35 (1H, q, J = 5.1 Hz, H_{11}), 3.95 (1H, s, OH), 3.75 (6H, s, OCH_3), 3.60 (2H, t, J = 6.9 Hz, $C\underline{H}_2OH$), 3.35 (3H, s, H_3), 3.31-3.28 (2H, m, CH_2OSi), 3.25-3.20 (2H, m, H_{12}), 2.35-2.32 (2H, m, H_8), 1.50-1.20 (12H, m, CH_2, CHSi), 0.80-0.11 (24H, m, $C\underline{H}_3CHSi$)

^{13}C ($CDCl_3$): δ = 164.2 (C_4), 158.8 (C_{17}), 150.6 (C_6), 144.4 (C_{18}), 136.2 (C_{14}), 135.5 (C_1), 130.3, 128.3, 127.3, 123.9 ($C_{Ph,\ 15}$), 113.4 (C_{16}), 111.3 (C_2), 87.1 (C_7), 85.3 (C_{13}), 84.9 (C_9), 72.7 (C_{11}), 63.7 (CH_2OH), 62.8 (C_{12}), 55.4 (OCH_3), 46.1 (CH_2OSi), 41.8 (C_8), 32.8 (CH_2), 25.7 (CH_2), 17.6, 17.4, 17.3 ($\underline{C}H_3CHSi$), 13.5, 13.2, 13.0 ($CH_3\underline{C}HSi$), 11.9 (C_3)



$C_{58}H_{89}N_4O_{11}PSi_2$
Mol. Wt.: 1105.49
C, 63.01; H, 8.11; N, 5.07; O, 15.92; P, 2.80; Si, 5.08

5'-O-(4,4'-Dimethoxytrityl)-2'-deoxythymidyl-3'-O-(1,1,3,3-tetra-isopropyl-disiloxyl -3'O-cyanoethoxy)-*N,N*-diisopropylamino) phosphine [10][145]

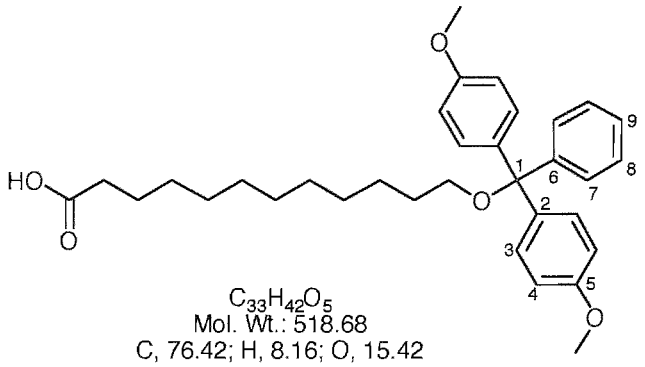
To a stirring solution of **[9]** (1.10g, 1.2mmol) in CH_2Cl_2 (10mL) was added anhydrous *N,N*-diisopropylethylamine (0.52g, 0.77mL, 4.5mmol) under argon. 2-Cyanoethyl-*N,N*-diisopropylchlorophosphoramidite (0.32g, 0.3mL, 1.4mmol) was added to the mixture dropwise, and the reaction allowed to stir at room temperature, under argon, for 1.5 hrs. EtOAc (50mL) was added, the organic layer washed with saturated potassium chloride solution (2 x 50mL), dried ($MgSO_4$) and the solvents removed *in vacuo*. The resulting oil

was purified by silica gel column chromatography, under argon, eluting with EtOAc. The clear oil obtained was dried over P_2O_5, *in vacuo*, for 24 hrs (1.32g, 1.2mmol, 98%).

R_f (G): 0.54; LRMS (ES^+ mode): *m/z* 1127.6 (MNa^+)

^1H (DMSO): δ = 11.40 (1H, s, H_5), 7.50 (1H, s, H_1), 7.25-7.15 (9H, m, H_{Ph}, H_{15}), 6.75 (4H, d, J = 8.6 Hz, H_{16}), 6.35 (1H, t, J = 6.0 Hz, H_7), 4.62-4.60 (1H, m, H_9), 4.00 (1H, q, J = 5.1 Hz, H_{11}), 3.86-3.84 (2H, m, $C\underline{H}_2CH_2CN$), 3.70 (6H, s, OCH_3), 3.62-3.58 (4H, m, CHN, CH_2OP), 3.35 (3H, s, H_3), 3.34-3.28 (2H, m, CH_2OSi), 3.25-3.20 (2H, m, H_{12}), 2.60 (2H, t, J = 5.0 Hz, CH_2CN), 2.35-2.32 (2H, m, H_8), 1.52-1.22 (12H, m, CH_2, CHSi), 1.20-1.15 (12H, m, $C\underline{H}_3CHN$), 0.90-0.11 (24H, m, $C\underline{H}_3CHSi$)

^{31}P (DMSO): δ = 147.9, 147.8



$C_{33}H_{42}O_5$
Mol. Wt.: 518.68
C, 76.42; H, 8.16; O, 15.42

12-O'-(4,4'-Dimethoxytrityloxy)dodecanoic acid [11]

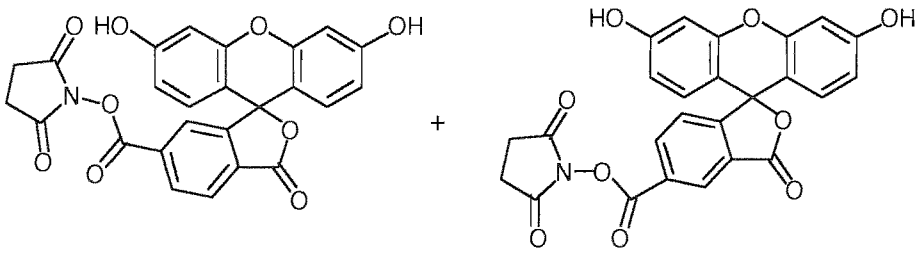
12-hydroxydodecanoic acid (3.00g, 13.9mmol) was dissolved in pyridine (10mL), and a solution of 4,4'-dimethoxytrityl chloride (5.16g, 15.2mmol) in pyridine (20mL) was added dropwise over a period of 0.5hr. The reaction was left stirring at room temperature, under argon, for 24 hrs. The mixture was concentrated *in vacuo*, extracted with CH_2Cl_2 (200mL), washed with saturated sodium bicarbonate solution (2x200mL), brine (200mL), dried ($MgSO_4$), and evaporated to dryness. The resulting cloudy yellow oil was purified by silica gel column chromatography, using silica pre-equilibrated with Et_3N: CH_2Cl_2 (95: 5) and eluted with CH_2Cl_2: MeOH (95: 5 then 90:10) giving a clear oil, which was dried over P_2O_5 under vacuum for 24 hrs (6.27g, 12.1mmol, 87%).

R_f (I): 0.61; LRMS (APCI$^-$ mode): *m/z* 517.4 (M^-), (ES$^-$ mode): *m/z* 631.5 ($MTFA^-$); HRMS: $C_{33}H_{42}O_5$ requires 518.30322, found 518.30322; λ_{max} (MeOH): 232nm

IR: ν = 2925.2 (m), 2853.23 (m), 1712.7 (w), 1507.94 (m) cm^{-1}

^1H ($CDCl_3$):δ = 8.50 (1H, br s, OH), 7.45-7.10 (9H, m, *J* = 8.6 Hz, $H_{3,\ 7\text{-}9}$), 6.65 (4H, d, *J* = 8.6 Hz, H_4), 3.70 (6H, s, CH_3O), 3.00 (2H, t, *J* = 7.1 Hz, CH_2O), 2.12 (2H, t, *J* = 6.4 Hz, CH_2CO), 1.60-1.45 (4H, m, $C\underline{H}_2CH_2O$, $C\underline{H}_2CH_2CO$), 1.24-1.21 (14H, m, CH_2)

^{13}C ($CDCl_3$): δ = 179.1 (CO), 158.4 (C_5), 145.6 (C_6), 136.9 (C_2), 130.2 (C_3), 128.4 (C_8), 127.8 (C_7), 126.7 (C_9), 113.1 (C_4), 85.7 (C_1), 63.7 (CH_2O), 55.3 (CH_3O), 35.9 ($\underline{C}H_2CO$), 30.3 ($\underline{C}H_2CH_2O$), 29.8, 29.7, 29.6, 26.5, (CH_2) 25.8 ($\underline{C}H_2CH_2CO$)

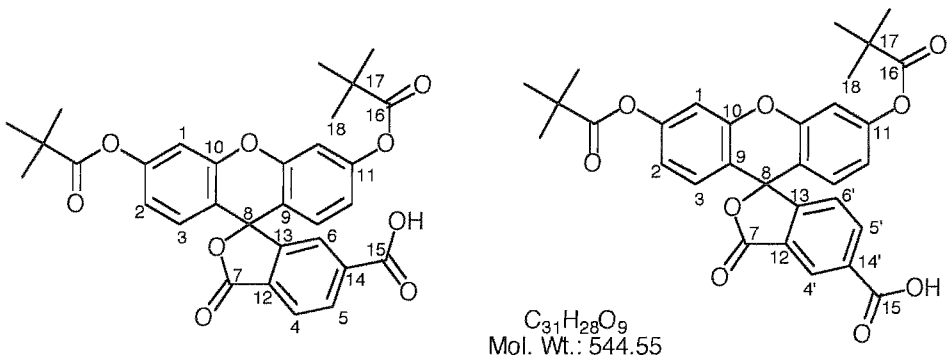


$C_{25}H_{15}NO_9$
Mol. Wt.: 473.39
C, 63.43; H, 3.19; N, 2.96; O, 30.42

5(6)-Carboxyfluorescein-*N*-hydroxysuccinimide ester [15][196,197]

To a suspension of 5(6)-carboxyfluorescein (1.00g, 2.7mmol) in EtOAc (30mL) was added EDC (0.51g, 2.7mmol) followed by *N*-hydroxysuccinimide (0.34g, 2.9mmol). The orange solution was stirred at room temperature, under argon, for 3 hrs. The solvent was removed *in vacuo* and the resulting viscous red oil was dissolved in CH_2Cl_2 (100mL), washed with saturated potassium chloride solution and dried ($MgSO_4$). The crude mixture was purified by silica gel column chromatography eluting with MeOH: CH_2Cl_2 (1:19). The product was dried over P_2O_5 *in vacuo* for 12 hrs to give an crude orange solid that was used directly in a labelling reaction (0.76g, 1.6mmol, 61%).

R_f (D): 0.55; LRMS (ES$^-$ mode): *m/z* 472.2 (M^-), λ_{max} (MeOH): 276, 466nm



$C_{31}H_{28}O_9$
Mol. Wt.: 544.55
C, 68.37; H, 5.18; O, 26.44

5(6)-Carboxyfluorescein Dipivalate [18][154]

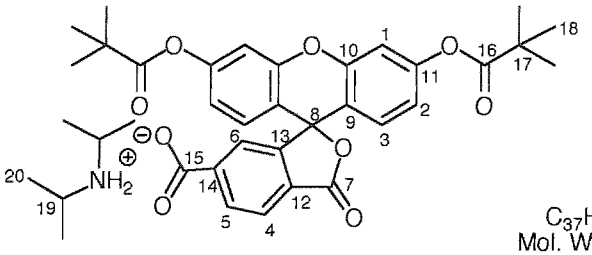
Anhydrous pyridine (80mL) was co-evaporated three times from 5(6)-carboxyfluorescein (19.01g, 50.5mmol) and the residue suspended in pyridine (40mL). Trimethylacetyl chloride (24.50g, 25mL, 0.2mol) was added dropwise to the stirring solution at 0°C, under argon. After 3 hrs the solvents were removed *in vacuo*. The residue was dissolved in CH_2Cl_2 (50mL), washed with saturated potassium chloride solution (3 x 50mL) then dried (Na_2SO_4). The solvents were removed *in vacuo* affording a yellow oil, which was purified by silica gel column chromatography eluting with CH_2Cl_2: Et_3N: MeOH (1:0:0 then 80:10:10). The title compounds were obtained as a yellow foam that was dried over P_2O_5, *in vacuo*, for 24 hrs (16.78g, 30.8mmol, 64%).

R_f (B): 0.52, 0.38; LRMS (ES^- mode): *m/z* 657.2 (M^-), 1201.3 ($2MTFA^-$); HRMS: $C_{31}H_{29}O_9$ requires 545.18116, found 545.18116; λ_{max} (MeOH): 276, 229nm; mp: 105-110°C

IR (CH_2Cl_2): ν = 3053.1 (w), 2983.0 (w), 1754.8 (s), 1694.7 (w), 1419.1 (m), 1258.7 (m) cm^{-1}

^1H ($CDCl_3$): δ = 8.60 (1H, s, $H_{4'}$), 8.26 (1H, d, J = 8.1 Hz, $H_{6'}$), 8.23 (1H, d, J = 8.1 Hz, H_4), 8.10 (1H, d, J = 8.1 Hz, H_5), 7.86 (1H, s, J = 8.1 Hz, H_6), 7.28 (1H, s, $H_{5'}$), 7.08 (4H, s, H_1), 6.80-6.78 (8H, m, $H_{2,3}$), 1.38 (36H, s, H_{19})

^{13}C ($CDCl_3$): δ = 176.7 (C_{16}), 168.0 (C_{15}), 165.5 (C_7), 156.9, 153.4, 152.9, 151.7, 151.6 ($C_{10\text{-}14}$), 136.8, 136.4, 132.7, 131.5, 129.5, 128.9, 127.0, 126.7 ($C_{3\text{-}6}$), 125.5, 125.4 (C_9), 118.0 (C_2), 115.7, 115.6 (C_8), 110.6 (C_1), 39.4 (C_{17}), 27.2 (C_{18})

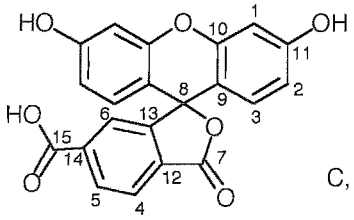


$C_{37}H_{42}NO_9$
Mol. Wt.: 644.73
C, 68.93; H, 6.57; N, 2.17; O, 22.33

6-Carboxyfluorescein dipivalate diisopropylamine salt [19][154]

5(6) Carboxyfluorescein (20.00g, 53.2mmol) was dissolved in anhydrous pyridine at 0°C. Trimethylacetyl chloride (28mL, 0.2mol) was added to the dark orange solution dropwise, and the reaction allowed to stir for 3 hrs, whilst warming to room temperature. The pale yellow mixture was diluted with CH_2Cl_2 (200mL), washed with saturated potassium chloride solution (2 x 250mL) and dried (Na_2SO_4). Removal of the solvents *in vacuo* gave a dark brown oil, which was converted to a stable foam, by coevaporation of CH_2Cl_2 (5x30mL). The foam (25.00g) was dissolved in boiling EtOH (55mL) to give a clear brown solution to which diisopropylamine (2.5mL) was added. The solution was cooled to room temperature then at -20°C for 18 hrs. A fine white precipitate was formed, which was removed by filtration and dried over P_2O_5 *in vacuo* for 24 hrs to give the desired compound as white solid (5.22g, 9.6mmol, 19%).

R_f (C): 0.73; LRMS (ES^- mode): *m/z* 543.0 (M^-), 657.0 ($MTFA^-$); HRMS: $C_{31}H_{29}O_9$ requires 545.18116, found 545.18116; λ_{max} (MeOH): 282, 233nm; mp: 222-227°C
IR (CH_2Cl_2): ν = 3052.1 (w), 2980.0 (w), 1754.8 (m), 1694.7 (w), 1609.5 (m), 1419.1 (m), 1258.7 (m) cm^{-1}
Found C: 68.78, H: 6.47, N: 1.82%, $C_{37}H_{42}NO_9$ requires C: 68.93, H: 6.57, N: 2.17%
^1H ($CDCl_3$): δ = 8.25 (1H, d, J = 8.1 Hz, H_4), 7.94 (1H, d, J = 8.1 Hz, H_5), 7.62 (1H, s, H_6), 6.97 (2H, d, J = 2.2 Hz, H_1), 6.80 (2H, d, J = 8.8 Hz, H_3), 6.68 (2H, dd, J = 8.8, 2.2 Hz, H_2), 3.15 (2H, sept, J = 6.0 Hz, H_{19}), 1.30 (18H, s, H_{18}), 1.16-1.14 (12H, m, H_{20})
^{13}C ($CDCl_3$): δ = 176.7 (C_{16}), 170.0 ($C_{7,15}$), 169.4 ($C_{12,14}$), 153.1, 152.6, 151.6 ($C_{13,11,10}$), 131.3, 129.2, 124.7, 124.6 ($C_{3\text{-}6}$), 127.1 (C_9), 117.8 (C_2), 116.4 (C_8), 110.3 (C_1), 46.6 (C_{19}), 39.3 (C_{17}), 27.2 (C_{18}), 19.2 (C_{20})



$C_{21}H_{12}O_7$
Mol. Wt.: 376.32
C, 67.02; H, 3.21; O, 29.76

6-Carboxyfluorescein [20][154]

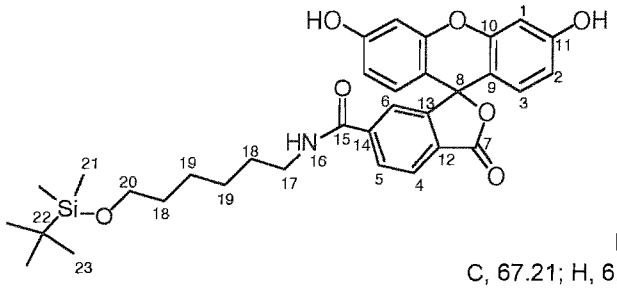
Method 1

To a solution of MeOH: concentrated aqueous ammonia (50: 5, 20mL) was added 6-carboxyfluorescein dipivalate (1.00g, 1.8mmol, isolated by silica gel column chromatography of **[18]**). The mixture was stirred under argon, for 18 hrs, at room temperature. The solution was evaporated to dryness and dried over P_2O_5 *in vacuo* to yield an orange solid (0.59g, 1.6mmol, 85%).

Method 2

To a solution of aqueous sodium hydroxide (1.00g, 25.0mmol) in wet MeOH (40mL) was added 6-carboxyfluorescein dipivalate diisopropylamine salt **[19]** (3.80g, 7.0mmol). The reaction was stirred at room temperature for 16 hrs. Removal of the solvents *in vacuo* gave a deep red oil, which was dissolved in water (100mL) and acidified with aqueous hydrochloric acid (2M, 200mL). The resulting orange precipitate was removed by filtration, washed with Et_2O (100mL), and dried (60°C) for 18 hrs to give the title compound as an orange solid (2.40g, 6.4mmol, 92%).

R_f (A): 0.69; LRMS (ES^- mode): *m/z* 375.3 (M^-), 489.2 ($MTFA^-$); HRMS: $C_{21}H_{13}O_7$ requires 377.06613, found 377.06613; λ_{max} (MeOH): 490nm; mp: 352-356°C
Found C: 67.09, H: 3.69%, $C_{21}H_{12}O_7$ requires C: 67.03, H: 3.21%
IR (CH_2Cl_2): ν = 2923.9 (s), 2842.7(s), 1628.6, 1460.0 (m), 1374.0 (m) cm^{-1}
^1H (d_6-DMSO): δ = 8.25 (1H, d, J = 8.1 Hz, H_4), 8.15 (1H, d, J = 8.1 Hz, H_5), 7.70 (1H, s, H_6), 6.85 (2H, s, H_1), 6.82-6.78 (4H, m, $H_{2,3}$), 5.50 (br s, OH)
^{13}C (d_6-DMSO): δ = 167.8 ($C_{7,15}$), 166.1, 160.8, 152.5 ($C_{10,11,13}$), 137.1 ($C_{12,14}$), 131.0 (C_3), 130.0 (C_9), 129.6, 125.9, 125.1 ($C_{6\text{-}6}$), 113.5 (C_2), 109. 6 (C_8), 102.4 (C_1)

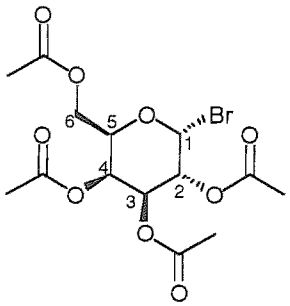


$C_{33}H_{39}NO_7Si$
Mol. Wt.: 589.75
C, 67.21; H, 6.67; N, 2.38; O, 18.99; Si, 4.76

6-(*t*-Butyldimethylsilyloxy)-1-hexyl-6-carboxyfluoresceinamide [21]

To a solution of 6-carboxyfluorescein (5.0g, 13.3mmol) in dry pyridine (20mL) was added 6-(*t*-butyldimethylsilyloxy)-1-hexylamine **[13]** (3.37g, 14.6mmol) and EDC (2.80g, 14.6mmol). The reaction was stirred at room temperature, under argon for 24 hrs. The solvents were removed *in vacuo* giving a red oil, which was dissolved in CH_2Cl_2 (200mL), washed with water (2 x 125mL), and dried (Na_2SO_4). The solvents were removed *in vacuo* producing a red foam, which was purified by chromatography on silica gel eluting with MeOH: CH_2Cl_2 (1:100 then 1:10). Drying over P_2O_5 *in vacuo*, for 24 hrs, gave the title compound as an orange solid (5.20g, 8.8mmol, 66%).

R_f(D): 0.38; LRMS (ES^+ mode): *m/z* 590.5 (MH^+); λ_{max} (MeOH) 461, 234nm
IR (CH_2Cl_2): ν = 3315.9 (w), 2928.1 (w), 2850.5 (w), 1627.3 (m), 1572.2 (m), 1241.8 (s) cm^{-1}
^1H ($CDCl_3$): δ = 10.20 (2H, br s, OH), 8.65 (1H, t, J = 5.2 Hz, H_{16}), 8.22 (1H, d, J = 8.1 Hz, H_4), 8.12 (1H, s, H_6), 7.25 (1H, d, J = 8.1 Hz, H_5), 6.70 (2H, s, H_1), 6.61-6.58 (4H, m, $H_{2,3}$), 3.60 (2H, t, J = 5.9 Hz, H_{20}), 3.26-3.23, (2H, m, H_{17}), 1.42-1.39 (8H, m, H_{18}, H_{19}), 0.83 (9H, s, H_{23}), 0.00 (6H, s, H_{21})
^{13}C ($CDCl_3$): δ = 168.2 ($C_{15,7}$), 164.4 (C_{10}), 160.1 (C_{11}), 152.0 ($C_{12,14}$), 140.7 (C_{13}), 129.3 (C_3), 128.6 (C_9), 125.0, 122.5 (C_{4-6}), 113.0 (C_2), 109.3 (C_8), 102.3 (C_1), 62.4 (C_{20}), 32.2 (C_{17}), 29.0, 27.0, 26.3, 25.1 ($C_{18,19}$), 25.9 (C_{23}), 18.0 (C_{22})



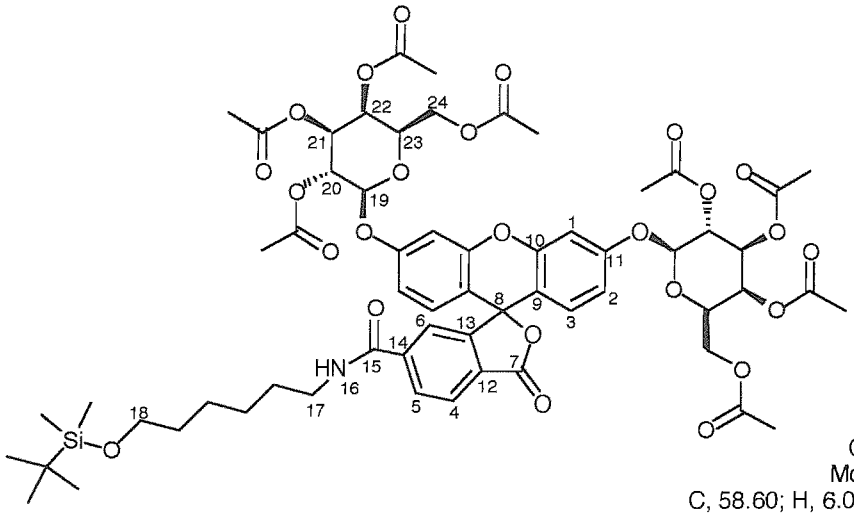
$C_{14}H_{19}BrO_9$
Mol. Wt.: 411.20
C, 40.89; H, 4.66; Br, 19.43; O, 35.02

α-D-Tetraacetate galactosyl bromide [22] [198,199]

To β-D-galactose pentaacetate (13.80g, 35.4mmol) was added a solution of 30% (w: w) hydrogen bromide in acetic acid (27.60g, 340.0mmol, 20.4mL). The reaction mixture was stirred, under argon, at 0°C for 2.5 hrs. CH_2Cl_2 (100mL) was added and the reaction poured onto ice water (100mL), washed further with ice water (2 x100mL), dried (Na_2SO_4), and the solvents removed *in vacuo*. The resulting white foam was dissolved in a minimal amount of Et_2O (10mL) and petroleum ether (40-60) was added dropwise until a persistent cloudiness was observed. Standing at 0°C for 1 hr afforded a white solid which was removed by filtration, washed with cold hexane and dried over P_2O_5 for 18 hrs (12.86g, 31.3mmol, 88%).

