Non-radioactive Labelling of Oligonucleotides

by

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To
My parents for their support,
Anne for encouragement,
but most especially to my late grandfather,
Walter Burns,
a man with great ability but never the opportunity.
Acknowledgements

I wish to thank:

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Abstract

Non-radioactive labelling of oligonucleotides now presents biomedical research with considerable advantages over conventional radioactive labelling techniques. Highly sensitive detection of oligonucleotide probes can be achieved by the addition of non-radioactive reporter groups, thereby precluding the hazardous, expensive and time-consuming introduction of radiolabels. The 2,4-dinitrophenyl (DNP) group is an example of a hapten with potential use as a non-radioactive labelling group for oligonucleotides. This label is highly antigenic, inexpensive, chemically simple, sterically undemanding, and is not found in vivo.

(1) A series of non-nucleoside-based DNP phosphoramidites have been prepared and used in the multiple labelling of oligonucleotides during solid phase synthesis. Oligonucleotides labelled in this way were synthesised in very high yield and easily purified by reversed-phase HPLC. The lengths of spacer arms between the DNP label and the oligonucleotide phosphate backbone have been varied in order to determine the optimum length for anti-DNP antibody binding. The optimum number of DNP labels for maximum signal strength is also reported. The labelled oligonucleotides were detected using standard ELISA methodology, employing a monoclonal IgG mouse anti-DNP antibody, giving sensitivities equivalent to those obtainable in the visualisation of biotinylated oligonucleotides. Horseradish peroxidase and alkaline phosphatase anti-DNP conjugates have also been prepared and used to simplify the detection procedure. The latter conjugate gave detection sensitivities comparable to those obtained in the visualisation of digoxigenin labels.

(2) DNP labelled phosphoramidite and triphosphate derivatives of 2'-deoxyuridine have also been synthesized and used to label oligonucleotides.
(3) As an alternative to the above DNP based non-radioactive labelling system, single and multiple dansyl based phosphoramidites have been synthesized and used to fluorescently label oligonucleotides. Protein-dansyl conjugates have been prepared for the production of anti-dansyl antibodies which would allow the immunogenic detection of this label.