R_f (L): 0.27; LRMS (EI^+ mode): *m/z* 332.56 ($M\text{-}Br^+$); λ_{max} (MeOH): 202nm; mp: 83-85°C
Found C: 40.87, H: 4.53%, $C_{14}H_{19}BrO_9$ requires C: 40.89, H: 4.66%
IR (CH_2Cl_2): ν = 2987.9 (w), 2966.9 (w), 2948.2 (w), 1739.5 (s), 1210.4 (s), 749.0 (m) cm^{-1}

^1H ($CDCl_3$): δ = 6.70 (1H, d, J = 3.7 Hz, H_1), 5.52 (1H, dd, J = 4.4, 1.4 Hz, H_4), 5.41 (1H, dd, J = 10.3, 2.9 Hz, H_3), 5.05 (1H, dd, J = 10.3, 3.7 Hz, H_2), 4.49 (1H, t, J = 6.5 Hz, H_5), 4.19 (2H, dd, J = 11.0, 5.9 Hz, H_6), 4.11 (1H, dd, J = 11.0, 7.3 Hz, H_6) 2.01 (3H, s, CH_3), 2.06 (3H, s, CH_3), 2.12 (3H, s, CH_3), 2.16 (3H, s, CH_3)
^{13}C ($CDCl_3$): δ = 170.4, 170.1, 169.9, 169.8 (CO), 88.1 (CHBr), 71.1 (CH), 68.0 (CH), 67.8 (CH), 66.9 (CH), 60.9 (CH), 20.8 (CH_3), 20.7 (CH_3), 20.6 (CH_3), 20.5 (CH_3)



$C_{61}H_{75}NO_{25}Si$
Mol. Wt.: 1250.33
C, 58.60; H, 6.05; N, 1.12; O, 31.99; Si, 2.25

6-(*t*-Butyldimethylsilyloxy)-1-hexyl-6-carboxyfluoresceinamide-(3,6)-*bis*-β-D-galactose tetraacetate [23]

Method 1[148]
To a suspension of the fluorescein derivative **[21]** (1.00g, 1.7mmol), freshly prepared silver oxide (0.39g, 1.7mmol), freshly distilled quinoline (0.5mL) in anhydrous benzene (15mL) was added α-D-tetraacetyl galactosyl bromide **[22]** (2.79g, 6.8mmol). The reaction was stirred in the dark, under argon, at room temperature for 72 hrs. The mixture was filtered through celite, CH_2Cl_2 (100mL) added, and the organic layer washed with water (150mL). Removal of the solvents *in vacuo* gave an orange foam, which was purified by silica gel column chromatography eluting with cyclohexane: EtOAc (1:0 then 4:1 then 2:1 then 1:1 then 0:1). The crude product was repurified by silica gel column chromatography eluting with a gradient of hexane: EtOAc (1:0 then 2:1 then 5:3 then 0:1), yielding the desired compound as a light yellow foam (0.40g, 0.3mmol, 19%).

Method 2;[157,158,200]
A mixture of the fluorescein derivative **[21]** (6.00g, 10.1mmol) and silver silicate catalyst (20.00g) was taken up in anhydrous CH_2Cl_2 (250mL) and then the solvent removed *in vacuo*. To a suspension of the orange powder in CH_2Cl_2 (100mL) was added crushed molecular sieves (20.00g) and the mixture stirred, in the dark, at room temperature for 1 hr. The mixture was cooled to -20°C, and a solution of α-D-tetraacetyl galactosyl bromide **[22]** (16.70g, 40.8mmol) in CH_2Cl_2 (50mL) was added dropwise. The reaction mixture was

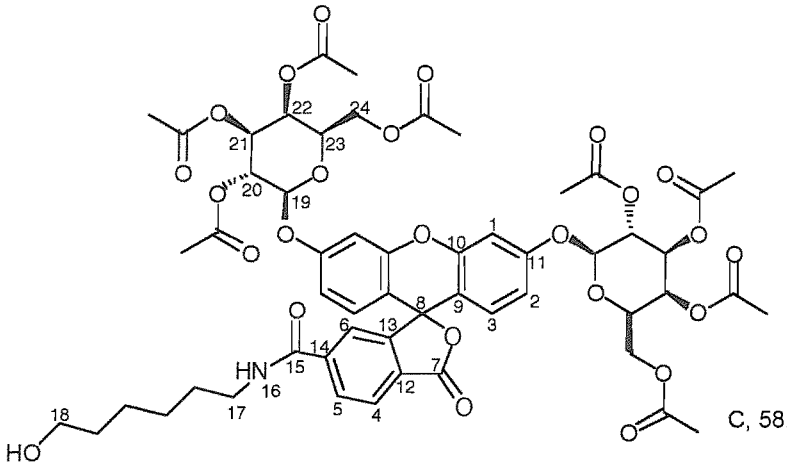
stirred in the dark, under argon, at room temperature for 24 hrs. A second portion of α-D-tetraacetyl-galactosyl bromide **[22]** (3.00g, 7.3mmol) and silver silicate catalyst (6.00g) were added and the reaction stirred for a further 24 hrs. The mixture was diluted with CH_2Cl_2 (200mL), filtered through celite; washed with CH_2Cl_2 (200mL) and the solvents removed *in vacuo* affording a pale brown foam (21.00g). This was purified by silica gel column chromatography eluting with EtOAc: Et_2O (0: 1 then 1: 1). The resulting pale yellow foam (10.80g) was purified by silica gel column chromatography eluting with MeOH: CH_2Cl_2 (0: 1 then 1: 9) to give an off white foam. This was recrystallised from a minimum amount of CH_2Cl_2 and Et_2O to give the title compound as white crystalline solid (6.70g, 5.4mmol, 55%).

R_f(D): 0.88; LRMS (ES^+ mode): *m/z* 1250.5 (MH^+); λ_{max} (MeOH): 264, 233nm; mp: 60-65°C

IR (CH_2Cl_2): ν = 2943.1 (m), 1752.5 (s), 1577.1 (w), 1227.6 (s), 1029.7 (s), 738.5 (s) cm^{-1}

^1H ($CDCl_3$): δ = 8.07 (1H, d, *J* = 8.1 Hz, H_4), 7.96 (1H, d, *J* = 8.1Hz, H_5), 7.50 (1H, s, H_6), 6.90 (2H, d, *J* = 4.4 Hz, H_1), 6.72 (4H, s, $H_{2,3}$), 6.23 (1H, t, *J* = 5.9 Hz, H_{16}), 5.48-5.46 (4H, m, H_{19}, H_{23}), 5.16-5.07 (4H, m, H_{24}), 4.27-4.06 (6H, m, $H_{20\text{-}22}$), 3.59 (2H, t, *J* = 5.9 Hz, H_{18}), 3.48 (2H, q, *J* = 7.4 Hz, H_{17}), 2.20-2.00 (24H, m, CH_3), 1.60-1.20 (8H, m, CH_2), 0.88 (6H, s, CHSi), 0.05 (9H, s, $(CH_3)_3Si$)

^{13}C ($CDCl_3$): δ = 170.5, 170.2, 169.5 ($CH_3\underline{C}O$) 168.6 ($C_{15,7}$) 165.7 (C_{10}) 158.6 (C_{11}), 152.3 ($C_{12,14}$), 137.8 (C_{13}), 129.4 (C_3), 128.8 (C_9), 125.7, 123.4, 122.8 ($C_{4\text{-}6}$), 113.5 (C_2), 104.4 (C_8), 99.2 (C_1), 71.4, 70.8, 68.5, 66.9 ($C_{19\text{-}23}$) 62.2 (C_{18}), 61.4 (C_{24}), 40.6 (C_{17}), 32.9, 27.0, 26.1. 25.7 (CH_2), 20.8 ($\underline{C}H_3CO$) 18.5 $(CH_3)_3\underline{C}$, -3.5 (CH_3Si)



$C_{55}H_{61}NO_{25}$
Mol. Wt.: 1136.06
C, 58.15; H, 5.41; N, 1.23; O, 35.21

1-*O*-Hexyl-6-carboxyfluoresceinamide -(3,6)-*bis*-β-galactose tetraacetate [24][201]

A solution of the fluorescein derivative **[23]** (2.00g, 1.6mmol) in 3HF•Et_3N: NMP: Et_3N (2: 3: 1.5, 20mL) was stirred at 60°C under argon, for 1 hr, in the dark. The mixture was diluted with CH_2Cl_2 (100mL), washed with water (3 x 100mL) and dried (Na_2SO_4), to give a yellow oil. Purification by silica gel column chromatography eluting with MeOH: CH_2Cl_2 (0:100 then 1:10) gave a pale yellow solid. The solid was dissolved in a minimal amount of CH_2Cl_2 and precipitated from cold Et_2O to afford the desired compound as a white powder (1.47g, 1.3mmol, 81%).

R_f (D): 0.67; LRMS (ES^+ mode): *m/z* 1136.4 (MH^+); λ_{max} (MeOH): 264, 226nm; mp: 148-152°C

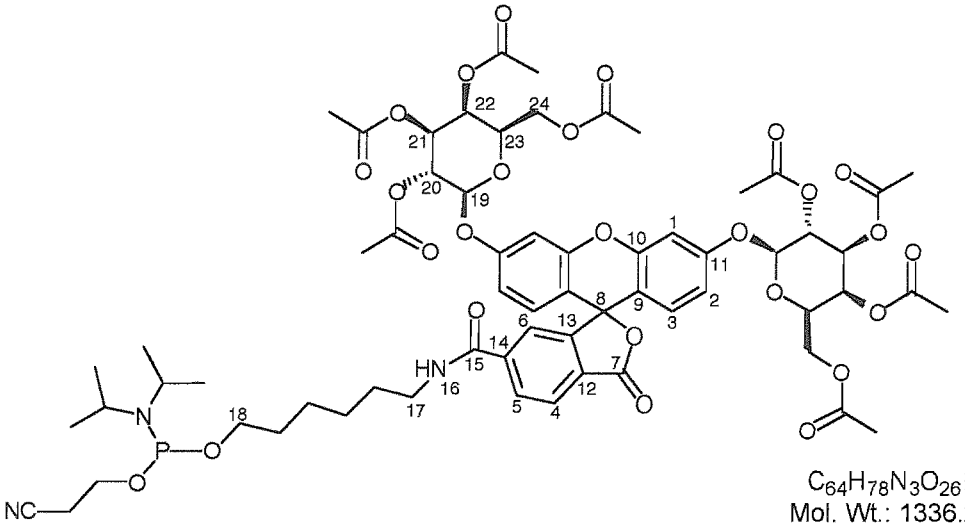
IR (CH_2Cl_2): ν = 3417.9 (br, m), 2936.5 (m), 1752.3 (s), 1667.1 (m), 1576.6 (w), 1370.8 (m), 1224.3 (s), 1194.4 (s), 737.9 (s) cm^{-1}

Found C: 57.40, H: 5.68, N: 1.49%, $C_{55}H_{61}NO_{25}$ requires C: 58.05, H: 5.68, N: 1.23%

^1H ($CDCl_3$): δ = 8.07 (1H, d, J = 8.1 Hz, H_4), 7.98 (1H, d, J = 8.1Hz, H_5), 7.50 (1H, s, H_6), 6.82 (2H, d, J = 4.4Hz, H_1), 6.72 (4H, s, $H_{2,3}$), 6.40 (1H, t, J = 5.9 Hz, H_{16}), 5.46-5.42 (4H, m, $H_{19,23}$), 5.16-5.08 (4H, m, H_{24}), 4.28-4.08 (6H, m, $H_{20\text{-}22}$), 3.62 (2H, t, J = 6.6 Hz, H_{18}), 3.38 (2H, q, J = 7.4 Hz, H_{17}), 2.30 (1H, t, J = 8.1 Hz, OH) 2.22 (6H, s, CH_3), 2.13 (3H, s, CH_3), 2.08 (9H, s, CH_3), 2.05 (6H, s, CH_3), 1.80-1.20 (8H, m, CH_2)

^{13}C ($CDCl_3$): δ = 170.5, 170.3, 170.2, 169.5 (CO_{Ac}) 168.5 ($C_{15,7}$) 165.8 (C_{10}) 158.5 (C_{11}), 152.0 ($C_{12,14}$), 141.6 (C_{13}), 129.5 (C_3), 128.8 (C_9), 128.4, 126.0, 122.8 ($C_{4\text{-}6}$), 113.9 (C_2),

104.8 (C_8), 99.1 (C_1), 71.4, 70.8, 68.5, 66.9 (C_{19-23}) 62.7 (C_{18}), 61.4 (C_{24}), 40.4 (C_{17}), 32.6, 30.8, 29.5, 26.7, 25.4 (CH_2), 20.9, 20.8, 20.7 (CH_3CO)



$C_{64}H_{78}N_3O_{26}P$
Mol. Wt.: 1336.28
C, 57.52; H, 5.88; N, 3.14; O, 31.13; P, 2.32

1-*O*-(Cyanoethoxy-*N,N*-diisopropylamino phosphine) hexyl-6-carboxyfluoresceinamide-(3,6)-*bis*-β-galactose tetraacetate [25]

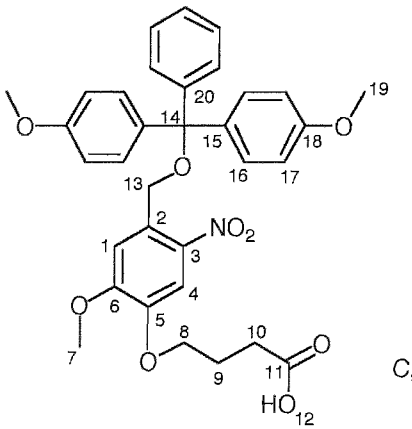
To a stirring solution of the alcohol **[24]** (0.50g, 0.4mmol) in anhydrous THF (3mL), was added anhydrous *N,N*-diisopropylethylamine (154μL, 0.9mmol) and 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite (115μL, 0.5mmol, Link Technologies), under argon. The reaction was allowed to stir at room temperature, under argon, for 0.5hrs. The reaction was diluted with EtOAc (30mL), washed with saturated potassium chloride solution (30mL), dried (Na_2SO_4), and the solvents removed *in vacuo*. The residue was dissolved in anhydrous CH_2Cl_2 (3mL) and dropped slowly onto rapidly stirring hexane (-20°C, 300mL). The hexane was removed, filtered through glasswool, and residue was washed with cold hexane (200mL). The residue was dissolved in CH_2Cl_2 (5mL) and filtered through the glasswool. Removal of the solvents *in vacuo* afforded a white foam. The foam was dissolved in anhydrous MeCN (4mL), filtered and dried *in vacuo* over P_2O_5 for 4 hrs (0.52g, 0.4mmol, 82%).

R_f (E): 0.77; LRMS (ES^- mode): *m/z* 1334.1 (M^-), 1449.2 ($MTFA^-$); (ES^+ mode): *m/z* 1337.0 (MH^+), 1359.9 (MNa^+), 1375.9 (MK^+); λ_{max} (MeOH): 250nm

IR (CH_2Cl_2): ν = 2934.3 (w), 2254.8 (w), 1752.2 (s), 1370.7 (m), 1226.3 (s), 1076.9 (m), 910 (s), 735.2 (s) cm^{-1}

^1H ($CDCl_3$): δ = 8.10 (1H, d, J = 8.1 Hz, H_4), 7.99-7.96 (1H, m, H_5), 7.50 (1H, s, H_6), 6.92 (2H, d, J = 4.4Hz, H_1), 6.67 (4H, s, $H_{2,3}$), 5.50-5.40 (4H, m, $H_{19,23}$), 5.15-5.00 (4H, m, H_{24}), 4.28-4.02 (6H, m, $H_{20\text{-}22}$), 3.62 (2H, s, H_{18}), 3.52-3.40 (4H, m, $(C\underline{H}_2)_2CN$), 3.35-3.29 (2H, m, H_{17}), 2.78-2.65 (2H, m, $C\underline{H}N$), 2.22 (6H, s, CH_3), 2.13 (3H, s, CH_3), 2.08 (9H, s, CH_3), 2.05 (6H, s, CH_3), 1.80-1.30 (8H, m, CH_2), 1.30-1.15 (12H, m, $C\underline{H}_3CHN$)

^{31}P ($CDCl_3$): δ = 147.86



$C_{33}H_{33}NO_9$
Mol. Wt.: 587.62
C, 67.45; H, 5.66; N, 2.38; O, 24.50

4-(4-(O-4, 4'-Dimethoxytrityl)-hydroxmethyl-2-methoxy-5-nitro-phenoxy)-butyric acid [65][176]

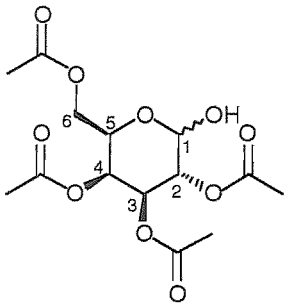
To a stirring solution of 4-(4-hydroxmethyl-2-methoxy-5-nitro-phenoxy)-butyric acid (0.30g, 1.1mmol) in anhydrous pyridine (3mL) was added 4-dimethylaminopyridine (3mg) followed by dropwise addition of a solution of 4,4'-dimethoxytrityl chloride (0.36g, 1.1mmol) in pyridine (3mL) was dropped onto the stirring acid. The reaction was diluted with CH_2Cl_2 (200mL), washed with water (200mL), brine (200mL) and the solvents removed *in vacuo* affording in a viscous yellow oil that was purified by silica gel column chromatography eluting with EtOAc: hexane (1:1) then EtOAc: MeOH (9:1). This gave a pale yellow solid that was dried over P_2O_5 *in vacuo* for 12 hours (0.54g, 0.9mmol, 86%).

R_f (E): 0.7, (M) 0.73; LRMS (ES^- mode): *m/z* 700.3 ($MTFA^-$); λ_{max} (MeOH): 344nm, mp: 62-66°C

IR (CH_2Cl_2): ν = 3286.0 (m), 2926.2 (m), 2853.4 (m), 1622.6 (s), 1570.9 (s), 1247.5 (s) cm^{-1}

^1H ($CDCl_3$): δ = 7.70 (1H, s, H_4), 7.68 (1H, s, H_1), 7.50-7.20 (9H, m, H_{16}, H_{Ph}), 6.85 (4H, d, J = 8.8 Hz, H_{17}), 4.60 (2H, s, H_{13}), 4.18-4.10 (2H, m, H_8), 4.00 (3H, s, H_7), 3.78 (6H, s, H_{19}), 2.56 (2H, t, J = 7.4 Hz, H_{10}), 2.20 (2H, quintet, J = 7.4 Hz, H_9)

^{13}C ($CDCl_3$): δ = 178.1 (C_{11}), 158.8 ($C_{5,18}$), 154.2 (C_6), 146.7 (C_3), 145.0 (C_{20}), 136.0 (C_{15}), 132.1, 130.2, 129.3, 128.2, 128.2 (C_{16}), 127.2 (C_2), 113.4 (C_{17}), 109.9, 109.4 ($C_{1,4}$), 87.3 (C_{14}), 68.6 (C_{13}), 63.2 (C_8), 56.4 (C_7), 55.4 (C_{19}), 31.2 (C_{10}), 24.6 (C_9)



$C_{14}H_{20}O_{10}$
Mol. Wt.: 348.30
C, 48.28; H, 5.79; O, 45.94

2,3,4,6-Tetra-*O*-acetyl-D-galactose [70][185]

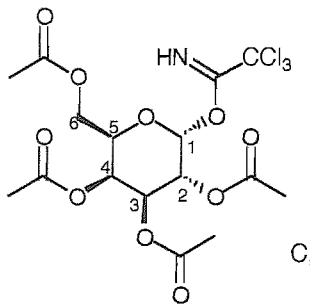
Gaseous ammonia was bubbled through a solution of β-D-galactose pentaacetate (27.00g, 69.2mmol) in THF: MeOH (7:3, 150mL) for 10 minutes at 0°C, followed by warming to room temperature over 30 minutes. The solvents were removed *in vacuo* and the resulting colourless oil purified by silica gel column chromatography eluting with EtOAc: hexane (1:1 then 4:1). This gave the title compound as a white foam, which was dried over P_2O_5 *in vacuo* for 24 hrs (17.30g, 49.7mmol, 73%).

R_f (M): 0.37, 0.32; LRMS (ES^- mode): *m/z* 461.2 ($MTFA^-$); (ES^+ mode): *m/z* 387.2 (MK^+), 371.2 (MNa^+), 366.2 (MNH_4^+); HRMS: $C_{14}H_{21}O_{10}$ requires 349.11347, found 349.11347; λ_{max} (MeOH): 271nm; mp: 100-104°C

IR (CH_2Cl_2): ν = 3053.8 (w), 2985.6 (w), 2304.7 (w), 1752.8 (s), 1421.9 (w), 1368.9 (m), 1265.0 (s) cm^{-1}

^1H ($CDCl_3$): δ = 5.52 (1H, t, J = 3.7 Hz, H_1), 5.48 (1H, dd, J = 3.7, 1.5 Hz, H_4), 5.42 (1H, dd, J = 10.3, 3.0 Hz, H_3), 5.16 (1H, dd, J = 11.7, 3.6 Hz, H_2), 4.48 (1H, t, J = 6.6 Hz, H_5), 4.10 (2H, dd, J = 6.6, 1.5 Hz, H_6) 3.44 (1H, d, J = 3.5 Hz, OH)

^{13}C ($CDCl_3$): δ = 170.8, 170.6, 170.5, 170.3 (CO), 96.8 ($CH_{anomeric}$), 68.4, 68.3, 67.4, 66.4 (CH), 62.0 (CH_2), 21.0, 21.0, 20.8 (CH_3CO)



$C_{16}H_{20}Cl_3NO_{10}$
Mol. Wt.: 492.69
C, 39.00; H, 4.09; Cl, 21.59; N, 2.84; O, 32.47

_O_-(2,3,4,6-Tetra-_O_-acetyl)-α-D-galactosyl trichloroacetimidate [71][187]

To a solution of 2,3,4,6-tetra-*O*-acetyl-D-galactose **[70]** (16.40g, 47.09mol) in CH_2Cl_2 (30mL) under argon, was added trichloroacetonitrile (14.20mL, 0.14mol). Following gradual addition of 1,8-diazabicyclo(5.4.0)undec-7-ene (0.3mL, 1.9mmol) the reaction was allowed to stir for 30 minutes. A colour change from clear, colourless to yellow was observed. The reaction was diluted with CH_2Cl_2 (50mL), washed with aqueous hydrochloric acid (2M, 50mL), brine (2 x 50mL) and dried (Na_2SO_4). The solvents were removed *in vacuo*, to leave a white solid that was recrystallised from Et_2O and hexane affording a white crystalline solid that was dried over P_2O_5 *in vacuo* for 3 days (15.90g, 32.3mmol, 70%).

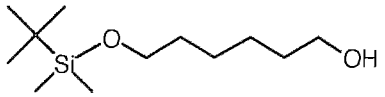
R_f (N): 0.735; LRMS (ES^- mode): *m/z* 492.1 ($MTFA^-$), (ES^+ mode): *m/z* 331.2 ($M-(NH_2COCCl_3)^+$); HRMS: $C_{16}H_{24}Cl_3N_2O_{10}$ requires 509.04965, found 509.04965; λ_{max} (MeOH): 271nm; mp: 52-55°C

Found C: 39.08, H: 3.99, N: 2.75%, $C_{16}H_{20}Cl_3NO_{10}$ requires C: 39.01, H: 4.09, N: 2.84%

IR (CH_2Cl_2): ν = 3341.9 (w), 3341.0 (w), 1750.0 (s), 1674.9 (m), 1617.0 (m) cm^{-1}

^1H ($CDCl_3$): δ = 8.60 (1H, s, NH), 6.61 (1H, d, *J* = 2.9 Hz, H_1), 5.57 (1H, d, *J* = 2.9 Hz, H_4), 5.43 (1H, dd, *J* = 11.0, 3.0 Hz, $H_{2\text{ or }3}$), 5.37 (1H, dd, *J* = 11.0, 3.0 Hz, $H_{2\text{ or }3}$), 4.44 (1H, t, *J* = 7.0 Hz, H_5), 3.45 (1H, dd, *J* = 10.3, 7.0 Hz, H_6) 3.43 (1H, dd, *J* = 11.0, 7.0 Hz, H_6), 2.45 (3H, s, CH_3), 2.18 (3H, s, CH_3), 2.03 (6H, s, 2(CH_3))

^{13}C ($CDCl_3$): δ = 170.5, 170.3, 170.2 (CO), 161.1 (C=NH), 93.6 ($CH_{anomeric}$), 69.1, 67.7, 67.5, 67.0 (CH), 61.4 (CH_2), 20.8, 20.7 (CH_3)



$C_{12}H_{28}O_2Si$
Mol. Wt.: 232.44
C, 62.01; H, 12.14; O, 13.77; Si, 12.08

6-(*t*-Butyldimethylsilyl)oxy-1-hexanol [72][188]

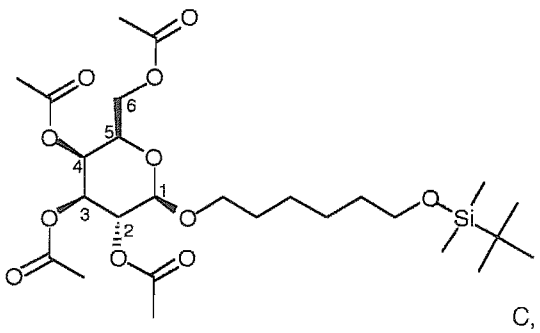
Sodium hydride (washed with anhydrous hexane, 2.03g, 84.6mmol) was suspended in anhydrous THF. Following addition of 1,6-hexandiol (10.00g, 84.7mmol) the solution was stirred under argon, at room temperature, for 45 minutes. *t*-Butyldimethylsilyl chloride (12.75g, 84.7mmol) was added and the mixture stirred for a further 1 hr. The reaction was diluted with Et_2O (50mL), washed with 10% potassium carbonate solution (200mL), brine (200mL) and dried (Na_2SO_4). Removal of the solvents *in vacuo* gave a viscous oil, which was purified by silica gel column chromatography eluting with EtOAc: hexane (1:10) to yield a yellow oil which was dried over P_2O_5 *in vacuo* for 8 hrs (12.20g, 52.5mmol, 62%).

R_f (D): 0.44; LRMS (ES^+ mode): *m/z* 202.2 (M-2CH_3); HRMS: $C_{12}H_{29}O_2Si$ requires 233.19368, found 233.19368; λ_{max} (MeOH): 270nm

IR (CH_2Cl_2): ν = 3386.4 (s), 2965.6 (s), 2926.6 (s), 1435.9 (m), 1373.5 (m), 1259.6 (s), 1102.1 (s), 876.8 (m) cm^{-1}

^1H ($CDCl_3$): δ = 3.60-3.50 (4H, m, CH_2O), 1.55-1.45 (4H, m, $\underline{C}H_2CH_2O$), 1.40-1.25 (4H, m, CH_2), 0.85 (9H, s, $(CH_3)_3Si$), 0.05 (6H, s, $(CH_3)_2Si$)

^{13}C ($CDCl_3$): δ = 62.3, 62.9 (CH_2O), 32.9, 32.8 ($\underline{C}H_2CH_2O$), 26.1 ($\underline{C}H_3)_3C$, 25.7, 25.7 (CH_2), 18.5 $(CH_3)_3\underline{C}$, -3.5 (CH_3Si)



$C_{26}H_{46}O_{11}Si$
Mol. Wt.: 562.72
C, 55.49; H, 8.24; O, 31.28; Si, 4.99

***O*-β-D-[(2,3,4,6-Tetra-*O*-acetyl)-galactosyl-[6-(*t*-butyldimethylsilyl)oxy hexane] [73]**[189,190,202]

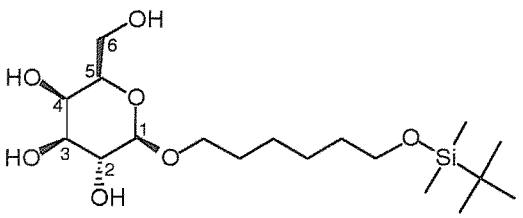
To a solution of 6-(*t*-Butyldimethylsilyl)oxy-1-hexanol **[72]** (5.15g, 22.0mmol) in anhydrous CH_2Cl_2 (150mL) at -15°C, under argon, was added *O*-(2,3,4,6-tetra-*O*-acetyl)-α-D-galactosyl trichloroacetimidate **[71]** (12.00g, 24.4mmol) and 4Å molecular sieves. Following the addition of trimethsilyl trifluoromethanesulfonate (170mg, 138mL, 0.7mmol) the reaction was allowed to stand for 10 minutes before quenching with saturated sodium bicarbonate solution (150mL). The organic layer was separated dried (Na_2SO_4) and the solvents removed *in vacuo* to give an oily white solid. This was purified by silica gel column chromatography eluting with EtOAc: hexane (1:4 then 2:4) to give a white solid that was dried over P_2O_5 *in vacuo* for 12 hrs (3.97g, 7.1mmol, 32%, improved to 57%).

R_f (M): 0.71; LRMS (APCI$^+$ mode): *m/z* 580.5 (MNH_4^+), 563.4 (M^+), 331.2 (M-$((CH_2)_6OTBS)^+$); λ_{max} (MeOH): 222nm; mp: 142-145°C

IR (CH_2Cl_2): ν = 3391.9 (w), 2932.2 (w), 2858.2 (w), 1751.1 (s), 1369.4 (m), 1225.4 (s), 1079.0 (m) cm^{-1}

^1H ($CDCl_3$): δ = 5.39 (d, 1H, *J* = 2.2 Hz, H_4), 5.20 (1H, dd, *J* = 10.3, 8.1 Hz, H_2), 5.02 (1H, dd, *J* = 10.3, 3.7 Hz, H_3), 4.45 (1H, d, *J* = 8.1 Hz, H_1), 4.20 (1H, dd, *J* = 11.0, 5.9 Hz, H_6), 4.13 (1H, dd, *J* = 11.0, 6.6 Hz, H_6), 3.95-3.84 (2H, m, OCH_2), 3.60 (2H, t, *J* = 6.6 Hz, CH_2OSi), 3.53-3.51 (1H, m, H_5), 2.16 (3H, s, CH_3CO), 2.06 (6H, s, CH_3CO), 1.98 (3H, s, CH_3CO), 1.65-1.45 (4H, m, $C\underline{H}_2CH_2O$), 1.40-1.25 (4H, m, CH_2), 0.90 (9H, s, $(CH_3)_3Si$), 0.05 (6H, s, $(CH_3)_2Si$)

^{13}C ($CDCl_3$):δ = 170.7, 170.5, 170.5, 169.6 (CO), 101.5 ($CH_{anomeric}$), 71.1, 70.7 (CH), 70.4 (CH_2OAc), 69.1, 67.2 (CH), 63.3 (CH_2OR), 61.5, 32.9, 29.5 (CH_2), 26.1, 25.8, 25.7 ($(\underline{C}H_3)_3C$), 20.9, 20.9, 20.8, (CH_3), 18.5 ($(CH_3)_3\underline{C}$), -3.6 (CH_3Si)

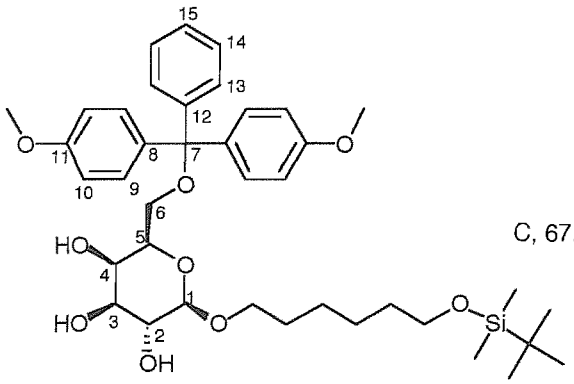


$C_{18}H_{38}O_7Si$
Mol. Wt.: 394.58
C, 54.79; H, 9.71; O, 28.38; Si, 7.12

***O*-β-D-[6-(*t*-Butyldimethylsilyl)oxy hexane]-galactose [74]**[203]

A solution the β-D-galactose derivative **[73]** (3.20g, 5.7mmol) in anhydrous MeOH (50mL) was cooled to 0°C and potassium carbonate (0.24g, 1.7mmol) added. The reaction was left standing for 2 hrs, the solvents removed *in vacuo* and the resulting foam dissolved in CH_2Cl_2 (50mL). The organic layer was washed with water (50mL), brine (3 x 50mL), dried (Na_2SO_4) and the solvents removed *in vacuo*. This gave the desired compound as a pale yellow foam, which was dried over P_2O_5 *in vacuo* for 12 hrs (2.15g, 5.5mmol, 96%).

R_f (O): 0.60; LRMS (ES^- mode): *m/z* 507.0 ($MTFA^-$); λ_{max} (MeOH): 219nm
IR (CH_2Cl_2): ν = 3054.3 (m), 2987.2 (w), 2305.4 (w), 1422.0 (m), 1265.0 (s) cm^{-1}
^1H ($CDCl_3$): δ = 4.20 (1H, d, J = 7.4 Hz, H_1), 3.95-3.85 (1H, m, H_4), 3.84 (1H, d, J = 2.9 Hz, H_3), 3.75-3.70 (2H, m, $H_{2,5}$), 3.63 (2H, t, J = 6.6 Hz, OCH_2), 3.55-3.45 (4H, m, H_6, CH_2OSi), 1.70-1.60 (2H, m, $C\underline{H}_2CH_2O$), 1.55-1.45 (2H, m, $C\underline{H}_2CH_2O$), 1.40-1.30 (4H, m, CH_2), 0.90 (9H, s, $(CH_3)_3Si$), 0.05 (6H, s, $(CH_3)_2Si$)
^{13}C ($CDCl_3$): 104.9 ($CH_{anomeric}$), 76.4, 74.9, 72.5, (CH), 70.7 (CH_2OAc), 70.4, 70.2, 64.2 (CH_2), 62.3, 33.8, 30.8 (CH_2), 26.8, 26.8, 26.4 ($(\underline{C}H_3)_3C$), 19.1 ($(CH_3)_3\underline{C}$), -3.5 (CH_3Si)



$C_{39}H_{56}O_9Si$
Mol. Wt.: 696.94
C, 67.21; H, 8.10; O, 20.66; Si, 4.03

O-β-D-(6-O-4,4'-Dimethyloxytrityl)-galactosyl-[6-(t-butyldimethylsilyl)oxy hexane] [75]

A solution of 4,4'-dimethyloxytrityl chloride (1.22g, 3.6mmol) in pyridine (4mL) was added dropwise to a solution of the β-D-galactose derivative **[74]** (1.41g, 3.6mmol) and 4-dimethylaminopyridine (43mg, 360.0μmol) in anhydrous pyridine (8mL), under argon. After stirring at room temperature for 15mins CH_2Cl_2 (300mL) was added, the organic layer washed with sodium bicarbonate solution (200mL), brine (200mL) and dried

(Na_2SO_4). Removal of the solvents *in vacuo* gave an orange oil, which was purified by silica gel column chromatography eluting with Et3N: CH_2Cl_2 (3: 100). This gave a yellow foam which was dried over P_2O_5 *in vacuo* for 18hrs (1.13g, 1.6mmol, 45%).

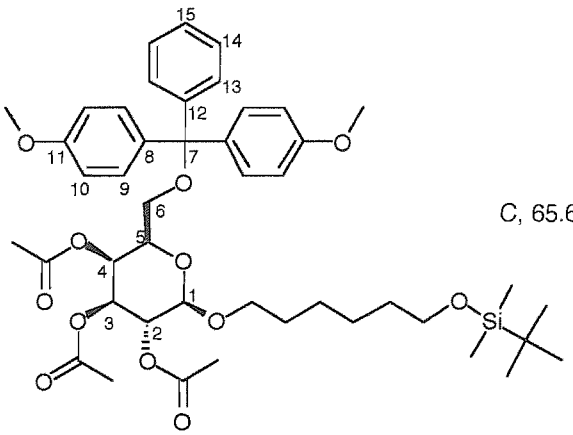
R_f (G): 0.46; LRMS (ES^+ mode): *m/z* 720.0 (MNa^+), 715.0 (MNH_4^+) 303.4 (DMT^+); HRMS: $C_{39}H_{56}O_9SiNa$ requires 719.35913, found 719.35913; λ_{max} (MeOH): 263, 241nm; mp: 62-65°C

Found C: 66.95, H: 8.08%, $C_{39}H_{56}O_9Si$ requires C: 67.21, H: 8.10%

IR (CH_2Cl_2): ν = 3054.3 (w), 2360.2 (w), 1653.5 (w), 1265.0 (s), 738.9 (s) cm^{-1}

^1H ($CDCl_3$): δ = 7.50-7.20 (9H, m, $H_{9, 13-15}$), 6.83 (4H, d, J = 8.8 Hz, H_{10}), 4.23 (1H, d, J = 6.6 Hz, H_1), 4.05 (1H, d, J =2.2 Hz, H_4), 3.95-3.85 (1H, m, H_5), 3.80 (6H, s, CH_3O), 3.65-3.50 (6H, m, $H_{2, 3}$, OCH_2, CH_2OSi), 3.43 (1H, dd, J =9.6, 5.9 Hz, H_6), 3.38 (1H, dd, J = 9.6, 6.6 Hz, H_6), 1.70-1.60 (2H, m, $C\underline{H}_2CH_2O$), 1.57-1.48 (2H, m, $C\underline{H}_2CH_2O$), 1.40-1.32 (4H, m, CH_2), 0.90 (9H, s, $(CH_3)_3Si$), 0.05 (6H, s, $(CH_3)_2Si$)

^{13}C ($CDCl_3$):δ = 158.7 (C_{11}), 144.9 (C_{12}), 136.1, 136.0 (C_8), 130.2, 128.2, 128.0, 127.0 ($C_{9, 13-15}$), 113.3 (C_{10}), 103.1 (C_1), 86.6 (C_7), 73.7, 72.4 (C_{2-5}), 70.1 (C_6), 69.3 (C_{2-5}), 63.3, 62.5 (CH_2O), 55.4 (CH_3O), 32.9, 29.8, 26.0, 25.8 (CH_2), 26.2 (($\underline{C}H_3)_3CSi$), 18.6 (($CH_3)_3C$), -3.4 (CH_3Si)



$C_{45}H_{62}O_{12}Si$
Mol. Wt.: 823.05
C, 65.67; H, 7.59; O, 23.33; Si, 3.41

O-β-D-[(2,3,4-Tri-O-acetyl)-6-O-(4,4'-dimethyloxytrityl)]-galactosyl-[6-(*t*-butyldimethylsilyl)oxy] hexane [76][204]

To a solution of the β-D-galactose derivative **[75]** (0.95g, 1.4mmol) in anhydrous CH_2Cl_2 (20mL) was added Et_3N (0.57mL, 4.1mmol), 4-dimethylaminopyridine (16.60mg, 140.0mol) and acetic anhydride (0.39mL, 4.1mmol). The reaction was stirred, at room temperature, under argon for 1.5 hrs. The reaction was diluted with CH_2Cl_2 (100mL), washed with saturated sodium bicarbonate solution (2 x 100mL), water (100mL), brine (2 x 100mL) and dried (Na_2SO_4). Removal of the solvents *in vacuo* gave a yellow foam, which was purified by silica gel column chromatography eluting with EtOAc: hexane. (3:1). The pale yellow foam obtained was dried over P_2O_5 *in vacuo* for 12 hrs (0.99g, 1.2mmol, 89%).

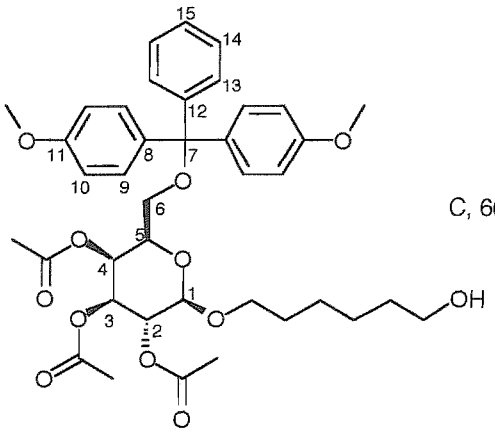
R_f (M): 0.77; LRMS (ES^+ mode): *m/z* 845.8 (MNa^+), 840.8 (MNH_4^+) 303.4 (DMT^+); HRMS: $C_{45}H_{63}O_{12}Si$ requires 823.40888, found 823.40888; λ_{max} (MeOH): 273, 234nm; mp: 60-64°C

Found C: 65.69, H: 7.68%, $C_{45}H_{62}O_{12}Si$ requires C: 65.67, H: 7.59%

IR (CH_2Cl_2):ν = 3054.2 (m), 2986.4 (w), 2359.9 (w), 1750.2 (m), 1608.1 (w), 1264.9 (s), 746.2 (s) cm^{-1}

^1H ($CDCl_3$): 7.40-7.19 (9H, m, $H_{9,\ 13\text{-}15}$), 6.85-6.78 (4H, m, *J* = 8.8 Hz, H_{10}), 5.60 (1H, d, *J* = 3.7 Hz, H_4), 5.10 (1H, dd, *J* = 10.3, 7.4Hz, $H_{2\ or\ 3}$), 5.12 (1H, dd, *J* = 10.3, 3.7 Hz, $H_{2\ or\ 3}$), 4.43 (1H, d, J = 7.4 Hz, H_1), 3.90-3.81 (2H, m, OCH_2), 3.80 (6H, s, CH_3O), 3.58 (2H, t, *J* = 6.6 Hz, CH_2OSi), 3.50-3.35 (2H, m, H_6), 3.10 (1H, t, *J* = 8.8 Hz, H_5), 2.05 (3H, s, CH_3CO), 2.00 (3H, s, CH_3CO), 1.90 (3H, s, CH_3CO), 1.60-1.43 (4H, m, $C\underline{H_2}CH_2O$), 1.38-1.25 (4H, m, CH_2), 0.90 (9H, s, $(CH_3)_3Si$), 0.05 (6H, s, $(CH_3)_2Si$)

^{13}C ($CDCl_3$): δ = 170.5, 170.2, 169.6 (CO), 158.7 (C_{11}), 144.3 (C_{12}), 135.8, 135.5 (C_8), 130.2, 130.1, 128.3, 128.0, 127.0 ($C_{9,\ 13\text{-}15}$), 113.3 (C_{10}), 101.4 (C_1), 86.4 (C_7), 72.2, 71.4 ($C_{2\text{-}5}$), 70.2 (C_6), 69.4 ($C_{2\text{-}5}$), 67.5, 63.3, 60.6 (CH_2), 55.4 (CH_3O), 32.9, 29.5, 25.8, 25.7 (CH_2), 26.1 (($\underline{C}H_3)_3CSi$), 20.9, 20.9, 20.8 ($\underline{C}H_3CO$) 18.5 (($CH_3)_3\underline{C}$), -3.2 (CH_3Si)



$C_{39}H_{48}O_{12}$
Mol. Wt.: 708.79
C, 66.09; H, 6.83; O, 27.09

***O*-β-D-[(2,3,4-Tri-*O*-acetyl)-6-*O*-(4,4'-dimethyloxytrityl)]-galactosyl hexan-6-ol [77]**[201]

The silyl ether **[76]** (0.92g, 1.1mmol) was suspended in 3HF•Et_3N: NMP: Et_3N (2: 3: 1.5, 3.5mL) and left standing at 65°C for 20minutes. The reaction was diluted with Et_2O (70mL), washed with water (50mL), saturated sodium bicarbonate solution (3 x 50mL), water (50mL), brine (70mL) and dried (Na_2SO_4). The solvents were removed *in vacuo* giving a white foam which was purified by silica gel column chromatography eluting with EtOAc: hexane (3:2 then 3:1 then 1:0). The resulting white foam was dried over P_2O_5 *in vacuo* for 14 hrs (0.77g, 1.1mmol, 97%).

R_f (N): 0.40; LRMS (APCI$^+$ mode): *m/z* 731.7 (MNa^+), 708.8 (M^+) 303.4 (DMT^+), 138.9 ($(CH_3)_3C(CH_2)_2SiF^+$); HRMS: $C_{39}H_{48}O_{12}$ requires 708.31458, found 708.31458; λ_{max} (MeOH): 272, 234nm; mp: 63-66°C

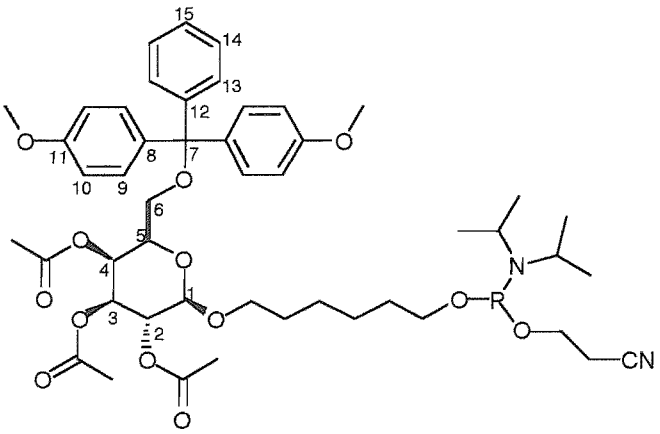
Found C: 66.38, H: 6.65%, $C_{39}H_{48}O_{12}$ requires C: 66.09, H: 6.83%

IR (CH_2Cl_2): ν = 3054.2 (w), 2986.4 (w), 1750.2 (s), 1607.9 (w), 1265.5 (s), 739.8 (s) cm^{-1}

^1H ($CDCl_3$): δ = 7.40-7.20 (9H, m, $H_{9,\ 13\text{-}15}$), 6.85-6.78 (4H, m, H_{10}), 5.58 (1H, d, J = 2.9 Hz, H_4), 5.10 (1H, dd, J = 10.3, 8.1 Hz, H_2), 5.12 (1H, dd, J = 10.3, 2.9 Hz, H_3), 4.43 (1H, d, J = 7.4 Hz, H_1), 3.90-3.81 (2H, m, OCH_2), 3.80 (6H, s, CH_3O), 3.63 (2H, t, J = 6.6 Hz, CH_2OH), 3.50-3.35 (2H, m, H_6), 3.10 (1H, t, J = 8.8 Hz, H_5), 2.05 (3H, s, CH_3CO), 2.00 (3H, s, CH_3CO), 1.90 (3H,s, CH_3CO), 1.60-1.50 (4H, m, $C\underline{H}_2CH_2O$), 1.40-1.35 (4H, m, CH_2)

^{13}C ($CDCl_3$): δ = 170.5, 170.2, 169.7 (CO), 158.7 (C_{11}), 144.3 (C_{12}), 135.8, 135.6 (C_8), 130.2, 130.1, 128.3, 128.0, 127.0 ($C_{9,\ 13\text{-}15}$), 113.3 (C_{10}), 101.4 (C_1), 86.4 (C_7), 72.3, 71.4,

($C_{2\text{-}5}$), 70.2 (C_6), 69.4 ($C_{2\text{-}5}$), 67.5, 62.9, 60.7 (CH_2), 55.4 (CH_3O), 32.8, 29.5, 25.8, 25.6 (CH_2), 21.0, 20.9, 20.8 ($\underline{C}H_3CO$)

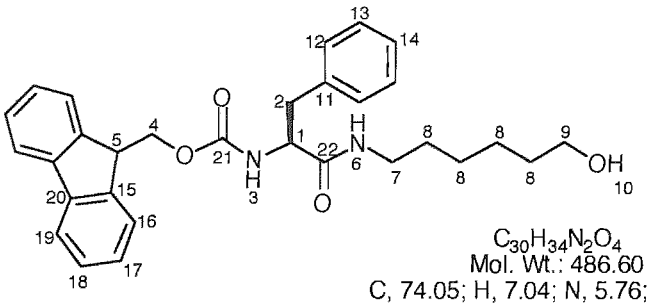


$C_{48}H_{65}N_2O_{13}P$
Mol. Wt.: 909.01
C, 63.42; H, 7.21; N, 3.08; O, 22.88; P, 3.41

_O_-β-D-[(2,3,4-Tri-_O_-acetyl)-6-_O_-(4,4'-dimethyloxytrityl)]-galactosyl-[6-(2-cyanoethyl diisopropylphosphoramidyl)oxy hexane] [78]

To the alcohol [77] (300mg, 0.4mmol) in anhydrous CH_2Cl_2 (5mL) was added *N,N*-diisopropylethylamine (295μL, 1.7mmol) and 2-cyanoethyl-diisopropyl chlorophosphoramidite (114μL, 510.0μmol) and the reaction stirred under argon. After stirring at room temperature, for 20 minutes the clear solution was diluted with Et_2O (25mL), washed with saturated potassium chloride solution (25mL), dried (Na_2SO_4) under argon, and then the solvents removed *in vacuo*. The oil obtained was purified by silica gel column chromatography, under argon, eluting with Et_2O to give the title compound as white foam, which was dried over P_2O_5 for 24 hrs (370mg, 0.4mmol, 96%).