(4) Oligonucleotide probes attached directly to alkaline phosphatase have been prepared using inexpensive, commercially available reagents and used in hybridisation experiments to detect 0.3fmoles of target DNA.
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<td>A</td>
<td>adenosine</td>
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<tr>
<td>AAAF</td>
<td>N-acetoxy-N(^2)-acetylaminofluorene</td>
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<tr>
<td>AEDANS</td>
<td>5-(aminoethyl)-napthalene-1-sulphonic acid</td>
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<td>AMP</td>
<td>adenosine 5'-monophosphate</td>
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<td>AMPPD</td>
<td>adamantyl monopyridinium phosphate dioxetane</td>
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<td>AP</td>
<td>alkaline phosphatase</td>
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<tr>
<td>ATP</td>
<td>adenosine 5'-triphosphate</td>
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<tr>
<td>BCIP</td>
<td>5-bromo-4-chloro-3-indolyl phosphate</td>
</tr>
<tr>
<td>BOC</td>
<td>t-butyloxy carbonyl</td>
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<tr>
<td>BODIPY</td>
<td>N-(4,4-difluoro-5,7-dimethyl-4-bora-3(\alpha),4(\alpha)-diaza-s-indacene-3-propionyl</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>C</td>
<td>cytidine</td>
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<tr>
<td>CCD</td>
<td>charge coupled device</td>
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<td>C(\gamma)G</td>
<td>chicken gamma globulin</td>
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<tr>
<td>conc.</td>
<td>concentrated</td>
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<td>CPG</td>
<td>controlled pore glass</td>
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<td>DAB</td>
<td>3,3'-diaminobenzidine</td>
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<td>dATP</td>
<td>2'-deoxyadenosine 5'-triphosphate</td>
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<tr>
<td>DCCI</td>
<td>1,3-dicyclohexylcarbodiimide</td>
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<td>ddATP</td>
<td>2',3'-dideoxyadenosine 5'-triphosphate</td>
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<td>dGTP</td>
<td>2'-deoxyguanosine 5'-triphosphate</td>
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<td>Dig</td>
<td>digoxigenin</td>
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<tr>
<td>DIPA</td>
<td>diisopropylamine</td>
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<td>DIPEA</td>
<td>N,N-diisopropylethylamine</td>
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<tr>
<td>DMF</td>
<td>N,N-dimethylformamide</td>
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<td>DMSO</td>
<td>dimethylsulphoxide</td>
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<td>DMTr</td>
<td>4,4'-'-dimethoxytrityl</td>
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<tr>
<td>DNA</td>
<td>oligodeoxyribonucleic acid</td>
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<tr>
<td>DNFB</td>
<td>2,4-dinitrofluorobenzene</td>
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<td>DNP</td>
<td>2,4-dinitrophenyl</td>
</tr>
<tr>
<td>DSP</td>
<td>dithiobis-(succinimidyldipropionate)</td>
</tr>
<tr>
<td>DSS</td>
<td>disuccinimidyl suberate</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>dUTP</td>
<td>2'-deoxyuridine 5'-triphosphate</td>
</tr>
<tr>
<td>EDC</td>
<td>1-ethyl-3-(3-dimethylaminopropyl) carbodiimide</td>
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<tr>
<td>EDTA</td>
<td>ethylene diamine tetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol bis-(β-aminoethyl ether) N,N,N',N'-tetracetic acid</td>
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<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
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<tr>
<td>eq.</td>
<td>equivalents</td>
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<td>ETFA</td>
<td>ethyl trifluoroacetate</td>
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<tr>
<td>EtOAc</td>
<td>ethyl acetate</td>
</tr>
<tr>
<td>FAB</td>
<td>fast atom bombardment</td>
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<td>FPLC</td>
<td>fast protein liquid chromatography</td>
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<tr>
<td>G</td>
<td>guanosine</td>
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<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
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<tr>
<td>hr(s)</td>
<td>hour(s)</td>
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<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
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<tr>
<td>JOE</td>
<td>Applied Biosystems fluorescent dye</td>
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<tr>
<td>K&lt;sub&gt;d&lt;/sub&gt;</td>
<td>dissociation constant</td>
</tr>
<tr>
<td>MBS</td>
<td>3-maleimidobenzoic acid N-hydroxysuccinimide ester</td>
</tr>
<tr>
<td>MES</td>
<td>4-morpholineethane sulphonic acid</td>
</tr>
<tr>
<td>min(s)</td>
<td>minute(s)</td>
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<tr>
<td>NaOAc</td>
<td>sodium acetate</td>
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<td>NAP</td>
<td>nucleic acid purification</td>
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<tr>
<td>NBT</td>
<td>nitro blue tetrazolium</td>
</tr>
<tr>
<td>NH&lt;sub&gt;4&lt;/sub&gt;OAc</td>
<td>ammonium acetate</td>
</tr>
<tr>
<td>NHS</td>
<td>N-hydroxysuccinimide</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
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<tr>
<td>NOBA</td>
<td>3-nitrobenzyl alcohol</td>
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<tr>
<td>p</td>
<td>phosphate</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PD</td>
<td>protein desalting</td>
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<td>PROXYL</td>
<td>2,2,5,5-tetramethyl-1-pyrollidinyloxy free radical</td>
</tr>
<tr>
<td>Px</td>
<td>9-phenylxanthene (pixyl)</td>
</tr>
<tr>
<td>RNA</td>
<td>oligoribonucleic acid</td>
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<td>Definition</td>
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<td>------------------------------------------------</td>
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<tr>
<td>SPDP</td>
<td>3-(2-pyridylthio) propionic acid N-hydrosuccinimide ester</td>
</tr>
<tr>
<td>T</td>
<td>thymidine</td>
</tr>
<tr>
<td>TAMRA</td>
<td>tetramethyl rhodamine</td>
</tr>
<tr>
<td>TBDMS</td>
<td>t-butyl-dimethylsilyl</td>
</tr>
<tr>
<td>TEG</td>
<td>tetraethylene glycol</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
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<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
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<tr>
<td>tlc</td>
<td>thin layer chromatography</td>
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<td>Tosyl</td>
<td>4-toluene sulphonyl</td>
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<tr>
<td>Tween-20</td>
<td>polyoxyethylenesorbitan monolaurate</td>
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<tr>
<td>U</td>
<td>uridine</td>
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<td>UV</td>
<td>ultraviolet</td>
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General Introduction