R_f (N): 0.81; LRMS (ES^+ mode): *m/z* 909.7 (M^+), 931.6 (MNa^+)
^1H ($CDCl_3$): δ = 7.40-7.15 (9H, m, $H_{9,\ 13\text{-}15}$), 6.90-6.80 (4H, m, J = 8.8 Hz, H_{10}), 5.60 (1H, d, J = 2.2 Hz, H_4), 5.20-5.05 (2H, m, $H_{2,\ 3}$), 4.43 (1H, d, J = 7.4 Hz, H_1), 3.90-3.81 (2H, m, OCH_2), 3.80 (6H, s, CH_3O), 3.62-3.58 (4H, m, CHN, $C\underline{H}_2CH_2CN$), 3.50-3.35 (4H, m, H_6 CH_2OP), 3.10 (1H, t, J = 8.8 Hz, H_5), 2.65 (2H, t, J = 6.6 Hz, $CH_2C\underline{H}_2CN$), 2.05 (3H, s, CH_3CO), 2.00 (3H, s, CH_3CO), 1.90 (3H, s, CH_3CO), 1.40-1.21 (8H, m, $C\underline{H}_2CH_2O$, CH_2), 1.20 (12H, dd, J = 6.6, 5.9 Hz, $C\underline{H}_3CHN$)
^{31}P ($CDCl_3$): δ = 147.90

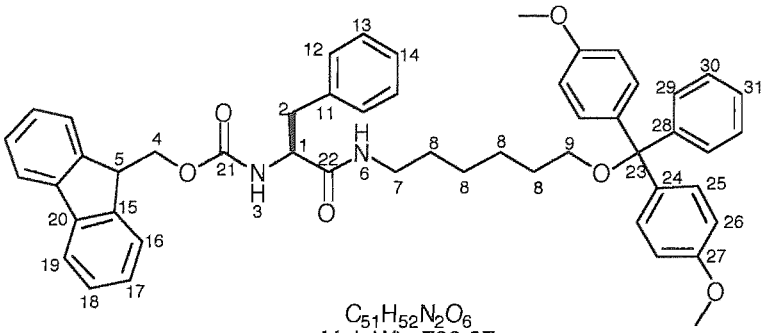


$C_{30}H_{34}N_2O_4$
Mol. Wt.: 486.60
C, 74.05; H, 7.04; N, 5.76; O, 13.15

Fmoc-phenylalanine-6-hydroxyhexylamide [81]

To a solution of Fmoc-phenylalanine (9.68g, 25.0mmol) and 6-amino-1-hexanol (2.93g, 25.0mmol) in anhydrous THF: DMF (5:1, 50mL) was added EDC (5.00g, 26.0mmol) and the mixture stirred, under argon, at room temperature for 24 hrs. The reaction was diluted with EtOAc (200mL), washed with sodium bicarbonate (200mL), water (200mL), brine (200mL) and then dried (Na_2SO_4). Removal of the solvents *in vacuo* gave a white, waxy solid that was purified by silica gel column chromatography eluting with MeOH: CH_2Cl_2 (0:1 then 1:10). The white powder obtained was dried over P_2O_5 *in vacuo* for 24 hrs (7.50g, 15.4mmol, 62%).

R_f (D): 0.58; LRMS (ES^+ mode): *m/z* 509.5 (MNa^+), 487.6 (MH^+); HRMS: $C_{30}H_{35}N_2O_4$ requires 487.25968, found 487.25968; λ_{max} (MeOH): 305, 295, 265nm; mp: 142-146°C
IR (Nujol mull): ν = 3295.9 (s), 2725.8 (w), 1689.3 (s), 1646.6 (m), 1460.6 (m), 1136.6 (w), 739.0 (m) cm^{-1}
^1H ($CDCl_3$): δ = 7.95 (1H, t, J = 5.2 Hz, H_6), 7.87 (2H, d, J = 8.1 Hz, H_{12}), 7.67-7.60 (2H, m, H_{13}), 7.45-7.18 (9H, m, $H_{14, 16\text{-}19}$), 4.36 (1H, t, J = 5.2 Hz, H_{10}), 4.24-4.10 (4H, m, $H_{2, 4}$), 3.40-3.35 (2H, m, H_9), 3.15-2.90 (3H, m, $H_{7, 5}$), 2.18 (1H, dd, J = 10.3, 9.5 Hz, H_1), 1.43-1.33 (4H, m, H_8), 1.29-1.17 (4H, m, H_8)
^{13}C ($CDCl_3$): δ = 171.2 (C_{22}), 155.8 (C_{21}), 143.8 (C_{11}), 140.7 (C_{20}), 138.3 (C_{15}), 129.3, 129.0, 128.1, 127.7, 127.4, 127.1, 126.3, 125.5, 125.4, 121.5, 120.2 ($C_{12\text{-}14, 16\text{-}19}$), 65.7 ($C_4$), 60.8 ($C_9$), 56.3 ($C_5$), 38.6 ($C_7$), 37.9 ($C_2$), 32.6, 29.3, 26.8, 26.1 (C_8)



$C_{51}H_{52}N_2O_6$
Mol. Wt.: 788.97
C, 77.64; H, 6.64; N, 3.55; O, 12.17

Fmoc-phenylalanine-6-hydroxy(-*O*-4,4'dimethoxytrityl-)hexylamide [82]

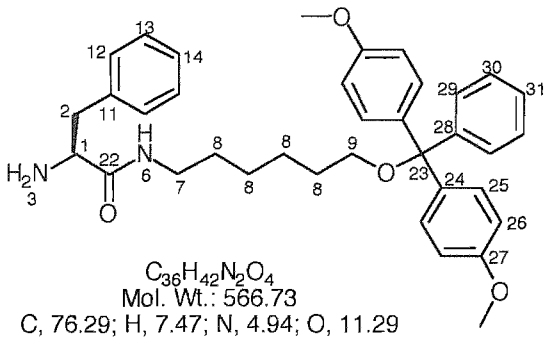
To a solution of the alcohol **[81]** (4.00g, 8.2mmol) in anhydrous pyridine (20mL) was added a solution of 4,4'-dimethoxytrityl chloride (3.07g, 9.1mmol) in pyridine (20mL) dropwise over a period of 10 minutes. The mixture was stirred under argon at room temperature for 18 hrs. The solvents were removed *in vacuo* and the residue dissolved in CH_2Cl_2 (200mL), washed with saturated sodium bicarbonate solution (200mL), water (200mL), brine (200mL) and then dried (Na_2SO_4). Removal of the solvents *in vacuo* gave a pale yellow solid, which was recrystallised from CH_2Cl_2 and petroleum ether. The resulting white crystals were dried over P_2O_5 for 24 hrs (5.95g, 7.5mmol, 92%).

R_f (D): 0.86; LRMS (ES^+ mode): *m/z* 812.2 (MNa^+), 487.8 (M-DMT^+), 303.4 (DMT^+); λ_{max} (MeOH): 305, 290, 265nm; mp: 94-98°C

IR (Nujol mull): ν = 3297.5 (m), 1689.3 (m), 1646.6 (m), 1607.0 (w), 1460.7 (m), 1175.2 (w), 737.2 (m), 706.7 (m) cm^{-1}

^1H ($CDCl_3$): δ = 7.78 (2H, d, *J* =7.4 Hz, H_{12}), 7.56 (2H, m, H_{13}), 7.48-7.20 (18H, m, $H_{14, 16\text{-}19, 25, 29\text{-}31}$), 6.85 (4H, d, *J* = 8.8 Hz, H_{26}), 5.65-5.40 (2H, m, $H_{3, 6}$) 4.45-4.30 (3H, m, $H_{4, 5}$), 4.20 (1H, t, *J* = 6.6 Hz, H_1), 3.80 (6H, s, OCH_3), 3.20-3.05 (4H, m, $H_{2, 7}$), 3.00 (2H, t, *J* = 6.6 Hz, H_9), 1.55 (2H, q, *J* = 6.6 Hz, H_8), 1.40-1.25 (4H, m, H_8), 1.20-1.05 (2H, m, H_8)

^{13}C ($CDCl_3$): δ = 170.6 (C_{22}), 158.5 ($C_{21, 27}$), 145.6 (C_{11}), 143.9 ($C_{20, 28}$), 141.5 (C_{15}), 136.8 (C_{24}), 130.2, 129.5, 128.9, 128.3, 127.9, 127.9, 127.3, 126.8, 125.2, 120.2 ($C_{12\text{-}14, 16\text{-}19, 25, 29\text{-}31}$), 113.1 ($C_{26}$), 85.8 ($C_{23}$), 67.2 ($C_4$), 63.4 ($C_9$), 56.7 ($OCH_3$), 55.4 ($C_1$), 47.3 ($C_5$), 39.7 ($C_7$), 39.1 ($C_2$), 30.1, 29.4, 26.3, 25.4 (C_8)



$C_{36}H_{42}N_2O_4$
Mol. Wt.: 566.73
C, 76.29; H, 7.47; N, 4.94; O, 11.29

Phenylalanine-6-hydroxy(-*O*-4,4'dimethoxytrityl-)hexylamide [83]

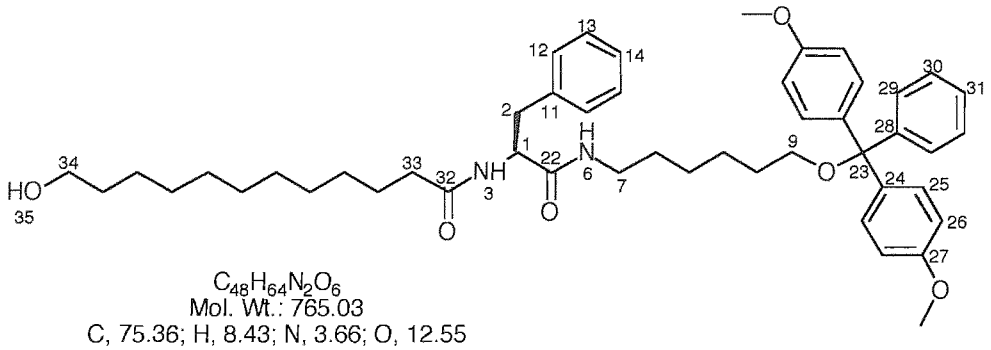
To a solution of piperidine in DMF (20%, 40mL) was added to the peptide **[82]** (5.00g, 6.3mmol) and the mixture stirred, under argon, for 2 hrs. This gave a cloudy yellow suspension. The solvents were removed *in vacuo* to give a viscous oil, was diluted with CH_2Cl_2 (200mL), washed with saturated sodium bicarbonate solution (200mL), water (200mL), brine (200mL) and then dried (Na_2SO_4). Removal of the solvents *in vacuo* afforded a yellow oil which was purified by silica gel column chromatography eluting with MeOH: CH_2Cl_2 (0:1 then 1:20) to give a yellow oil that was dried over P_2O_5 for 24 hrs (3.44g, 6.1mmol, 96%).

R_f (D): 0.54; LRMS (ES^+ mode): *m/z* 589.0 (MNa^+), 303.4 (DMT^+); λ_{max} (MeOH): 270, 240nm

IR (Nujol mull):ν = 3361.1 (m), 3059.9 (m), 3028.7 (m), 1652.2 (s), 1607.0 (m), 1459.5 (m), 1176.8 (m), 702.3 (m) cm^{-1}

^1H ($CDCl_3$): δ = 7.46 (2H, d, J =7.4 Hz, H_{12}), 7.37-7.20 (12H, m, $H_{13,14,\ 25,\ 29\text{-}31}$), 6.85 (4H, d, J = 8.8 Hz, H_{26}), 3.80 (6H, s, OCH_3), 3.60 (1H, dd, J = 4.4, 3.7 Hz, H_6), 3.32-3.20 (3H, m, $H_{3,\ 7}$), 3.05 (2H, t, J = 6.6 Hz, H_9), 2.70 (1H, dd, J = 9.5, 3.5 Hz, H_1), 1.55 (2H, q, J = 7.4 Hz, H2), 1.54-1.25 (8H, m, H_8)

^{13}C ($CDCl_3$): δ = 174.2 (C_{22}), 158.5 ($C_{21,\ 27}$), 145.6 (C_{11}), 138.2 (C_{28}), 136.9 (C_{24}), 130.2, 129.5, 128.9, 128.3, 127.9, 126.9, 126.7 ($C_{12\text{-}14}$, $C_{25,\ 29\text{-}31}$), 113.1 (C_{26}), 85.8 (C_{23}), 63.5 (C_9), 56.6 (OCH_3), 55.4 (C_1), 41.3 (C_7), 39.2 (C_2), 30.2, 29.7 27.0, 26.2 (C_8)



$C_{48}H_{64}N_2O_6$
Mol. Wt.: 765.03
C, 75.36; H, 8.43; N, 3.66; O, 12.55

12-Hydroxy dodecanamido-phenylalanine-6-hydroxy(-*O*-4,4'dimethoxytrityl-) hexylamide [84]

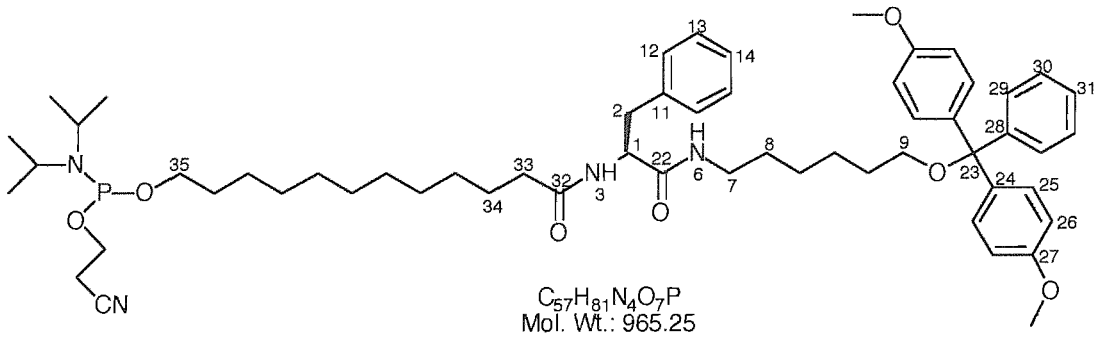
To a solution of the amine **[83]** (3.32g, 5.9mmol) and 12-hydroxydodecanoic acid (1.27g, 5.9mmol) in CH_2Cl_2 (30mL) was added EDC (1.13g, 5.9mmol). The mixture was stirred, under argon, for 2 hrs. The reaction was diluted with CH_2Cl_2 (200mL), washed with saturated sodium bicarbonate solution (200mL), water (200mL), brine (200mL) and then dried (Na_2SO_4). Removal of the solvents *in vacuo* afforded a pale yellow solid, which was recrystallised from CH_2Cl_2 and petroleum ether. The title compound was obtained as a white solid, which was dried, *in vacuo*, over P_2O_5 for 24 hrs (3.42g, 4.5mmol, 76%).

R_f (D): 0.72; LRMS (ES^+ mode): *m/z* 303.2 (DMT^+), 787.5 (MNa^+), 1551.9 ($2MNa^+$); λ_{max} (MeOH): 275, 240nm; mp: 70-74°C

IR (CH_2Cl_2): ν = 3280.5 (m), 2361.6 (w), 1633.9 (m), 1461.1 (s) cm^{-1}

^1H ($CDCl_3$): δ = 7.44 (2H, d, J =7.4 Hz, H_{12}), 7.36-7.20 (12H, m, $H_{13,14,\ 25,\ 29\text{-}31}$), 6.83 (4H, d, J = 8.8 Hz, H_{26}), 6.46 (1H, d, J = 8.1 Hz, H_3), 6.00 (1H, t, J = 5.9 Hz, H_6), 4.68-4.62 (1H, m, H_1), 3.80 (6H, s, OCH_3), 3.65 (2H, t, J = 6.6 Hz, H_{34}), 3.00-2.70 (6H, m, $H_{2,\ 7,\ 9}$), 2.17 (2H, t, J = 5.4 Hz, H_{33}), 1.60 (1H, s, H_{35}), 1.45-1.30 (8H, m, CH_2), 1.20-1.00 (18H, m, CH_2)

^{13}C ($CDCl_3$): δ = 173.3 (C_{22}), 170.9 (C_{32}), 158.4 (C_{27}), 145.5 (C_{11}), 137.0 (C_{28}), 136.8 (C_{24}), 130.2, 129.4, 128.7, 128.3, 127.9, 127.1, 126.7 ($C_{12\text{-}14,\ 25,\ 29\text{-}31}$), 113.1 ($C_{26}$), 85.8 ($C_{23}$), 63.4 ($C_9$), 63.1 ($C_{34}$), 55.4 ($OCH_3$), 54.8 ($C_1$), 39.6 ($C_7$), 38.9 ($C_2$), 36.7, 32.9, 30.1, 29.6, 29.5, 29.5, 29.4, 29.2, 26.9, 26.1, 25.9, 25.7 (C_{33}, CH_2)

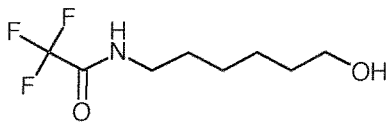


$C_{57}H_{81}N_4O_7P$
Mol. Wt.: 965.25
C, 70.93; H, 8.46; N, 5.80; O, 11.60; P, 3.21

12-(2-Cyanoethyl diisopropylphosphoramidyl)oxy-dodecanamido-phenylalanine-6-hydroxy(-*O*-4,4'dimethoxytrityl-)hexylamide [85]

To a solution of the alcohol **[84]** (0.50g, 650.0μmol) in anhydrous CH_2Cl_2 (5mL) was added *N,N*-diisopropylethylamine (453μL, 2.6mmol) and 2-cyanoethyl diisopropyl chlorophosphoramidite (175μL, 0.8mmol), under argon. After stirring at room temperature for 40 minutes the clear solution was diluted with EtOAc (30mL) washed with saturated potassium chloride solution (30mL), dried (Na_2SO_4) and the solvents removed *in vacuo*. The residue was dissolved in anhydrous CH_2Cl_2 (3mL) and the solution dropped slowly onto rapidly stirring hexane (-20°C, 250mL) to form a white precipitate. The hexane was removed with filtration through glass wool. The residue was washed with cold hexane (150mL). The residue was dissolved in CH_2Cl_2 (5mL), passed through the glasswool and the solvents removed *in vacuo*. Anhydrous MeCN was added (10mL), the solution filtered, the solvents removed *in vacuo* and the resultant solid dried over P_2O_5 for 24 hrs (0.58g, 0.6mmol, 92%).

R_f (D): 0.80; LRMS (ES^+ mode): *m/z* 988.1(MNa^+)
^{31}P ($CDCl_3$): δ = 147.87, 14.91



$C_8H_{14}F_3NO_2$
Mol. Wt.: 213.20
C, 45.07; H, 6.62; F, 26.73; N, 6.57; O, 15.01

1-Hexanol-6-trifluoroacetamide [90][205]

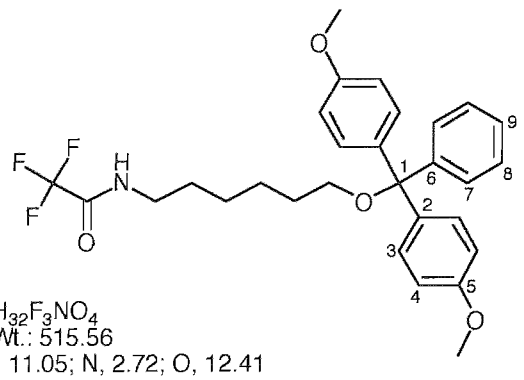
To a solution of 6-amino-1-hexanol (13.30g, 0.11mol) in CH_2Cl_2 (100mL) was added ethyl trifluoroacetate (14.16mL, 16.91g, 0.1mmol) and the mixture stirred at room temperature, under argon, for 12 hrs. Et_3N (2mL) was added to raise the pH to 8.0, followed by a second amount of ethyl trifluoroacetate (3mL, 3.58g, 25.2mmol). After stirring for 4 hrs, the solvents were removed *in vacuo*. The yellow oil obtained was dissolved in CH_2Cl_2 (200mL), washed with 10% citric acid solution (2 x 200mL), dried (Na_2SO_4), and the solvents removed *in vacuo*. The clear yellow oil was recrystallised from a minimal amount of toluene: chloroform (4:1). This afforded a white crystalline solid that was dried over P_2O_5 *in vacuo* for 12 hrs (16.95g, 79.5mmol, 71%).

R_f (J): 0.61; LRMS (ES^- mode): *m/z* 326.2($MTFA^-$); HRMS: $C_8H_{18}F_3N_2O_2$ requires 231.13204, found 231.13204; λ_{max} (MeOH): 268nm; mp: 41-43°C

IR: ν = 3386.9 (m), 3305.7 (m), 2931.8 (m), 2858.1 (m), 1696.8 (s), 1549.6 (s), 1176.2 (s), 1151.9 (s) cm^{-1}

^1H ($CDCl_3$): δ = 6.80 (1H, s, NH), 3.63 (2H, t, *J* = 7.6 Hz, CH_2O), 3.35 (2H, q, *J* = 7.5 Hz, $C\underline{H}_2NH$), 1.95 (1H, s, OH), 1.65-1.52 (4H, m, $C\underline{H}_2CH_2OH$, $C\underline{H}_2CH_2NH$), 1.43-1.35 (4H, m, CH_2)

^{13}C ($CDCl_3$): δ = 157.8 (CF_3), 114.1 (CO), 62.7 (CH_2OH), 39.9 (CH_2NH), 32.5 ($\underline{C}H_2CH_2NH$), 26.4 ($\underline{C}H_2CH_2OH$), 25.3, 26.2 (CH_2)



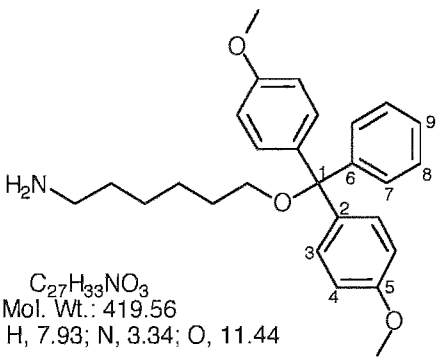
$C_{29}H_{32}F_3NO_4$
Mol. Wt.: 515.56
C, 67.56; H, 6.26; F, 11.05; N, 2.72; O, 12.41

1- O'-(4,4'-Dimethoxytrityloxy) hexyl-6-trifluoroacetamide [91]

A solution of recrystallised 4,4'-dimethoxytrityl chloride (19.00g, 56.1mmol) in pyridine (30mL) and added portionwise to a solution of 1-hexanol-6-trifluoroacetamide **[90]**

(11.95g, 56.1mmol) and 4-dimethylaminopyridine (137mg, 1.12mol) in pyridine (18mL). The mixture was stirred at room temperature, under argon, for 15 hrs and quenched with MeOH (50mL). The solvents were removed *in vacuo* and the oil obtained dissolved in CH_2Cl_2 (200mL), washed with saturated sodium bicarbonate solution (2 x 200mL), dried (Na_2SO_4), and the solvents removed *in vacuo*. The yellow oil was purified by silica gel column chromatography eluting with hexane: EtOAc (10:0 then 9:1 then 8:2 then 5:5 then 0: 10) Drying over P_2O_5 *in vacuo*, for 24hrs, afforded the title compound as a yellow oil (23.54g, 45.7mmol, 81%).

R_f (K): 0.1; LRMS (ES^- mode): *m/z* 628.2 ($MTFA^-$), (ES^+ mode): *m/z* 537.5 (MNa^+); HRMS: $C_{29}H_{32}F_3NO_4$ requires 515.22834, found 515.22834; λ_{max} (MeOH): 274, 233nm
IR: ν = 3301.2 (w), 2934.8 (m), 2860.78 (m), 1704.8 (s), 1507.7 (s), 1247.3 (s), 1172.0 (s), 1153.8 (s) cm^{-1}
^1H ($CDCl_3$): δ = 7.47-7.42 (4H, m, H_4) 7.36-7.17 (9H, m, $H_{3,\ 7\text{-}9}$), 6.50 (1H, s, NH), 3.79 (6H, s, OCH_3), 3.34 (2H, q, *J* = 5.1 Hz, $C\underline{H}_2NH$), 3.05 (2H, t, *J* = 6.0 Hz, CH_2O), 1.66-1.51 (4H, m, $C\underline{H}_2CH_2O$, $C\underline{H}_2CH_2NH$), 1.43-1.35 (4H, m, CH_2)
^{13}C ($CDCl_3$): δ = 207.3 (CF_3), 171.4 (CO), 158.5 (C_5), 145.5 (C_2), 136.8 (C_6), 130.1 (C_8), 128.3 (C_3), 127.9 (C_7), 126.7 (C_9), 113.1 (C_4), 85.8 (C_1), 63.2 (CH_2O), 55.3 (OCH_3), 40.1 (CH_2NH), 30.0 ($\underline{C}H_2CH_2O$), 29.0 ($\underline{C}H_2CH_2NH$), 26.7, 26.0 (CH_2)



$C_{27}H_{33}NO_3$
Mol. Wt.: 419.56
C, 77.29; H, 7.93; N, 3.34; O, 11.44

1-*O*-(4,4'-Dimethoxytrityloxy)-6-hexylamine [92]

Ammonia gas was bubbled through MeOH (200mL) for 20 minutes. To the saturated solution was added 1-*O*-(4,4'-dimethoxytrityloxy) hexyl-6-trifluoroacetamide **[91]** (9.56g, 18.6mmol) and ammonia gas continuously bubbled through the stirred solution for 18 hrs.

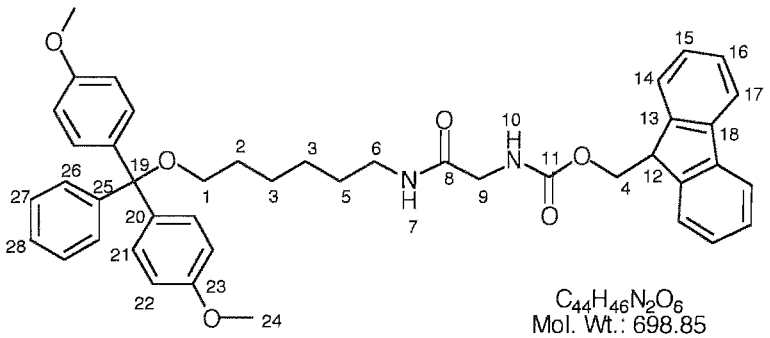
The solvents were removed *in vacuo* and the residue coevaporated with anhydrous CH_2Cl_2 (50mL). Drying over P_2O_5 *in vacuo* for 24hrs to yield a clear oil which was purified by silica gel column chromatography eluting with EtOAc: Et_3N: MeOH (10:0:0 then 8:1:1) to give a colourless oil, after drying over P_2O_5 (6.89g, 16.4mmol, 88%).

R_f (D): 0.51; LRMS (ES^+ mode): *m/z* 839.8 ($2M^{+}$); λ_{max} (MeOH): 272, 230nm

IR: ν = 2934.3 (w), 2361.5 (m), 1606.5 (m), 1507.3 (s), 1247.2 (s) cm^{-1}

^1H ($CDCl_3$): δ = 7.40-7.10 (9H, m, $H_{3,\ 7\text{-}9}$), 6.80 (4H, d, J = 8.6 Hz, H_4), 3.70 (6H, s, OCH_3), 3.30 (2H, q, J = 5.1 Hz, $C\underline{H}_2NH_2$), 2.95 (2H, t, J = 4.2 Hz, CH_2O), 1.60-1.40 (4H, m, $C\underline{H}_2CH_2O$, $C\underline{H}_2CH_2NH_2$), 1.34-1.12 (4H, m, CH_2)

^{13}C ($CDCl_3$): δ = 158.4 (C_5), 145.6 (C_2), 136.9 (C_6), 130.2 (C_8) 128.3 (C_3), 127.8 (C_7), 126.7 (C_9), 113.1 (C_4), 85.8 (C_1), 63.5 (CH_2O), 55.4 (OCH_3), 41.9 (CH_2NH_2), 32.9 ($\underline{C}H_2CH_2O$), 30.2 ($\underline{C}H_2CH_2NH_2$), 26.9, 26.3 (CH_2)



$C_{44}H_{46}N_2O_6$
Mol. Wt.: 698.85
C, 75.62; H, 6.63; N, 4.01; O, 13.74

Fmoc-glycine-6-hydroxy(-*O*-4,4'-dimethoxytrityl)hexylamide [93]

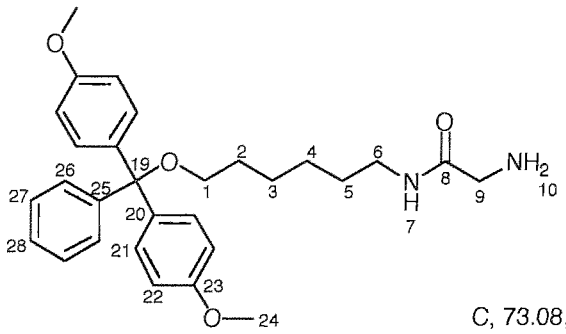
To a solution of 6-hydroxy(-*O*-4,4'-dimethoxytrityl)hexylamine **[92]** (8.89g, 21.2mmol) in anhydrous CH_2Cl_2 (15mL) was added a solution of Fmoc-glycine-OH (NovaBiochem, 6.31g, 21.2mmol) in CH_2Cl_2: DMF (3: 1, 20mL). EDC (4.47g, 23.3mmol) was added to the yellow solution and the reaction stirred under argon at room temperature for 4 hrs. The reaction was diluted with CH_2Cl_2 (200mL), washed with water (200mL), saturated sodium bicarbonate solution (200mL), brine (200mL) and dried (Na_2SO_4). Removal of the solvents *in vacuo* afforded a yellow oil that was purified by silica gel column chromatography eluting with CH_2Cl_2. The yellow oil obtained was dried over P_2O_5 *in vacuo* for 14 hrs (7.80g, 11.2mmol, 53%).

R_f (P): 0.71; LRMS (ES^+ mode): *m/z* 800.1 (MEt_3N^+), 721.0 (MNa^+); λ_{max} (MeOH): 271, 232nm; decomposed: 155-160°C

IR: ν = 2932.6 (w), 2896.7 (w), 2855.7 (w), 2365.3 (m), 2341.3 (m), 1652.4 (m), 1606.4 (m), 1506.8 (m), 1247.6 (s), 749.6 (s) cm^{-1}

^1H ($CDCl_3$): δ = 7.40-7.08 (17H, m, $H_{14\text{-}17,\ 21,\ 26\text{-}28}$), 6.70 (4H, d, J = 9.0 Hz, H_{22}), 4.35-4.30 (2H, m, H_4), 4.20 (1H, t, J = 7.0 Hz, H_{12}), 3.73 (6H, s, H_{24}), 3.27 (2H, s, H_9), 3.23 (2H, q, J = 6.6 Hz, H_6), 2.98 (2H, t, J = 6.6 Hz, H_1), 1.80-1.20 (8H, m, $H_{2,\ 3,\ 5}$)

^{13}C ($CDCl_3$): δ = 158.4 (C_{23}), 145.6 (C_{20}), 143.9 (C_{11}), 141.5 (C_8), 136.8 (C_{25}), 130.2 (C_{27}), 128.9 (C_{13}), 128.3 (C_{21}), 127.9 (C_{26}), 127.3 ($C_{17,\ 14}$), 126.7 (C_{28}), 125.2 (C_{16}), 121.2 (C_{18}), 120.2 (C_{15}), 113.1 (C_{22}), 85.8 (C_{19}), 67.3 (C_4), 63.4 (C_1), 55.4 (C_{24}), 47.3 (C_6), 43.3 (C_{12}), 39.8 (C_9), 30.1, 29.9 ($C_{5,\ 2}$), 26.9, 26.2 (C_3)



$C_{29}H_{36}N_2O_4$
Mol. Wt.: 476.61
C, 73.08; H, 7.61; N, 5.88; O, 13.43

Glycine-6-hydroxy(-*O*-4,4'-dimethoxytrityl)hexylamide [94]

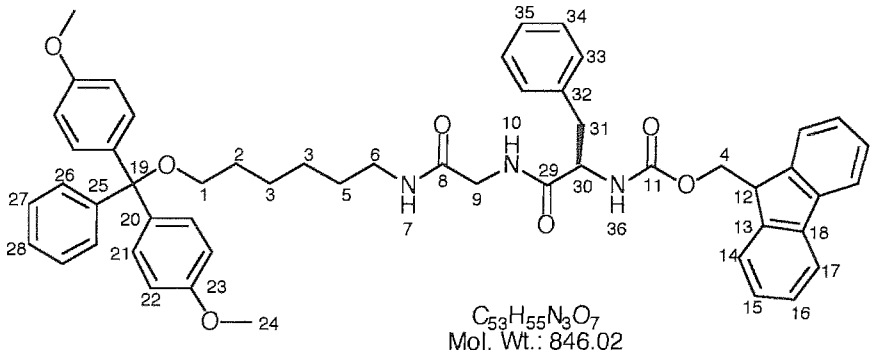
To a solution of piperidine in DMF (20%, 50mL) was added the Fmoc-protected compound **[93]** (6.00g, 8.6mmol) and the mixture stirred under argon, at room temperature for 24 hrs. The solvents were removed *in vacuo* and the resulting oil dissolved in CH_2Cl_2 (200mL), washed with water (200mL), saturated sodium bicarbonate solution (200mL), brine (200mL) and dried (Na_2SO_4). Removal of the solvents *in vacuo* gave a pale yellow solid which was purified by silica gel column chromatography eluting with MeOH: CH_2Cl_2 (0:1 then 1:10) to give the desired compound as a colourless oil that was dried over P_2O_5 *in vacuo* (2.75g, 5.8mmol, 67%).

R_f (D): 0.44; LRMS (ES^+ mode): *m/z* 499.4 (MNa^+), 303.3 (DMT^+); HRMS: $C_{29}H_{36}N_2O_4$ requires 476.26751, found 476.26751; λ_{max} (MeOH): 269, 234nm

IR: ν = 3329.1 (w), 2931.7 (w), 2364.2 (m), 2341.3 (m), 1654.2 (m), 1606.6 (m), 1507.4 (m), 1246.1 (s), 1032.1 (s), 730.7 (s) cm^{-1}

^1H ($CDCl_3$): δ = 7.40-7.13 (9H, m, $H_{21, 26-28}$), 6.77 (4H, d, J = 9.0 Hz, H_{22}), 3.71 (6H, s, H_{24}), 3.27 (2H, s, H_9), 3.25 (2H, q, J = 6.6 Hz, H_6), 3.05 (2H, t, J = 6.6 Hz, H_1), 1.60-1.16 (10H, m, $H_{2-5, 10}$)

^{13}C ($CDCl_3$): δ = 172.8 (C_8), 158.5 (C_{23}), 145.6 (C_{20}), 136.8 (C_{25}), 130.2 (C_{27}), 128.3 (C_{21}), 127.9 (C_{26}), 126.7 (C_{28}), 113.1 (C_{22}), 85.8 (C_{19}), 63.5 (C_1), 55.4 (C_{24}), 44.9 (C_9), 39.1 (C_6), 30.1, 29.8 ($C_{2, 5}$), 27.0, 26.2 ($C_{3,4}$)



$C_{53}H_{55}N_3O_7$
Mol. Wt.: 846.02
C, 75.24; H, 6.55; N, 4.97; O, 13.24

Fmoc-phenylalanine-glycine-6-hydroxy(-*O*-4,4'-dimethoxytrityl)hexylamide [95]

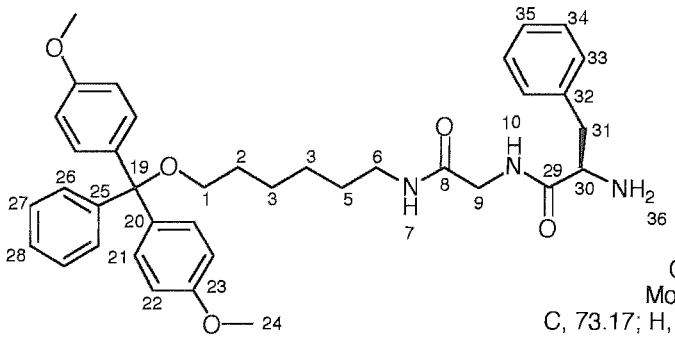
To a solution of the amine **[94]** (1.10g, 2.3mmol) in anhydrous CH_2Cl_2 (10mL) was added Fmoc-phenylalanine-OH (NovaBiochem, 0.90g, 2.3mmol) and EDC (0.49g, 2.5mmol). The solution was stirred at room temperature, under argon, for 14 hrs. The reaction was diluted with CH_2Cl_2 (50mL), washed with water (50mL), saturated sodium bicarbonate solution (50mL), brine (50mL) and then dried (Na_2SO_4). Removal of the solvents *in vacuo* gave a pale yellow foam (1.92g, 98%), that was taken through to the next step crude. A small amount (100mg) was purified by silica gel column chromatography, for analysis, eluting with MeOH: CH_2Cl_2 (0:1 then 1:50) to give an off white solid which was dried over P_2O_5 *in vacuo* (83mg, 83%).

R_f (D): 0.67, (E) 0.65; LRMS (ES^+ mode): *m/z* 868.9 (MNa^+), 543.4 (M-DMT^+), 303.3 (DMT^+); HRMS: $C_{53}H_{55}N_3O_7$ requires 845.4040017, found 845.4040017; λ_{max} (MeOH): 265nm; mp: 78-82°C

IR: ν = 3388.2 (w), 2930.2 (w), 2858.7 (w), 2363.5 (m), 2341.6 (m), 1653.9 (m), 1607.0 (m), 1506.6 (m), 1246.7 (s), 1031.5 (s), 748.5 (s) cm^{-1}

^1H ($CDCl_3$): δ = 7.69-7.07 (22H, m, $H_{14\text{-}17,\ 21,\ 26\text{-}28,\ 33\text{-}35}$), 6.87 (1H, t, J = 8.8 Hz, H_{10}), 6.75 (4H, d, J = 7.9 Hz, H_{22}), 6.36-6.32 (1H, m, H_7), 5.12-5.08 (1H, m, H_{36}), 4.40-4.30 (2H, m, H_4), 4.23 (1H, t, J = 7.0 Hz, H_{12}), 4.08 (1H, t, J = 6.6 Hz, H_{30}), 3.89-3.72 (2H, m, H_9), 3.71 (6H, s, H_{24}), 3.14-3.03 (2H, m, H_6), 2.95 (2H, t, J = 6.6 Hz, H_1), 1.57-1.53 (2H, m, H_{31}), 1.42-1.12 (8H, m, $H_{2,\ 3,\ 5}$)

^{13}C ($CDCl_3$): δ = 171.8 (C_8), 168.5 (C_{29}), 158.4 (C_{23}), 156.4 (C_{11}), 145.6 (C_{20}), 143.7 (C_{13}), 141.4 (C_{32}), 136.9 (C_{25}), 136.4 (C_{18}), 130.2, 129.4, 128.9, 128.4, 128.0, 127.9, 127.3, 127.3, 126.8, 125.1, 120.2 ($C_{14\text{-}17,\ 21,\ 26\text{-}28,\ 33\text{-}35}$), 113.1 ($C_{22}$), 85.8 ($C_{19}$), 67.2 ($C_4$), 63.4 ($C_1$), 56.7 ($C_{30}$), 55.4 ($C_{24}$), 47.2 ($C_6$), 43.4 ($C_{12}$), 39.8 ($C_9$), 38.3 ($C_{31}$), 30.2, 29.5 ($C_{5,\ 2}$), 27.0, 26.2 ($C_3$)



$C_{38}H_{45}N_3O_5$
Mol. Wt.: 623.78
C, 73.17; H, 7.27; N, 6.74; O, 12.82

Phenylalanine-glycine-6-hydroxy(-*O*-4,4'-dimethoxytrityl)hexylamide [96]

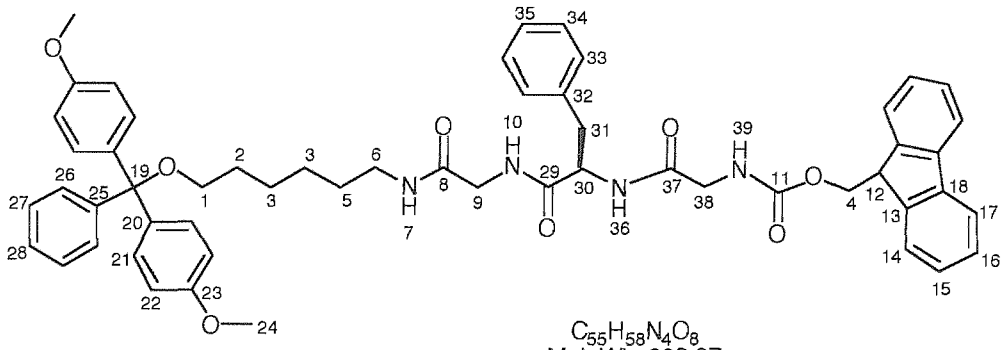
To a solution of piperidine in DMF (20%, 25mL) was added the Fmoc protected amide **[95]** (1.90g, crude) and the mixture stirred under argon, at room temperature for 12 hrs. The solvents were removed *in vacuo* and the resulting oil dissolved in CH_2Cl_2 (150mL), washed with water (150mL), saturated sodium bicarbonate solution (150mL), brine (150mL) and then dried (Na_2SO_4). Removal of the solvents *in vacuo* gave a pale yellow solid (2.14g), which was taken through to the next step crude. A small amount (100mg) was purified by silica gel column chromatography eluting with MeOH: CH_2Cl_2 (0:1 then 1:10) to give the desired compound as a white waxy solid, which was dried over P_2O_5 *in vacuo* (50mg, 91% based on yield of last step).

R_f (D): 0.70, (E): 0.13; LRMS (ES^+ mode): *m/z* 646.5 (MNa^+), 303.3 (DMT^+); λ_{max} (MeOH): 262nm

IR: ν = 3313.7 (w), 2932.1 (w), 2854.6 (w), 2359.9 (m), 1649.9 (m), 1606.6 (m), 1507.7 (s), 1248.0 (s), 1031.4 (s), 753.2 (s) cm^{-1}

^1H ($CDCl_3$): δ = 8.82-8.79 (1H, m, H_{10}), 7.40-7.10 (14H, m, $H_{21, 26\text{-}28, 33\text{-}35}$), 6.75 (4H, d, J = 9.0 Hz, H_{22}), 6.34-6.31 (1H, m, H_7), 3.84 (2H, d, J = 6.5 Hz, H_9), 3.71 (6H, s, H_{24}), 3.62-3.56 (1H, m, H_{30}), 3.21-3.10 (2H, m, H_6), 2.97 (2H, t, J = 6.6 Hz, H_1), 1.83-1.72 (2H, m, H_{31}), 1.56-1.12 (8H, m, $H_{2, 3, 5}$)

^{13}C ($CDCl_3$): δ = 175.2 (C_8), 166.8 (C_{29}), 158.4 (C_{23}), 145.4 (C_{20}), 137.5 (C_{32}), 136.8 (C_{25}), 130.0, 129.3, 128.8, 128.2, 127.7, 126.9, 126.6 ($C_{21, 26\text{-}28, 33\text{-}35}$), 113.0 ($C_{22}$), 85.7 ($C_{19}$), 63.3 ($C_1$), 56.4 ($C_{30}$), 55.2 ($C_{24}$), 43.4 ($C_9$), 41.0 ($C_{31}$), 39.6 ($C_6$), 30.0, 29.5 ($C_{2, 5}$), 26.8, 26.0 ($C_3$)



$C_{55}H_{58}N_4O_8$
Mol. Wt.: 903.07
C, 73.15; H, 6.47; N, 6.20; O, 14.17

Fmoc-glycine-phenylalanine-glycine-6-hydroxy(-*O*-4,4'-dimethoxytrityl)hexylamide [97]

To a solution of the amine **[96]** (2.14g, crude) in anhydrous CH_2Cl_2 (10mL) was added Fmoc-phenylalanine-OH (NovaBiochem, 0.90g, 2.3mmol) and EDC (0.49g, 2.5mmol). The solution was stirred at room temperature, under argon, for 1 hr. The reaction was diluted with CH_2Cl_2 (50mL), washed with water (50mL), saturated sodium bicarbonate solution (50mL), brine (50mL) and then dried (Na_2SO_4). Removal of the solvents *in vacuo* gave a pale yellow foam (2.27g), which was taken through to the next step crude. A small amount (110mg) was purified by silica gel column chromatography eluting with MeOH: CH_2Cl_2

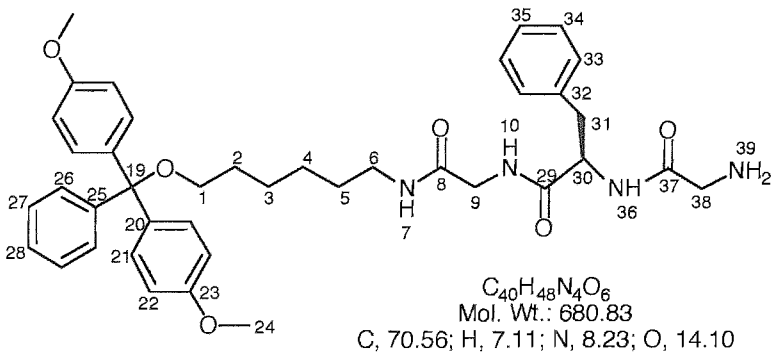
(0:1 then 1:50) to give the title compound as a white foam that was dried over P_2O_5 *in vacuo* (63mg, 83%, based on yields of last two steps).

R_f (D): 0.86 (E): 0.54; LRMS (ES^+ mode): *m/z* 926.0 (MNa^+), 224.1 ($FMOC^+$); λ_{max} (MeOH): 280, 261nm; mp: 55-57°C

IR: ν = 3299.6 (w), 2934.5 (w), 2359.6 (m), 2341.4 (m), 1650.2 (m), 1608.4 (m), 1506.9 (m), 1259.5 (s), 1030.8 (s), 749.9 (s) cm^{-1}

^1H ($CDCl_3$): δ = 7.77-7.00 (22H, m, $H_{14\text{-}17,\ 21,\ 26\text{-}28,\ 33\text{-}35}$), 7.10-7.00 (1H, m, H_{10}), 6.80 (4H, d, J = 7.9 Hz, H_{22}), 6.60-6.56 (1H, m, H_7), 5.86-5.83 (1H, m, H_{39}), 4.72 (1H, d, J = 6.5 Hz, H_{36}), 4.35 (2H, d, J = 7.0 Hz, H_4), 4.17 (1H, t, J = 7.0 Hz, H_{12}), 3.93-3.84 (3H, m, $H_{30,\ 38}$), 3.78 (6H, s, H_{24}), 3.14 (2H, q, J = 6.5 Hz, H_6), 3.08-3.05 (2H, m, H_9), 2.99 (2H, t, J = 6.5 Hz, H_1), 1.92-1.88 (2H, m, H_{31}), 1.57-1.20 (8H, m, $H_{2,\ 3,\ 5}$)

^{13}C ($CDCl_3$): δ = 171.9 (C_{11}), 169.6 (C_{37}), 168.5 ($C_{8,\ 29}$), 158.4 (C_{23}), 145.5 (C_{20}), 143.7 (C_{13}), 141.4 (C_{32}), 136.8 (C_{25}), 136.3 (C_{18}), 130.2, 129.3, 128.9, 128.3, 128.0, 127.9, 127.3, 126.7, 125.1, 120.2 ($C_{14\text{-}17,\ 21,\ 26\text{-}28,\ 33\text{-}35}$), 113.1 ($C_{22}$), 85.8 ($C_{19}$), 67.6 ($C_4$), 63.4 ($C_1$), 55.4 ($C_{24}$), 55.0 ($C_{30}$), 47.1 ($C_{12}$), 44.7 ($C_{38}$), 43.3 ($C_6$), 39.8 ($C_9$), 38.5 ($C_{31}$), 30.1, 29.5, 27.0, 26.1 ($C_{2\text{-}5}$)



$C_{40}H_{48}N_4O_6$
Mol. Wt.: 680.83
C, 70.56; H, 7.11; N, 8.23; O, 14.10

Glycine-phenylalanine-glycine-6-hydroxy(-*O*-4,4'-dimethoxytrityl)hexylamide [98]

To a solution of piperidine in DMF (20%, 25mL) was added the peptide **[97]** (2.17g, crude) and the mixture stirred under nitrogen, at room temperature for 1.5 hrs. The solvents were removed *in vacuo* and the resulting white waxy solid dissolved in CH_2Cl_2 (200mL), washed with saturated sodium bicarbonate solution (2 x 200mL), brine (200mL) and then dried (Na_2SO_4). Removal of the solvents *in vacuo* gave a white solid which was purified by silica

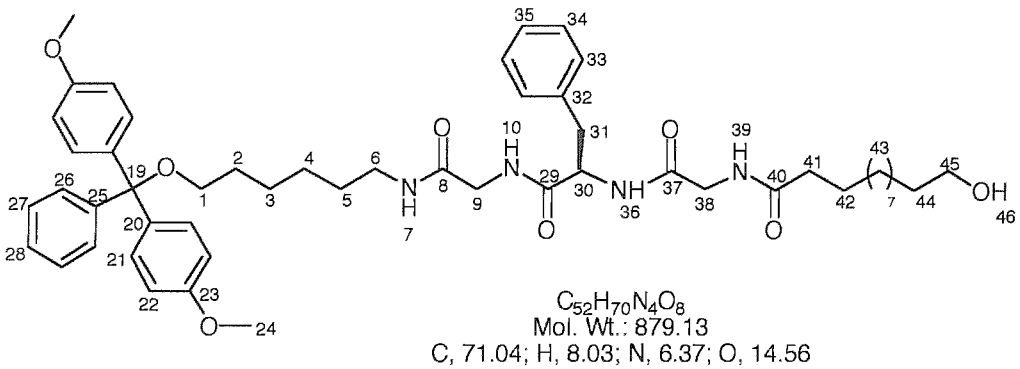
gel column chromatography eluting with MeOH: CH_2Cl_2 (0:1 then 1:10) to give the desired compound as a white crystalline solid, which was dried over P_2O_5 *in vacuo* (0.90g, 1.3mmol, 92%, 57% overall yield of last 4 steps,).

R_f (D): 0.31; LRMS (ES^+ mode): *m/z* 703.6 (MNa^+), 303.3 (DMT^+); HRMS: $C_{40}H_{48}N_4O_6Na$ requires 703.34716, found 703.34716; λ_{max} (MeOH): 273, 234nm; mp: 63-68 °C

IR: ν = 3298.9 (w), 2928.6 (w), 2855.7 (w), 2363.1 (m), 2341.6 (m), 1648.8 (m), 1606.7 (m), 1506.8 (m), 1245.8 (s), 1030.6 (s), 750.2 (s) cm^{-1}

^1H ($CDCl_3$): δ = 7.87 (1H, t, J = 7.4 Hz, H_{10}), 7.40-7.05 (14H, m, $H_{21, 26\text{-}28, 33\text{-}35}$), 6.75 (4H, d, J = 8.8 Hz, H_{22}), 6.60-6.56 (1H, m, H_7), 4.50 (1H, d, J = 6.5 Hz, H_{36}), 3.88-3.72 (1H, m, H_{30}), 3.73 (6H, s, H_{24}), 3.20 (2H, s, H_{39}), 3.12-3.06 (2H, m, H_6), 2.95 (2H, t, J = 6.0 Hz, H_1), 2.08-1.98 (2H, m, H_{38}), 1.60-1.10 (12H, m, $H_{2\text{-}5, 9, 31}$)

^{13}C ($CDCl_3$): δ = 173.7 (C_{37}), 171.8 (C_8), 168.9 (C_{29}), 158.4 (C_{23}), 145.5 (C_{20}), 136.8 (C_{32}), 136.6 (C_{25}), 130.2, 129.3, 128.9, 128.3, 127.8, 127.3, 126.7, ($C_{21, 26\text{-}28, 33\text{-}35}$), 113.1 ($C_{22}$), 85.8 ($C_{19}$), 63.5 ($C_1$), 55.4 ($C_{24}$), 55.1 ($C_{30}$), 46.5 ($C_{38}$), 3.3 ($C_6$), 39.8 ($C_9$), 37.8 ($C_{31}$), 30.2, 29.5, 27.0, 26.2 ($C_{2\text{-}5}$)



$C_{52}H_{70}N_4O_8$
Mol. Wt.: 879.13
C, 71.04; H, 8.03; N, 6.37; O, 14.56

12-Hydroxydodecanamido-glycine-phenylalanine-glycine-6-hydroxy(-*O*-4,4'-dimethoxytrityl)hexylamide [99]

To a solution of the amine **[98]** (0.50g, 740.0μmol) in anhydrous CH_2Cl_2 (5mL) was added 12-hydroxydodecanoic acid (0.90g, 740.0μmol) and EDC (155mg, 0.8mmol). The solution was stirred at room temperature, under argon, for 12 hrs. The reaction was diluted with

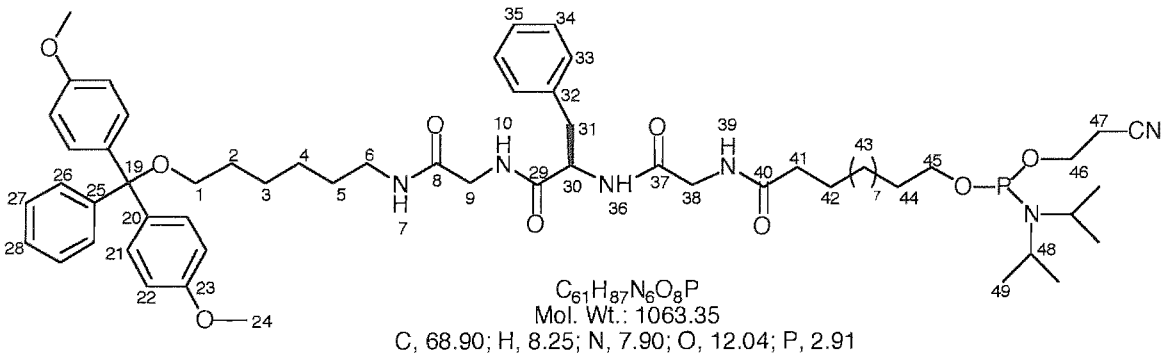
CH_2Cl_2 (100mL), washed with water (100mL), saturated sodium bicarbonate solution (100mL), brine (100mL) and then dried (Na_2SO_4). Removal of the solvents *in vacuo* produced a white foam that was purified by silica gel column chromatography eluting with MeOH: CH_2Cl_2 (1:10) to give the title compound as an off white waxy solid that was dried over P_2O_5 *in vacuo* (0.53g, 0.6mmol, 83%).

R_f (D): 0.46; LRMS (ES^+ mode): *m/z* 902.3 (MNa^+), 303.4 (DMT^+); HRMS: $C_{52}H_{70}N_4O_8$ requires 878.51937, found 878.51937; λ_{max} (MeOH): 267nm

IR (CH_2Cl_2): ν = 3282.8 (s, br), 2924.2 (m), 2852.9 (m), 2359.1 (w), 1624.4 (s), 1508.2 (s), 1247.8 (s), 1032.1 (s), 749.5 (s) cm^{-1}

^1H ($CDCl_3$): δ = 8.30 (1H, d, *J* = 7.4 Hz, H_{10}), 7.73-7.62 (2H, m, $H_{7, 39}$), 7.40-7.05 (14H, m, $H_{21, 26-28, 33-35}$), 6.75 (4H, d, *J* = 8.8 Hz, H_{22}), 5.06-4.95 (1H, m, H_{36}), 3.95-3.83 (1H, m, H_{30}), 3.73 (6H, s, H_{24}), 3.60 (2H, t, *J* = 6.6 Hz, H_{45}), 3.15-3.06 (2H, m, H_6), 3.00 (2H, t, *J* = 6.6 Hz, H_1), 2.90 (2H, d, *J* = 5.2 Hz, H_{41}), 2.23-2.18 (2H, m, H_{38}), 1.65-1.10 (30H, m, $H_{2-5, 9, 31, 42-44}$)

^{13}C ($CDCl_3$): δ = 174.2, 171.1, 168.7, 168.5 ($C_{8, 29, 37, 40}$), 158.4 (C_{23}), 145.5 (C_{20}), 136.8 (C_{32}), 136.6 (C_{25}), 130.1, 129.7, 128.5, 128.3, 127.8, 127.1, 126.7, ($C_{21, 26-28, 33-35}$), 113.1 (C_{22}), 85.8 (C_{19}), 63.5 (C_1), 63.0 (C_{45}), 55.3 (C_{24}), 55.3 (C_{30}), 46.6, 43.3, 40.0, 39.8, 36.0 ($C_{6, 9, 31, 38, 41}$), 32.9, 30.3, 29.7, 29.6, 29.6, 29.4, 27.2, 26.3, 25.9, 25.8 ($C_{2-5, 42-44}$)



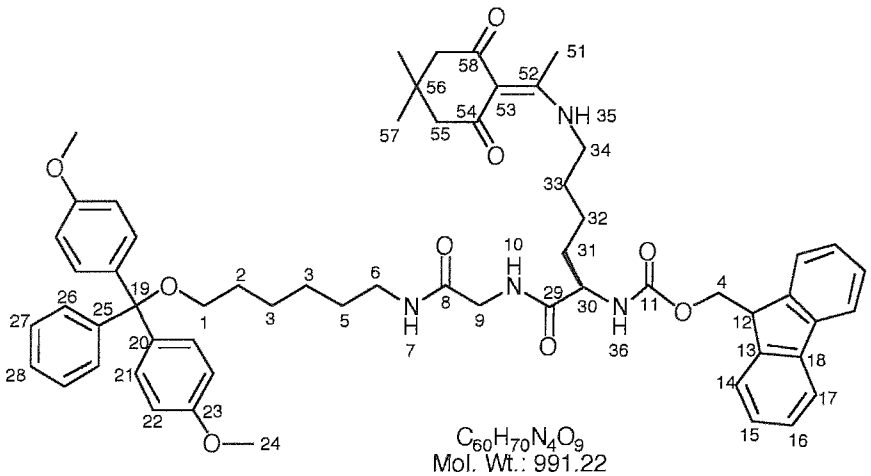
12-(2-Cyanoethyl diisopropylphosphoramidyl)oxy-dodecanamido-glycine-phenylalanine-glycine-6-hydroxy (-*O*-4,4'-dimethoxytrityl)hexylamide [100]

To a solution of the alcohol **[99]** (0.40g, 460.0μmol) in anhydrous CH_2Cl_2 (5mL) was added *N,N*-diisopropylethylamine (317μL, 1.8mmol) and 2-cyanoethyldiisopropylchloro-

phosphoramidite (111μL, 118mg, 0.5mmol), under argon. After stirring at room temperature for 1 hr *N,N*-diisopropylethylamine (100μL, 0.6mmol) and 2-cyanoethyldiisopropylchloro- phosphoramidite (100μL, 106mg, 450.0μmol) were added. After another 1 hr the pale yellow solution was diluted with EtOAc (25mL), washed with saturated potassium chloride solution (30mL), dried (Na_2SO_4), and the solvents removed *in vacuo*. Anhydrous CH_2Cl_2 (3mL) was added and this solution dropped slowly onto rapidly stirring hexane (-20°C, 250mL) forming a white precipitate. The hexane was removed with filtration, through glasswool. The residue was washed with cold hexane (150mL) and then dissolved in CH_2Cl_2 (5mL), passed through the glasswool and the solvents removed *in vacuo*. Anhydrous MeCN was added (10mL), the solution filtered, the solvents removed *in vacuo* and dried over P_2O_5 for 24 hrs (0.45g, 420.0μmol, 92%).

R_f (D): 0.64, 0.51 (Q): 0.81, 0.7; LRMS (ES^+ mode): *m/z* 1086.6 (MNa^+); λ_{max} (MeOH): 276 nm

^{31}P ($CDCl_3$): δ = 147.2



Fmoc-lysine(Dde)-glycine-6-hydroxy(-*O*-4,4'-dimethoxytrityl)hexylamide [105]

To a solution of the amine **[94]** (0.89g, 1.9mmol) in anhydrous CH_2Cl_2 (10mL) was added Fmoc-lysine(Dde)-OH (NovaBiochem, 1.00g, 1.9mmol) and EDC (0.39g, 2.0mmol). The solution was stirred at room temperature, under argon, for 1 hr. The reaction was diluted with CH_2Cl_2 (100mL), washed with water (150mL), saturated sodium bicarbonate solution

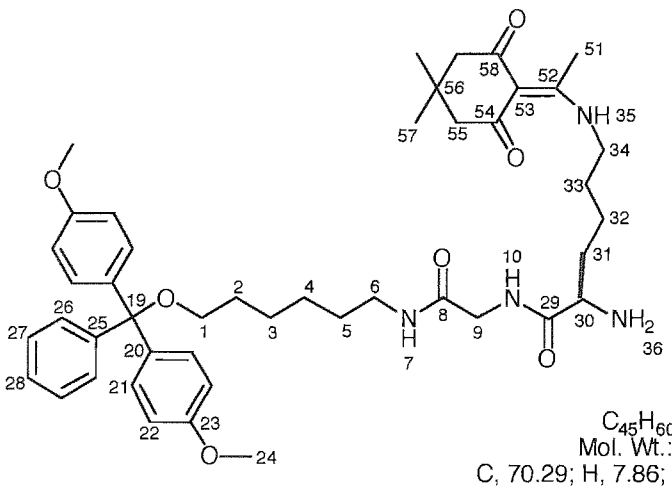
(150mL), brine (150mL) and then dried (Na_2SO_4). Removal of the solvents *in vacuo* gave a white foam (1.81g, crude). A small amount (76mg) was purified by silica gel column chromatography, for analysis, eluting with MeOH: CH_2Cl_2 (1: 20) to give an off white solid which was dried over P_2O_5 *in vacuo* (67mg, 88%).

R_f (D): 0.43; LRMS (ES^+ mode): *m/z* 1030.2 (MK^+), 991.2 (M^+), 303.3 (DMT^+); λ_{max} (MeOH): 294, 257nm; mp: 69-73°C

IR (CH_2Cl_2): ν = 3276.8 (w), 2933.2 (m), 2360.2 (m), 1650.0 (m), 1564.5 (m), 1506.9 (m), 1257.4 (s), 1028.8 (s), 749.8 (s) cm^{-1}

^1H ($CDCl_3$): δ = 7.80-7.20 (17H, m, $H_{14\text{-}17,\ 21,\ 26\text{-}28}$), 6.90 (1H, t, J = 8.3 Hz, H_{10}), 6.75 (4H, d, J = 7.9 Hz, H_{22}), 6.36-6.32 (1H, m, H_7), 5.65 (1H, d, J = 6.6 Hz, H_{36}), 4.40-4.30 (2H, m, H_4), 4.18 (1H, t, J = 7.0 Hz, H_{12}), 4.10-4.05 (1H, m, H_{30}), 3.85-3.77 (2H, m, H_9), 3.71 (6H, s, H_{24}), 3.35-3.25 (2H, m, H_{34}), 3.17-3.08 (2H, m, H_6), 2.95 (2H, t, J = 6.6 Hz, H_1), 2.41 (3H, s, H_{51}), 2.28 (4H, s, H_{55}), 1.95-1.10 (14H, m, $H_{2,\ 3,\ 5,\ 31\text{-}33}$), 0.93 (6H, s, H_{57})

^{13}C ($CDCl_3$): δ = 173.81 ($C_{58,\ 54}$), 172.10 (C_8), 170.03 (C_{29}), 158.43 (C_{23}), 156.66 (C_{11}), 145.53 (C_{20}), 143.8 ($C_{13,\ 53}$), 141.5 (C_{18}), 136.8 (C_{25}), 130.2, 128.3, 128.0, 127.8, 127.3, 126.7, 125.1, 120.2 ($C_{14\text{-}17,\ 21,\ 26\text{-}28}$), 113.1 ($C_{22}$), 108.1 ($C_{52}$), 85.8 ($C_{19}$), 67.2 ($C_4$), 63.4 ($C_1$), 55.4 ($C_{24}$), 47.3 ($C_{6,\ 34}$), 43.3 ($C_{12}$), 43.1 ($C_{33,\ 55}$) 39.8 ($C_9$), 30.3 ($C_{56}$), 30.1, 29.5 ($C_{2,\ 5}$), 28.4 ($C_{51}$), 26.9, 26.1 ($C_3$), 23.0 ($C_{31,\ 32}$), 18.1 ($C_{57}$)



$C_{45}H_{60}N_4O_7$
Mol. Wt.: 768.98
C, 70.29; H, 7.86; N, 7.29; O, 14.56

Lysine(Dde)-glycine-6-hydroxy(-*O*-4,4'-dimethoxytrityl)hexylamide [106]

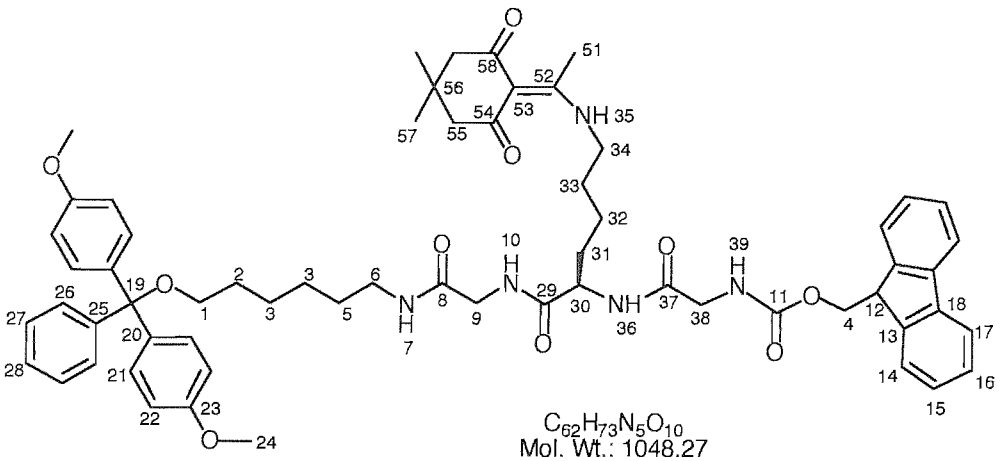
To a solution of piperidine in DMF (20%, 25mL) was added the peptide **[105]** (1.74g, 1.8mmol) and the mixture stirred under argon, at room temperature for 45 minutes. The solvents were removed *in vacuo* and the resulting oil dissolved in CH_2Cl_2 (200mL), washed with water (200mL), saturated sodium bicarbonate solution (200mL), brine (150mL) and then dried (Na_2SO_4). After removal of the solvents *in vacuo* an off white foam (1.35g crude) was obtained which was taken through to the next step crude. A small amount (96mg) was purified by silica gel column chromatography eluting with MeOH: CH_2Cl_2 (1:20 then 1:10) to give the title compound as a white foam which was dried over P_2O_5 *in vacuo* (59mg, 69% based on yield of last step).

R_f (D): 0.20; LRMS (ES^+ mode): *m/z* 767.8 (M^+), 790.9 (MNa^+); λ_{max} (MeOH): 297, 235nm

IR (CH_2Cl_2): ν = 3308.4 (w), 2930.3 (m), 2861.4 (m), 2359.9 (m), 1638.9 (m), 1567.1 (m), 1507.3 (m), 1257.1 (s), 1030.9 (s), 750.6 (s) cm^{-1}

^1H ($CDCl_3$): δ = 7.92-7.88 (1H, m, H_{10}), 7.45-7.15 (9H, m, $H_{21, 26-28}$), 6.80 (4H, d, J = 8.3 Hz, H_{22}), 6.65 (1H, t, J = 5.9 Hz, H_7), 3.80 (2H, d, J = 5.2 Hz, H_9), 3.76 (6H, s, H_{24}), 3.52-3.49 (1H, m, H_{30}), 3.40-3.30 (2H, m, H_{34}), 3.23-3.10 (4H, m, $H_{6, 36}$), 2.98 (2H, t, J = 6.6 Hz, H_1), 2.41 (3H, s, H_{51}), 2.28 (4H, s, H_{55}), 1.90-1.10 (14H, m, $H_{2-5, 31-33}$), 0.93 (6H, s, H_{57})

^{13}C ($CDCl_3$): δ = 198.0 (C_{54}), 175.2 (C_{53}), 173.7 (C_{58}), 169.0 (C_{29}) 162.7 (C_8), 158.4 (C_{23}), 145.5 (C_{20}), 136.8 (C_{25}), 130.1, 129.3, 128.8, 128.3, 127.8, 126.7, ($C_{21, 26-28}$), 113.1 (C_{22}), 108.0 (C_{52}), 85.8 (C_{19}), 63.4 (C_1), 55.3 (C_{24}), 44.8, 43.3, 39.8 ($C_{6, 9, 34}$), 36.7 (C_{30}), 34.26, 32.9 ($C_{33, 55}$), 30.3 (C_{56}), 30.1, 29.8 ($C_{2, 5}$), 28.4 (C_{51}), 27.0, 26.2 ($C_{3, 4}$), 22.5 ($C_{31, 32}$), 18.1 (C_{57})



$C_{62}H_{73}N_5O_{10}$
Mol. Wt.: 1048.27
C, 71.04; H, 7.02; N, 6.68; O, 15.26

Fmoc-glycine-lysine(Dde)-glycine-6-hydroxy(-*O*-4,4'-dimethoxytrityl)hexylamide [107]

To a solution of the amine **[106]** (1.55g, crude) in anhydrous CH_2Cl_2 (10mL) was added Fmoc-glycine-OH (NovaBiochem, 0.54g, 1.9mmol) in anhydrous DMF: CH_2Cl_2 (1:10. 10mL) and EDC (0.40g, 2.1mmol). After the reaction had been stirring at room temperature, under argon, for 45 minutes and a further portion of EDC was added (0.40g, 2.1mmol). After 1.75 hrs the reaction was diluted with CH_2Cl_2 (150mL), washed with water (150mL), sodium bicarbonate solution (150mL), brine (150mL) and then dried (Na_2SO_4). Removal of the solvents *in vacuo* produced a pale yellow foam (1.95g, crude). A small portion (100mg) was purified by silica gel column chromatography, for analysis, eluting with MeOH: CH_2Cl_2 (1:0 then 1:10) to give the title compound as a yellow foam which was dried over P_2O_5 *in vacuo* (36mg, 67% based on yields of last two steps).

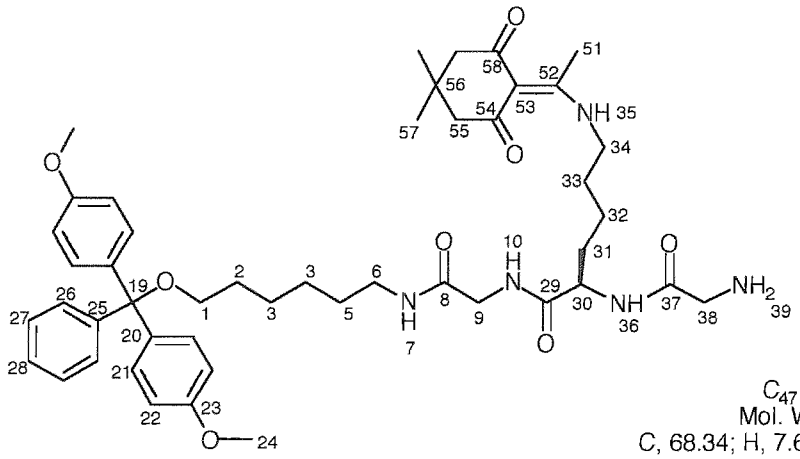
R_f (D): 0.37; LRMS (ES^+ mode): *m/z* 1071.1 (MNa^+), 1048.1 (MH^+) 303.4 (DMT^+); λ_{max} (MeOH): 257nm; mp: 96-101°C

IR (CH_2Cl_2): ν = 3276.2 (w), 2933.5 (m), 2360.8 (m), 2360.6 (m), 1628.2 (m), 1571.5 (s), 1506.8 (s), 1257.4 (s), 1032.1 (s), 748.0 (s) cm^{-1}

^1H ($CDCl_3$): δ = 13.32-13.28 (1H, m, H_{35}), 7.70-7.15 (17H, m, $H_{14\text{-}17,\ 21,\ 26\text{-}28}$), 6.80 (4H, d, J = 7.9 Hz, H_{22}), 6.71-6.69 (1H, m, H_{10}), 6.39-6.33 (1H, m, H_7), 4.55 (1H, t, J = 7.0 Hz, H_{12}), 4.35 (2H, d, J = 6.6 Hz, H_4), 5.65 (1H, d, J = 6.6 Hz, H_{36}), 4.20 (1H, t, J = 7.4 Hz, H_{30}), 4.05-3.90 (4H, m, $H_{9,\ 38}$), 3.73 (6H, s, H_{24}), 3.40-3.30 (2H, m, H_{34}), 3.17-3.08 (2H, m,

H_6), 3.00 (2H, t, J = 6.6 Hz, H_1), 2.50 (3H, s, H_{51}), 2.30 (4H, s, H_{55}), 1.90-1.10 (14H, m, $H_{2,3,5,31-33}$), 0.95 (6H, s, H_{57})

^{13}C ($CDCl_3$): δ = 173.9 ($C_{58,54}$), 171.8 (C_8), 169.9 (C_{29}), 168.5 (C_{37}), 158.4 (C_{23}), 156.1 (C_{11}), 145.5 (C_{20}), 143.8 ($C_{13,53}$), 141.4 (C_{18}), 136.8 (C_{25}), 130.2, 128.3, 128.0, 127.8, 127.3, 126.7, 125.1, 120.2 ($C_{14-17,21,26-28}$), 113.1 (C_{22}), 108.1 (C_{52}), 85.8 (C_{19}), 67.5 (C_4), 63.4 (C_1), 55.4 (C_{24}), 55.2, 52.9 (C_{55}), 47.2, 44.8, 43.1, 39.8, 31.8 ($C_{6,9,33,34,38}$), 43.3 (C_{12}), 30.3 (C_{56}), 30.1, 29.5 ($C_{2,5}$), 28.4 (C_{51}), 28.1 (C_{30}), 27.0, 26.1 (C_3), 22.8 ($C_{31,32}$), 18.2 (C_{57})



$C_{47}H_{63}N_5O_8$
Mol. Wt.: 826.03
C, 68.34; H, 7.69; N, 8.48; O, 15.50

Glycine-lysine-(Dde) -glycine-6-hydroxy (-*O*-4,4'-dimethoxytrityl)hexylamide [108]

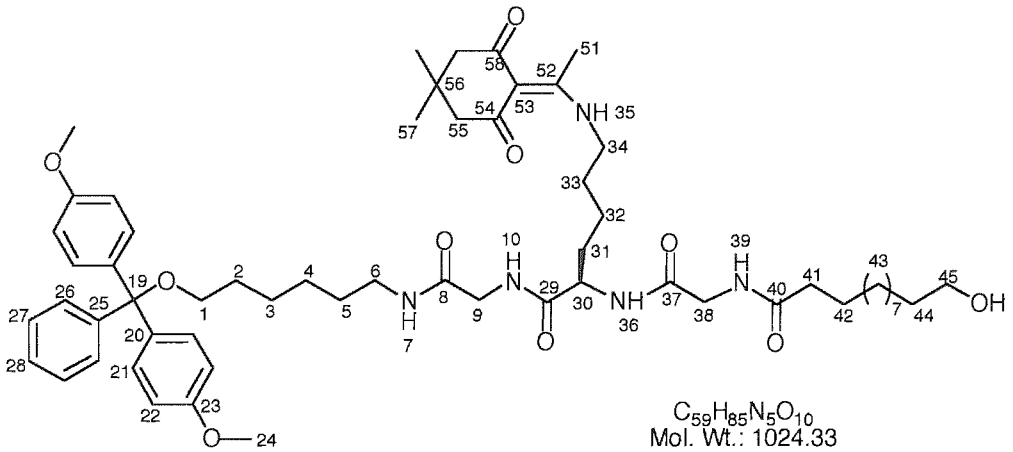
To a solution of piperidine in DMF (20%, 25mL) was added the peptide **[107]** (1.95g, crude) and the mixture stirred under argon, at room temperature for 1.5 hrs. The solvents were removed *in vacuo* and the resulting oil dissolved in CH_2Cl_2 (150mL), washed with sodium bicarbonate solution (150mL), brine (150mL) and dried (Na_2SO_4). After removal of the solvents *in vacuo* an off white foam was obtained which was purified by silica gel column chromatography eluting with MeOH: CH_2Cl_2 (1:20 then 3:20). The white foam produced was dried over P_2O_5 *in vacuo* (433mg, 82%, 29% overall yield of based on last 4 steps).

R_f (D): 0.18; LRMS (ES^+ mode): *m/z* 849.4 (MNa^+), 827.4 (MH^+), 303.4 (DMT^+); HRMS: $C_{47}H_{64}N_5O_8$ requires 826.47549, found 826.47549; λ_{max} (MeOH): 297, 261nm; mp: 74-78°C

IR (CH_2Cl_2): ν = 3274.5 (m), 2934.5 (m), 2361.9 (m), 2360.7 (m), 1627.8 (m), 1571.2 (s), 1507.8 (s), 1245.1 (s), 1032.3 (s), 739.0 (s) cm^{-1}

^1H ($CDCl_3$): δ = 13.12-13.08 (1H, m, H_{35}), 7.96-7.93 (1H, m, H_{10}), 7.40-7.15 (9H, m, $H_{21, 26\text{-}28}$), 6.75 (4H, d, J = 8.3 Hz, H_{22}), 6.55 (1H, t, J = 5.9 Hz, H_7), 4.37-4.25 (2H, m, H_{39}), 3.83-3.78 (2H, m, H_9), 3.75 (6H, s, H_{24}), 3.40-3.30 (3H, m, $H_{6, 36}$), 3.20-3.10 (2H, m, H_{34}), 3.00 (2H, t, J = 6.6 Hz, H_1), 2.45 (3H, s, H_{51}), 2.32 (4H, s, H_{55}), 2.15-2.10 (2H, m, H_{38}), 1.80-1.15 (14H, m, $H_{2, 3, 5, 31\text{-}33}$), 0.93 (6H, s, H_{57})

^{13}C ($CDCl_3$): δ = 173.8 ($C_{58, 54}$), 172.1 (C_{29}), 168.8 (C_{37}), 162.7 (C_8), 158.4 (C_{23}), 145.5 ($C_{20, 53}$), 136.8 (C_{25}), 130.2, 128.3, 127.8, 126.7, ($C_{21, 26\text{-}28}$), 113.1 (C_{22}), 108.0 (C_{52}), 85.8 (C_{19}), 63.4 (C_1), 55.4 (C_{24}), 53.3, 53.0, 43.3, 43.2, 39.8 ($C_{6, 9, 33, 34, 38}$), 31.2 (C_{55}), 30.3 (C_{56}), 30.1, 29.5 ($C_{2, 5}$), 28.4 (C_{51}), 28.1 (C_{30}), 27.0, 26.2 (C_3), 23.1 ($C_{31, 32}$), 18.2 (C_{57})



$C_{59}H_{85}N_5O_{10}$
Mol. Wt.: 1024.33
C, 69.18; H, 8.36; N, 6.84; O, 15.62

12-Hydroxydodecanamido-glycine-lysine-(Dde)-glycine-6-hydroxy (-*O*-4,4'-dimethoxytrityl)hexylamide [109]

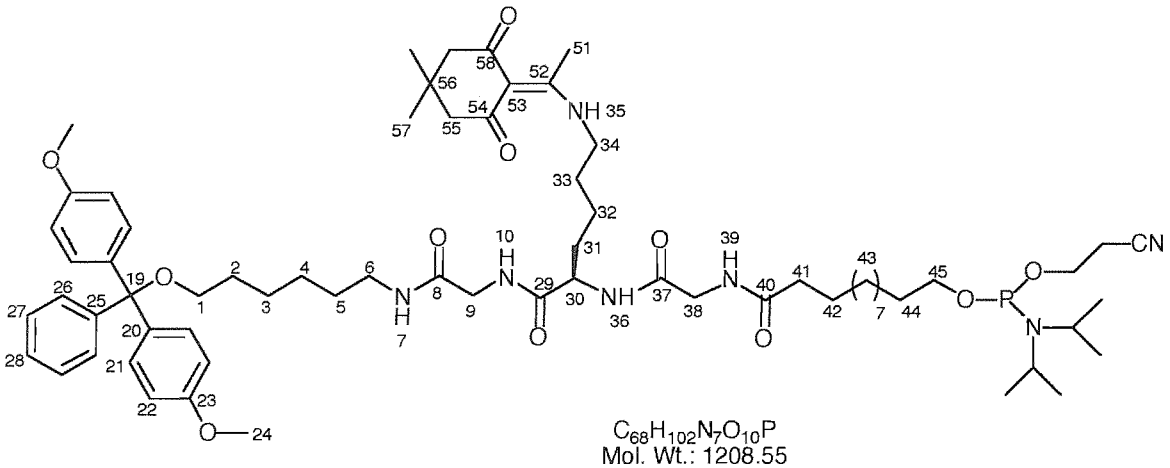
To a solution of the amine **[108]** (0.39g, 470.0μmol) in anhydrous CH_2Cl_2 (10mL) was added 12-hydroxydodecanoic acid (0.10g, 470.0μmol) and EDC (0.11g, 0.6mmol) The solution was stirred at room temperature, under argon, for 2.5 hrs. The reaction was diluted with CH_2Cl_2 (200mL), washed with water (200mL), saturated sodium bicarbonate solution (200mL), brine (100mL) and then dried (Na_2SO_4). Removal of the solvents *in vacuo* produced a yellow oil that was purified by silica gel column chromatography eluting with MeOH: CH_2Cl_2 (1:20 then 1:10) to give the title compound as an off white foam that was dried over P_2O_5 *in vacuo* (0.40g, 0.4mmol, 82%).

R_f (D): 0.86; LRMS (ES^+ mode): *m/z* 1024.8 (M^+), 303.3 (DMT^+); λ_{max} (MeOH): 252nm; mp: 126-130 °C

IR (CH_2Cl_2): ν = 3282.3 (m, br), 2925.4 (m), 2853.3 (m), 2364.0 (w), 1622.6 (s), 1571.4 (s), 1508.9 (s), 1257.7 (s), 1032.1 (s), 750.0 (s) cm^{-1}

^1H ($CDCl_3$): δ = 13.30-13.26 (1H, m, H_{35}), 7.96-7.93 (1H, m, H_{10}), 7.45-7.41 (1H, m, H_{39}), 7.40-7.15 (9H, m, $H_{21, 26-28}$), 6.97-6.91 (1H, m, H_7), 6.75 (4H, d, *J* = 8.8 Hz, H_{22}), 4.00-3.82 (5H, m, $H_{9, 30, 38}$), 3.75 (6H, s, H_{24}), 3.60 (2H, t, *J* = 6.6 Hz, H_{45}), 3.40-3.33 (2H, m, H_{34}), 3.25-3.15 (2H, m, H_6), 3.00 (2H, t, *J* = 6.6 Hz, H_1), 2.55 (3H, s, H_{51}), 2.32 (4H, s, H_{55}), 2.25 (2H, t, *J* = 8.3 Hz, H_{41}), 1.80-1.15 (32H, m, $H_{2-5, 31-33, 42-44}$), 0.93 (6H, s, H_{57})

^{13}C ($CDCl_3$): δ = 174.2, 173.7, 171.7, 169.2, 168.5 ($C_{8, 29, 37, 40, 54, 58}$), 158.4 ($C_{23}$), 145.5 ($C_{20, 53}$), 136.8 ($C_{25}$), 130.1, 128.3, 127.8, 126.7, ($C_{21, 26-28}$), 113.1 (C_{22}), 108.0 (C_{52}), 85.8 (C_{19}), 63.5 (C_1), 63.0 (C_{45}), 55.3 (C_{24}), 52.9, 43.7, 43.3, 39.8 ($C_{6, 9, 34, 38}$), 30.2 (C_{56}), 28.4 (C_{51}), 36.1, 33.2, 32.9, 29.6, 29.5, 29.3, 28.7, 27.2, 26.3, 25.9, 25.7, 22.8 ($C_{2-5, 30, 31-33, 41-44, 55}$), 18.1 ($C_{57}$)



$C_{68}H_{102}N_7O_{10}P$
Mol. Wt.: 1208.55
C, 67.58; H, 8.51; N, 8.11; O, 13.24; P, 2.56

12-(2-Cyanoethyl diisopropylphosphoramidyl)oxy-dodecanamido-glycine-lysine(Dde)-glycine-6-hydroxy (-O-4,4'-dimethoxytrityl)hexylamide [110]

To a solution of the alcohol **[109]** (0.40g, 0.4mmol) in anhydrous CH_2Cl_2 (5mL) was added *N,N*-diisopropylethylamine (265mg, 355μL, 2.3mmol) and 2-cyanoethyl diisopropyl chlorophosphoramidite (91μL, 91mg, 470.0μmol), under argon. After stirring at room temperature for 2.5 hrs the pale yellow solution was diluted with EtOAc (25mL) washed

with saturated potassium chloride solution (30mL) dried (Na_2SO_4), and the solvents removed *in vacuo*. Anhydrous CH_2Cl_2 (3mL) was added and this solution dropped slowly onto rapidly stirring hexane (-20°C, 250mL) forming a white precipitate. The hexane was removed, with filtration, through glass wool. The residue was washed with cold hexane (150mL) and then dissolved in CH_2Cl_2 (5mL), passed through the glasswool and the solvents removed *in vacuo*. Anhydrous MeCN was added (10mL), the solution filtered, the solvents removed *in vacuo* and dried over P_2O_5 for 24 hrs (0.43g, 360.0μmol, 91%).
R_f (D): 0.93, 0.89; LRMS (ES^+ mode): *m/z* 1247.0 (MK^+), 1165.0 (M-$CH(CH_3)_2$), 1123.8 (M-2$CH(CH_3)_2$); λ_{max} (MeOH): 296, 368nm
^{31}P ($CDCl_3$): δ = 147.92, 147.80

Silver oxide catalyst[206]

A solution of silver nitrate (3.00g, 17.7mmol) in water (30mL) was warmed to 85°C. A second solution of sodium hydroxide (0.69g, 11.3mmol) in water (30mL) warmed to 85°C and added to the silver nitrate solution. The mixture was shaken vigorously for 5 minutes and a dark brown precipitate of silver oxide formed. In the absence of light, the solid was removed by filtration, washed with hot water (5 x 20mL), Et_2O (3 x 20mL), dried *in vacuo* and stored in the dark for immediate use.

Silver silicate catalyst[157]

Procedure was carried out in the absence of light. Neutral alumina (15.00g) was suspended in a solution of silver nitrate (17.09g, 0.10mol) in water (100mL). A solution of sodium silicate (47.30g) in water (100mL) was added dropwise with stirring over 1 hr. The resulting mixture was heated (70°C) for 15 minutes with stirring, cooled to room temperature, filtered, and the residue washed with water (2 x100mL). The residue was stirred in a silver nitrate solution (10% w: v, 100mL) at 70°C for 0.5 hr. The solution was allowed to cool, washed with anhydrous acetone (2 x 100mL), toluene (2 x 100mL) and dried *in vacuo* (60°C, 3hrs). The powder was dried over P_2O_5 for 24 hrs, affording the silver silicate catalyst as a pale yellow powder (40g).

5.2 Preparation of Resins

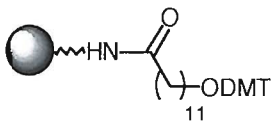
5.2.1 General Methods

Resins were supplied by Novabiochem, Link Technologies, Rapp Polymere and Argonaut Technologies. All other reagents were obtained from Aldrich/Sigma or Lancaster. Solvents were distilled as described previously. All glassware used was pre-soaked in trimethylsilyl chloride: CH_2Cl_2 (1:20) for 0.5 hour, rinsed thoroughly with CH_2Cl_2 and oven dried.

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

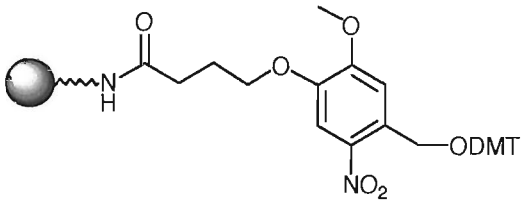
Synthesiser columns for packaging of the resins were obtained from Glen Research.

5.2.2 Resin Derivatisation



12-*O*-(4,4'-dimethoxytrityloxy)dodecanamide resins [12, 45-48]

The resin was washed thoroughly with anhydrous *N,N*-diisopropylethylamine in CH_2Cl_2 (1%, x 5) and dried. 12-*O*-(4,4'-Dimethoxytrityloxy)dodecanoic acid **[11]** was dissolved in a minimum amount of anhydrous *N,N*-diisopropylethylamine in CH_2Cl_2 (1%), HOBT (5eq), followed by DIC (5eq) were then added to this solution. The resin was soaked with this solution, and sufficient *N,N*-diisopropylethylamine in CH_2Cl_2 (1%) was added to cover the resin. The resin was agitated gently with a stream of nitrogen or argon gas for 1 hr. The resin was filtered, washed with *N,N*-diisopropylethylamine in CH_2Cl_2 (1%, x 5), CH_2Cl_2 (x 3), Et_2O (x 5) and dried *in vacuo*. A small sample of resin was removed to ascertain the loading (section 5.2.2.1). The remainder was soaked in a capping solution of acetic anhydride: 2,6-lutidine in THF: 1-methylimidazole in THF (1:1) for 1 hour. The resin was dried over P_2O_5 *in vacuo* and a small sample used to verify the loading.



UV Linker resin [66]

The LCAA-CPG (250mg) was washed thoroughly with anhydrous *N,N*-diisopropylethylamine in CH_2Cl_2 (1%, x 5) and dried. **[65]** (25mg, 40.0μmol) was dissolved in a minimum amount of anhydrous *N,N*-diisopropylethylamine in CH_2Cl_2 (1%), HOBT (10.1mg, 75.0μmol), followed by DIC (9.5mg, 750.0μmol) were then added to the solution. The resin was soaked with this solution, and sufficient *N,N*-diisopropylethylamine in CH_2Cl_2 (1%) added to cover the resin. The resin was agitated gently with a stream of nitrogen or argon gas for 2 hrs. The resin was filtered, washed with *N,N*-diisopropylethylamine in CH_2Cl_2 (1%, x 5), CH_2Cl_2 (x 3), Et_2O (x 5) and dried *in vacuo*. A small sample of resin was removed to ascertain the loading (section 5.2.2.1). The remainder was soaked a capping solution of acetic anhydride: 2,6-lutidine in THF: 1-methylimidazole in THF (1:1) for 1 hour. The resin was dried over P_2O_5 *in vacuo* and a small sample was used to verify the loading.

5.2.2.1 Determination of resin loading by trityl analysis

A sample of resin was weighed and suspended in hydrochloric acid: EtOH (3:2 v: v, 25mL) and shaken for 5 minutes. The absorbance of 1mL of the orange solution was measured at 495nm on a Perkin Elmer UV/VIS spectrometer. The amount of 4,4'-dimethoxytrityl cation present was calculated:

Absorbance for 1mL (at 495nm)		= **a**
For 25mL	= **a** × 25	= **b**
[The absorbance of 1μmol of DMT^+ at 495nm in = 71.7]		
For **c** g of resin	= **b** ÷ 71.7	= **d**μmol
Resin Loading (amount of DMT^+ present)	= **d** ÷ **c**	= **e μmolg^{-1}**

Example:

Absorbance for 1mL (at 495nm)		= 0.8897
For 25mL	= 0.8897 × 25	= 22.2417
[The absorbance of 1 μmol of DMT^+ at 495nm in = 71.7]		
For **c** g of resin	= 22.2417 ÷ 71.7	= 0.31 μmol
Resin Loading (amount of DMT^+ present)	= 0.31 ÷ 0.0086	= **36.47 μmolg^{-1}**

Cpd No.	Batch No.	Resin Type	Supplier	Resin (g)	DMT cpd (μmol)	Loading (μmolg^{-1})
12	1	LCAA-CPG	Link Technologies	2.16	600	36.47
45	1	Polystyrene	Link Technologies	0.05	390	48.89
45	2	Polystyrene	Link Technologies	0.05	96	10.02
45	3	Polystyrene	Link Technologies	0.1	390	34.00
45	4	Polystyrene	Link Technologies	0.07	200	52.00
46	1	Tentagel	NovaBiochem	1	240	67.00
47	1	Tentagel	Rapp Polymere	0.5	270	57.60
48	1	Macropore	Argonaut Technologies	0.5	0.27	46.20
66	1	LCAA-CPG	Link Technologies	0.25	0.04	22.00

5.3 Preparation of Synthetic Oligonucleotides

5.3.1 General Methods

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

Cleavage of the oligonucleotides from the solid support was achieved by exposure to concentrated ammonia, unless otherwise stated. All oligonucleotides requiring base deprotection were placed in concentrated ammonia or ammonia: ethanol (3:1) at 60°C for 4 hours and evaporated to dryness *in vacuo*

Purification was effected using reversed phase HPLC on a Gilson RPHPLC system, controlled by Gilson 7.12 software. Elution of oligonucleotides was monitored by UV detection at desired wavelengths (260-290nm). Buffer systems consisted of buffer A: 0.1M ammonium acetate), buffer B: acetonitrile: buffer A (4:6 or 3:7), with a typical gradient programme as described in figure 5.1 Analytical injections were monitored at 265nm, 0.1 AUFS, preparative injections were monitored at 280-290nm, 1.0 AUFS. The oligonucleotides were collected manually.

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

The resins were packaged into Glen Research columns suitable for an ABI 394 DNA synthesiser. The amount of resin used was determined by the resin loading and the scale of the synthesis (0.2μmol or 1.0μmol).

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

Figure 5. 1 Typical RPHPLC elution gradient

5.3.2 Oligonucleotide Sequences

Modifications:

F = fluorescein phosphoramidite
X = hexachlorinated fluorescein phosphoramidite
M = methyl red phosphoramidite
N = aminolink phosphoramidite
H = hexaethylene glycol phosphoramidite
D = **[10]** (disiloxyl phosphoramidite)
B = **[25]** (*bis*-(β-gal)-FAM phosphoramidite)
S = **[78]** (β-gal linker phosphoramidite)
P_1 = **[85]** (Phe linker 1 phosphoramidite)
P_2 = **[100]** (Phe linker 2 phosphoramidite)
K = **[110]** (Lys linker phosphoramidite)
R_1 = **[12]** CPG-LCAA resin
R_2 = **[45]** polystyrene resin
R_3 = **[46]** TentaGel™ resin
R_4 = **[47]** TentaGel™ resin
R_5 = **[48]** macropore resin
R_6 = **[66]** UV linker resin
R_7 = CPG (standard succinyl linker) resin

No.	Sequence	Molecular ion	
		Expected	Found
13	TTTTTTTTTTTTDR$_1$	———	———
17	NTTTTTTTTTTTT	3784.6	3782.9
26	TTTTTTTTTTTTTT	4500.9	4501.0
27	BTTTTTTTTTTTTTT	5360.9	5361.0
28	NCATCTGAACTTTGCCAGCTT	6222.2	6223.0
31	GATCTGACTTGGTAGHN	5174.0	5173.9
33	BHACGATGCGCCGATCG	5781.2	5778.8
34	CGATCGGCGCATCGTCTACCAAGTCAGATC	9147.6	9143.9
35	BHCTACTGCGCCGATCG	5732.1	5730.5
36	CGATCGGCGCACAAGTCAGATC	6726.2	6723.6
37	GATCTGACTTGGTAGACTATGCGCCGATCGN	10480.1	10485.4
38	GATCTGACTTGGTAGACTATGCGCCGATCG	9238.0	9242.0
39	CGATCGGCGCATAGTCTACCAAGTCAGATC	9176.0	9181.0
40	CGCACGCGATCGGCGCATAGTCTACCAAGTCGATCCGTGCGM	13971.4	13975.0
41	BHHTATTAAGCCTGCAAG	6123.3	6123.1
42	CTTGCAGGCTTCATACGATCGGCGCATAGTCTACCAAGTCAGATC	13798.3	13800.2
43	BHHCGATCGGCCTGCAAG	6125.4	6126.0

44	CTTGCAGGCGCGCATAGTCTACCAAGTCAGATC	10093.7	10095.0
49	FCGCACGCTTAAAGTCACTTCATTTTCGTGCGM MHHHR$_1$	_____	_____
50	CGAGATACGGTTTTCACAGCHHHHHHHHR$_2$	_____	_____
51	CGAGATACGGTTTTCACAGCR$_3$	_____	_____
52	CGAGATACGGTTTTCACAGCR$_4$	_____	_____
53	AAAATGAAGTGACTTTAAG	5881.1	5881.5
54	GCTGTGAAAACCGTATCTCGM	6213.0	6210.9
55	FCGCACGATGTAGCACATCAGAAGCGTGCGTM MHHHR$_1$	_____	_____
56	CTTCTGATGTGCTACAT	5151.4	5151.0
57	FCGCACGCTTAAAGTCACTTCATTTTCGTGCGM HHHR$_2$	_____	_____
58	FCGCACGCTTAAAGTCACTTCATTTTCGTGCGM HR$_2$	_____	_____
59	FCGCACGCTTAAAGTCACTTCATTTTCGTGCGM HHHHHR$_2$	_____	_____
60	FCGCACGCTTAAAGTCACTTCATTTTCGTGCGM HHHHHHHHHHHHR$_2$	_____	_____
61	FCGCACGCTTAAAGTCACTTCATTTTCGTGCGM HR$_4$	_____	_____
62	FCGCACGCTTAAAGTCACTTCATTTTCGTGCGM HR$_5$	_____	_____
63	TTTTTTTTTTTTTTTTTTR$_6$	_____	_____
64	CGAGATACGGTTTTCACAGCR$_6$	_____	_____

67	GCCTCCAGCTTAGTTMMHHR$_2$	———	———
68	FAACTAAGCTGGAGGCACGTACGA	7643.1	7640.1
69	TATGATCGTACGTGCCTCCAGCTTAGTTTTCTC	10031.7	10033.4
79	FSTTTTTTTTTTTT	5379.7	5379.0
80	FSSTTTTTTTTTTTTTT	5722.0	5726.0
86	XP$_1$TTTTTTTTTTTTTT	5771.8	5772.0
87	XHP$_1$HTTTTTTTTTTTTTT	6460.3	6459.9
88	CGTGACATCTGCAGTCP$_1$DR$_1$	———	———
89	FCAATGTTP$_1$TTCATTGM	5488.1	5380.0
101	FTTTTTTP$_2$TTTTTT	4763.1	4763.0
102	FP$_2$TTTTTT	2940.4	2940.0
103	FP$_2$TTTTTTR$_2$	———	———
104	TP$_2$HHTR$_7$	———	———
111	TTTTTTLTTTTTTTTTTTTTTT	7248.1	7251.1
112	TTCTTCTTLTCCTCTTCTTCTCCTTT	8025.6	8027.0
113	TLHHTR$_7$	———	———
114	TTGAAAGGCCP$_2$GCTCCAGGGT	6796.8	6795.6
115	TTGAAAGGCCLGCTCCAGGGT	6775.8	6778.0

5.4 Molecular Biology

5.4.1 General Methods

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

Fluorescence microscopy was performed using a LEITZ DM IL microscope and photographed with a ASHAI PENTAX camera using Kodak Ektachrome colour film 160T, 36exp obtained from Sigma/Aldrich Techware. Fluorescent spectra were measured on a Perkin Elmer LS50B Luminescence Spectrometer using quartz glass 3mL and 0.4mL cells.

Irradiation of samples was performed on a transilluminator platform at 260nm. Ultraviolet spectra and thermal melting experiments were carried out on a Perkin Elmer UV/Vis Lambda 2 spectrometer with PTP-1 temperature programmer.

Agarose gel electrophoresis was performed using a Hoefer 250 Power supply and mini gel equipment. Polyacrylamide gel electrophoresis on a Hoefer vertical electrophoresis sturdier SE-410 apparatus and power supply.

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

Centrifugation was performed in a microcentrifuge at 13,000 rpm for 30 minutes.

Ion exchange and gel filtration chromatography were performed on a Pharmacia LKB system and elution was monitored at 260/280nm

5.4.2 Experimental

Cleavage of oligonucleotides from the solid support using 3HF•Et_3N: NMP: Et_3N[201]

3HF•Et_3N: NMP: Et_3N (2: 3: 1.5, 20mL) was prepared and sealed under argon. This solution (200μL) was added to the dry resin sample (4mg) in a screw top vial. The resin solution was heated to 65°C for 1.5 hrs and allowed to cool to room temperature. The solution was then removed from the resin by filtration and the resin was washed with water (5 x 2mL). The HF solution and aqueous washings were combined and the solvents were removed *in vacuo*. The sample was dissolved in water (1mL) and loaded onto a NAP 10 sephadex column, elution was effected with water (1.5mL).

Cleavage of oligonucleotides from the solid support using 1.0M TBAF/THF

TBAF in THF (1.0M, 200μL) was added to the dry resin in a screw top vial and allowed to stand, at room temperature, for 4 hrs. The solution was then removed from the resin by filtration and the resin was washed with water (5 x 2mL). The fluoride solution and aqueous washings were combined and the solvents were removed *in vacuo*. The sample was dissolved in water (1mL), and loaded onto a NAP 10 sephadex column; elution was effected with water (1.5mL).

UV thermal melting of oligonucleotides

The two single stranded oligonucleotides to be melted were mixed (1:1, total 0.5OD) and diluted with buffer (sodium phosphate 10mM, EDTA 1mM, NaCl 0.1M or 1.0M, pH 7.0). The solution in an UV cuvette (pathlength 10mm) was heated to 80°C and cooled slowly to room temperature to effect efficient strand annealing. The sample was melted at a heating rate of 1° per minute, monitoring at 260nm on a PC with 10s intervals. The data curves were smoothed and the first derivatives obtained using the Perkin Elmer PECSS2 software.

The time peaks were converted to temperature manually, in order to ascertain Tm values. The melt process was repeated to acquire consistent results (± 0.25°C).

Preparation of oligonucleotides for electrospray mass spectroscopy

Method 1

The oligonucleotide sample was dissolved in ammonium acetate (5M, 100μL) and allowed to stand at room temperature for 1 hr. The oligonucleotide was then precipitated with cold EtOH (300μL) and placed in a MeOH/dry ice bath for 2 hrs. The solution was centrifuged at 14000g for 0.5hr, the supernatant removed, washed with cold EtOH (80%), centrifuged again at 14000g for 15 minutes and the supernatant removed. The sample was dissolved in a solution of water: isopropanol (1:1) containing tripropylamine (1%) for electrospray mass spectral analysis.

Method 2

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

β-D-Galactosidase assay for generation of fluorescence with $(\beta\text{-gal})_2$Fam labelled oligonucleotides

The activity of β-D-Galactosidase is defined as one unit causes the hydrolysis of one micromole of *o*-nitrophenol-β-D-galactopyranoside per minute at 25°C and pH 7.5.
The oligonucleotide substrate in buffer (1.5 OD, sodium phosphate 0.3M, magnesium chloride 0.003M, pH 7.5, 500μL) was placed in a cuvette (1mL, 10mm pathlength) and allowed to equilibrate at 37°C for 5 minutes. β-D-galactosidase (500μL of a 0.1mg mL^{-1} solution in the same buffer) was added to the cuvette and the reaction was monitored immediately at 37°C in UV/Vis spectrometer at 490nm, for 1 hr.

Preparation of an MBS derivatised oligonucleotide

A 3' amino-derivatised oligonucleotide was prepared by standard DNA synthesis (1.0μmol scale) and purified (RPHPLC). The oligonucleotide was desalted twice through a NAP 10 sephadex column (loaded in water (1.0mL), eluted with water (1.5mL)). The water was removed *in vacuo* and the oligonucleotide dissolved in sodium carbonate buffer (0.1M, pH 6.00, 50μL) and transferred to a vial (3mL). The flask was rinsed with sodium carbonate buffer (2 x 25μL) and added to the vial. A solution of MBS (5mg) in DMSO (120μL) was prepared and an aliquot (15μL) was added to the oligonucleotide. The solution was vortexed until almost clear. The reaction was allowed to stand, at room temperature, for 4 hrs after which a further aliquot of the MBS solution (15μL) was added. The solution was left overnight, at room temperature, in the dark. The reaction was diluted with water (870μL) and desalted through NAP 10 sephadex eluting with water (1.5mL). The resulting oligonucleotide was purified by RPHPLC; the MBS derivatised oligonucleotide was collected and analysed by electrospray mass spectrometry.

Conjugation of β-D-galactosidase with an MBS derivatised oligonucleotide

An MBS derivatised oligonucleotide was dried *in vacuo*. A solution of β-D-galactosidase (1mg) in potassium phosphate buffer (0.1M, pH 5.6, 500μL) was added to the oligonucleotide. The clear solution was allowed to stand, at room temperature, in the dark, overnight. The reaction was diluted with potassium phosphate buffer (0.1M, pH 5.6, 500μL) and purified by gel filtration eluting isocratically with potassium phosphate buffer (0.1M, pH 5.6). Detection of the peaks was achieved by monitoring the absorbance at 260/280nm. The major conjugate peak was collected, the solvents were removed *in vacuo*. The residue was dissolved in water (1.0mL) and desalted through NAP 10 sephadex eluting with water (1.5mL). The conjugate was purified by ion-exchange chromatography eluting with an increasing gradient of buffer B [buffer A: Tris.HCl (50.0mmol), pH 7.4, buffer B: buffer A, NaCl (1.0M), pH 7.4)]. Detection of the peaks was achieved by monitoring the absorbance at 260/280nm. All the major conjugate peaks were collected and assayed for β-D-Galactosidase activity and the presence of oligonucleotides (absorbance at 260nm).

Activity assay of β-D-galactosidase conjugate[207]

Assay buffer	sodium phosphate (0.3M), magnesium chloride (0.003M), pH 7.5
Substrate solution	*o*-nitrophenol-β-D-galactopyranoside (1mg) in assay buffer (1mL)
Enzyme conjugate solution	fraction from ion exchange chromatography (50μL)

The assay buffer (900μL) was placed into a cuvette (pathlength 10mm) and the enzyme conjugate (50μL) was added and shaken. The cuvette was placed in a UV/Vis spectrometer at 37°C and the absorbance measured at 405nm. The cuvette was removed from spectrometer and the substrate added (50μL), shaken and quickly placed back in the spectrometer. The absorbance at 405nm at 37°C was monitored for 5 minutes. The final colour of the solution and its absorbance at 405nm were recorded.

Testing homogeneous assay (as described section 2.3.7 and 2.3.12)

Signal probe and native β-galactosidase

The signal probe (**[33]**, **[41]** or **[43]**, 0.187nmol) in buffer (0.3M sodium phosphate, 0.003M $MgCl_2$, 0.1M, NaCl, pH 7.5, 350μL) was placed in a fluorimeter cuvette, and incubated at 37°C for 10mins. The fluorescence intensity was noted and β-D-galactosidase (2μL, 1mg mL^{-1} solution) was added to the cuvette. The change in fluorescence intensity was monitored over 1 hour at 37°C.

Signal probe and β-D-galactosidase conjugate

The signal probe (**[33]**, **[41]** or **[43]**, 0.187nmol) in buffer (0.3M sodium phosphate, 0.003M $MgCl_2$, 0.1M, NaCl, pH 7.5, 300μL) was placed in a fluorimeter cuvette, and incubated at 37°C for 10mins. The fluorescence intensity was noted. The β-D-galactosidase conjugate **[32]** or **[37]** was evaporated to dryness and dissolved in the same buffer (**[32]** or **[37]**, 0.187nmol, 50μL) then added to the cuvette. The change in fluorescence intensity was monitored over 1 hour at 37°C.

Signal probe and β-D-galactosidase conjugate and target

The signal probe (**[33]**, **[41]** or **[43]**, 0.187nmol) in buffer (0.3M sodium phosphate, 0.003M $MgCl_2$, 0.1M, NaCl, pH 7.5, 300μL) was placed in a fluorimeter cuvette, with the complementary target (**[34]**, **[42]** or **[44]**, 0.935nmol). The solution was heated to 70°C and cooled slowly to room temperature. The solution was incubated at 37°C for 10mins and the fluorescence intensity noted. The β-D-galactosidase conjugate **[32]** or **[37]** was evaporated to dryness, then dissolved in the same buffer (**[32]** or **[37]**, 0.187nmol, 50μL) and added to the cuvette. The change in fluorescence intensity was monitored over 1 hour at 37°C.

Dot blot assay

24 x SSC buffer	sodium chloride (3.6M), trisodium citrate (360.0mmol)
Denhardt's solution	bovine serum albumin (0.01% w:v), Ficoll (0.01% w:v), polyvinylpyrrolidone (0.01% w:v)
Prehybridisation solution	1x Denhardt's solution, SDS (0.1% w:v), 6x SSC, denatured herring sperm DNA ($10\mu gmL^{-1}$)
Hybridisation solution	SDS (0.01%), 6x SSC, 1x Denhardt's solution, β-D-galactosidase conjugate ($50ngmL^{-1}$)
Assay Buffer	sodium phosphate (0.3M), magnesium chloride (0.003M), pH 7.5

Serial dilutions, using water, of an oligonucleotide stock solution were performed producing six oligonucleotide solutions: 10pmol, 2pmol, 0.4pmol, 0.08pmol, and 0.016pmol. A piece of membrane (Hybond N, Amersham) was cut (5 x 2 cm) and marked into squares (1 x 1cm). Each solution was spotted onto a separate square. The DNA was crosslinked to the membrane by exposure to ultraviolet irradiation for 5 minutes at 260nm, on a transilluminator platform. Prehybridisation was achieved by sealing the membrane in a plastic bag with prehybridisation solution at 42°C for 0.5 hr. This solution was replaced with the hybridisation solution (containing the complementary target oligonucleotide) and left in the sealed bag at 42°C, in the dark, for a further 0.5 hr. The membrane was washed thoroughly with 6x SSC buffer, followed by PBS (200mL). The membrane was placed in

the assay buffer and a solution of xGal (100μL) was added, the membrane was observed for any colour changes over a 24hr period.

SDS page polyacrylamide gel electrophoresis of β-D-galactosidase conjugate[208]

Resolving gel	acrylamide (30% solution w:v, 1.5mL), Tris.HCl (1M, pH 8.8, 2.24mL), water (2.24mL), SDS (10% solution w:v, 60μL), TEMED (5μL), AMPS (10% solution w:v, 50μL)
Stacking Gel	acrylamide (30% solution w:v, 200μL), Tris.HCl (1M, pH 6.8, 250μL), water (1.5mL), SDS (10% solution w:v, 20μL), TEMED (3μL), AMPS (10% solution w:v, 20μL)
Running Buffer	Tris.HCl (0.025M), glycine (0.192M), SDS (0.1%), water
Loading Buffer	Tris.HCl (50.0mmol, pH 6.8), SDS (2%), DTT, glycerol (20%), bromophenol blue (0.01%)
Gel fix solution	MeOH (50%), acetic acid (12%), water
Sodium thiosulphate solution	0.2g/L
Silver nitrate solution	2g/L, formaldehyde (37% solution, 0.75mL/L)
Developing solution	sodium carbonate (60g/L), formaldehyde (37% solution, 0.5mL/L, sodium thiosulphate (4mg/L)

A resolving gel was poured between two glass plates, covered with a thin layer of water and left for 15 minutes. The gel was washed with water and the stacking gel poured on, the comb was added and the gel allowed to set for 20 minutes. The comb was removed, the gel placed in the electrophoresis equipment and covered with the running buffer. The samples: molecular markers in loading buffer (2μL), β-D-galactosidase (1mg/mL solution, 1μL in 9μL loading buffer, 2μL), conjugate (6μL in 6μL loading buffer, 5μL) were prepared by heating to 95°C for 5 minutes to allow denaturation. The samples were loaded into the lanes of the gel. The voltage was set to 80 volts ($8Vcm^{-1}$) for the first 20 minutes allowing

the samples to migrate through the stacking gel, and then raised to 150 volts for 2 hours. The gel was removed from the plates and soaked in gel fix solution (200mL).

Silver staining

The gel was washed twice with EtOH (50%) and once with EtOH (30%) for 20 minutes each, and then submerged in sodium thiosulphate solution for exactly 1 minute. Rinsing with water (x 3) for 20 seconds each was followed by incubation with the silver solution for 20 minutes at room temperature, then the gel was washed with water (x 3) and then placed in the developing solution, at room temperature, for 10 minutes. The gel was rinsed with water (x 2) for 2 minutes each, and then placed in gel fix solution for 10 minutes. Finally the gel was washed with MeOH (50%), MeOH (30%, 3% glycerol) and glycerol (3%) each for 20 minutes. The gel was dried overnight.

Annealing molecular beacon to β-D-galactosidase conjugate

The molecular beacon oligonucleotide (6μL, 0.045OD, **[40]**) was placed in a fluorimeter cuvette (3mL) in buffer (sodium phosphate 10mM, EDTA 1mM, NaCl 0.1M or 1.0M, pH 7.0, 3mL). The oligonucleotide was heated and cooled between 20 to 70°C over time and respective increases and decreases in fluorescence intensity monitored. A solution of β-D-galactosidase conjugate in water, was evaporated to dryness and dissolved in the same buffer (0.108OD, 2.4eq, 5μL) and added to the cuvette at room temperature. The cuvette was heated to 70°C over time and the fall in fluorescence intensity monitored as the solution cooled to 20°C over time.

3% Agarose gel electrophoresis (mini gel)

A solution of agarose (1.50g) in TBE buffer (1x, boric acid (1.10g), Trizma (2.40g), EDTA (0.19g) water (200mL), 50mL) was heated to boiling in a microwave oven for 4 minutes to give a clear colourless viscous solution. The solution was allowed to cool (50°C), SYBR™ green stain II (4μL) was added and the solution was poured slowly into the electrophoresis apparatus. The gel was left to set (20 minutes). The comb was removed and the gel covered in TBE buffer (200mL, with SYBR green stain 20μL). Each sample (5μL) was diluted with

a loading buffer (Tris.HCl (50.0mmol, pH 6.8), SDS (2%), DTT, glycerol (20%), bromophenol blue (0.01%), 1μL) and loaded onto the gel. The gel was exposed to a current of 100mA, 100V. When the bands had migrated by the required distance, the current was removed and the gelwas visualised on a transilluminator platform at 260nm.

Chymotrypsin cleavage assay

The activity of chymotrypsin is defined as; one unit causes the hydrolysis of one micromole of benzoyl-L-tyrosine ethyl ester (BTEE) per minute at 25°C at pH 7.5.

Resin cleavage- method 1

The resin (1-2mgs) was placed in an UV cuvette in buffer (1mL, Tris.HCl buffer 0.08M, calcium chloride 0.1M, pH 7.8) and shaken. After equilibration at 37°C for 5 minutes, chymotrypsin (100 μL of a 1mg mL^{-1} solution) was added and the cuvette shaken and placed in a UV/Vis spectrometer and the resin allowed to settle. The solution was monitored at 37°C at either 260nm or 490nm, according to the oligonucleotide released, for 1 hr.

Resin cleavage- method 2

The resin containing a trityl ON oligonucleotide (5.8mgs, **[104]**) was placed in an eppendorf in buffer (0.5mL, Tris.HCl buffer 0.08M, calcium chloride 0.1M, pH 7.8) and shaken. After equilibration at 37°C for 5 minutes, chymotrypsin (100 μL of a 1mg mL^{-1} solution) was added. The solution was incubated at 37°C in for 4 hrs. The resin was removed, washed thoroughly and dried. The resin was analysed for trityl content (see section 5.2.2.1)

Oligonucleotide cleavage (substrate limiting)

Into six tubes was placed oligonucleotide substrate (0.118OD, 5.92μg, 1.25nmol) in buffer (Tris.HCl buffer 0.08M, calcium chloride 0.1M, pH 7.8, 100μL). After equilibration at 37°C for 5 minutes, chymotrypsin (100 μL of a 1mg mL^{-1} solution in buffer) was added to each tube. After allotted periods of time (10mins, 0.5hr, 1hr, 3hrs, 4hrs, 19hrs) each tube was removed, placed at 95°C for 5 minutes, and the contents diluted with water (400μL)

and transferred to a Micron™ filter device (Mol wt cut off = 18,000) and centrifuged at 13,000 rpm for 0.5hrs. The resulting filtrate was diluted with water (0.7mL) and analysed by analytical RPHPLC.

Oligonucleotide cleavage (1:1 substrate: enzyme)

Into four tubes was placed oligonucleotide substrate (0.059OD, 2.96μg, 62nmol) in buffer (Tris.HCl buffer 0.08M, calcium chloride 0.1M, pH 7.8, 50μL). After equilibration at 37°C for 5 minutes, chymotrypsin (0.62×10^{-3} units, 1.25 μL of a 0.1mg mL^{-1} solution in buffer) was added to each tube. After allotted periods of time (1min, 10mins, 1hr, 4hrs) each tube was removed placed at 95°C for 5 minutes, diluted with water (400μL), transferred to a Micron™ filter device (Mol wt cut off = 18,000) and centrifuged at 13,000 rpm for 0.5hrs. The resulting filtrate was diluted with water (0.7mL) and analysed by analytical RPHPLC.

Removal of Dde lysine side-chain protection from resin-bound oligonucleotide

Hydrazine monohydrate in DMF (2%, 200μL) was added to the dry resin in a screw top vial and allowed to stand, at room temperature, for 10mins. The solution was then removed from the resin by filtration and a fresh portion of the hydrazine reagent (2%, 200μL) added to the resin and left standing for a further 10mins. The solution was removed from the resin by filtration and the resin washed with DMF (5 x 2mL) and Et_2O (3 x 5mL). The resin was dried *in vacuo*.

Trypsin cleavage assay

Resin cleavage

The resin (2.8mgs, **[113]**) was exposed to a solution of hydrazine monohydrate: DMF (1:50) for 15 minutes, washed thoroughly and dried. The resin was placed in an eppendorf in buffer (HEPES 0.1M, calcium chloride 0.5M, pH 7.30, 5mL) and shaken. After equilibration at 37°C for 5 minutes, trypsin (100 μL of a 1mg mL^{-1} solution in same buffer) was added. The solution was incubated at 37°C in for 4 hrs. The resin was removed, washed thoroughly and dried. The resin was analysed for trityl content (see section 5.2.2.1).

Oligonucleotide cleavage (substrate limiting)

Into six tubes was placed oligonucleotide substrate (0.616OD, 25.6μg, 3.54nmol) in buffer (HEPES 0.1M, calcium chloride 0.5M, pH 7.3, 100μL). After equilibration at 37°C for 5 minutes, trypsin (100 μL, of a 1mg mL^{-1} solution in buffer) was added to each tube. After allotted periods of time (10mins, 0.5hr, 1hr, 3hrs, 4hrs, 19hrs) each tube was removed placed at 95°C for 5 minutes and the contents diluted with water (400μL), transferred to a Micron™ filter device (Mol wt cut off = 18,000) and centrifuged at 13,000 rpm for 0.5hrs. The resulting filtrate was diluted with water (0.7mL) and analysed by analytical RPHPLC.

Oligonucleotide cleavage (1:1 substrate: enzyme)

Into four tubes was placed oligonucleotide substrate (0.616OD, 25.6μg, 3.54nmol) in buffer (HEPES 0.1M calcium chloride 0.5M, pH 7.3, 100μL). After equilibration at 37°C for 5 minutes, trypsin (3.54×10^{-3} units, 1.2μL, of a 0.01mg mL^{-1} solution in buffer) was added to each tube. After allotted periods of time (1min, 10mins, 1hr, 4hrs) each tube was removed, placed at 95°C for 5 minutes, diluted with water (400μL), transferred to a Micron™ filter device (Mol wt cut off = 18,000) and centrifuged at 13,000 rpm for 0.5hrs. The resulting filtrate was diluted with water (0.7mL) and analysed by analytical RPHPLC.

Annealing of solid supported molecular beacons with their solution targets

A molecular beacon oligonucleotide was assembled (standard phosphoramidite DNA synthesis (0.2μmol scale)) on resin (**[12]**, **[45]-[48]**) The resin was placed in concentrated ammonia: EtOH (4:1), at 55°C for 4 hours, removed, washed thoroughly with Et_2O (x 3) and allowed to dry. A target solution oligonucleotide (100μL, excess) was evaporated to dryness and dissolved in buffer (Na_2HPO_4/NaH_2PO_4 10.0mmol, EDTA 1.0mmol, NaCl 1M, pH 7.0, 50μL). In three separate wells was placed (i) dry resin (ii) resin and buffer (iii) resin and target in buffer. The plate was left for one hour, viewed and photographed under a fluorescence microscope.

Chapter 6

Publication

The following published papers were included in the bound thesis. These have not been digitised due to copyright restrictions, but the links are provided.

Brown, Lynda J., Cummins, Jon, Hamilton, Alan and Brown, Tom (2000)

Molecular Beacons Attached to Glass Beads Fluoresce upon Hybridisation to Target DNA

Chemical Communications : Vol. *7, pp.621-622*

http://dx.doi.org/10.1039/b000389l

Chapter 7

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7.0 References

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