The emergence of automated and commercialised oligonucleotide synthesis has, in the past decade, greatly influenced many areas of molecular biology and medical research. Fields as diverse as DNA sequencing, gene synthesis and the diagnosis of genetic and viral disease, now make substantial use of readily available synthetic DNA. Supplying this huge demand for custom made oligonucleotides has been made possible by the development of the automated DNA synthesizer, a timely combination of advanced technologies within engineering and computer science. However, the key to efficient oligonucleotide synthesis rests not only in state-of-the-art hardware, but moreover, in the underlying chemistry. The first major breakthrough in this field occurred in 1962 as Merrifield (1) carried out pioneering work on solid phase synthesis. Initially used for producing polypeptides, the successful transfer of this technique by Letsinger (2) in 1965 to oligonucleotide synthesis not only assisted purification of synthetic oligonucleotides, but more importantly has allowed full automation of the synthesis procedure. The second advance made in 1976, again by Letsinger (3), established the basis of what is now generally known as the "phosphoramidite method" of oligonucleotide synthesis. This system in its present form is capable of producing oligonucleotides containing in excess of 150 mononucleotides, an achievement attributable to the stability of deoxynucleotide phosphoramidites, short reaction times, and very high yields per nucleotide addition (>99% per cycle). The synthesis and purification of relatively large oligonucleotides is now possible in less than 24 hours.

The phosphoramidite approach to DNA synthesis is also very flexible, allowing for example, the production of oligoribonucleotides, phosphorothioate oligonucleotides, and a large range of other
sugar/base/phosphate backbone modified DNA. The therapeutic and diagnostic applications of these and other compounds continues to stimulate interest in both biochemical and biomedical research, and ensures that nucleic acid chemistry remains an intensely active field.

As the work described in this thesis involves this now well established phosphoramidite methodology, a description of the organic chemistry underlying DNA synthesis will not be given, although this may be found in a detailed review by Sonveaux (4). A description of the development of oligonucleotide synthesis is given in a review by Beaucage and Iyer (5), and standard oligonucleotide synthesis and purification procedures are covered by Brown (6).

The work described in this thesis deals with the non-radioactive labelling of oligonucleotides, beginning with the production of novel molecular probes using phosphoramidite methodology, and ending with their commercialisation and use in a range of practical applications.
1.0 Introduction

The ability of complementary strands of DNA to form a duplex has become popular knowledge since its discovery by Crick and Watson in 1953 (7). Few however appreciate the impact that this property has had on many areas of biomedical research such as the diagnosis of genetic abnormality or viral infection. Today, as our knowledge of the genes ultimately responsible for disease increases, so too does our ability to diagnose and possibly prevent a wider range of illnesses. In essence, diagnosis can now be achieved by the combination of modern DNA sequencing and DNA synthesis techniques. The former allows identification and characterisation of base sequences specific to particular genes, whilst the latter is used to produce oligonucleotide "probes" complementary to these sequences. If probes are modified in such a way as to allow their detection, duplex formation between the probe and target DNA in a clinical sample will effectively highlight the presence of the target base sequence.

Although this is a highly simplified description of modern diagnostic technology, the process outlined above is in theory limited only by our ability to detect small amounts of labelled oligonucleotides. Hence the development of sensitive labelling systems has attracted much interest in recent years. However, due to cost and safety considerations, originally established radioisotope labelling techniques are now losing favour among the majority biomedical research workers, a situation also prompted by the development of alternative, non-radioactive labelling strategies. The use of non-radioactive labels is particularly desirable for work which involves the repetitive handling of large numbers of radiolabelled probes. This is a situation found in many laboratories dealing with applications such as large scale DNA sequencing, or in medical establishments.
involved in routine screening of clinical samples for viral infection or genetic disease.

The radiolabelling of synthetic oligonucleotides with $^{32}\text{P}$ is commonly carried out by the action of polynucleotide kinase on [γ-$^{32}\text{P}$] dATP for 5'-end labelling, or alternatively by the use of terminal transferase and [α-$^{32}\text{P}$] ddATP for 3'-end labelling (8). Depending upon the application, other radioisotopes such as $^{3}\text{H}$, $^{14}\text{C}$, $^{125}\text{I}$ and $^{35}\text{S}$ are readily available and can also be used. Radiolabelled probes can then be detected with very high sensitivity by autoradiography. However, the use of radiolabelled oligonucleotides does suffer from several major disadvantages:

(i) The half-lives of the commonly used radioisotopes vary from 14.2 days to 87.4 days for $^{32}\text{P}$ and $^{35}\text{S}$ respectively. This does require, especially in the case of $^{32}\text{P}$, that experiments involving radiolabels be carried out as soon as possible after preparation of the probe. Long term storage is not possible and repeated labelling of DNA is necessary for routine work. This not only makes radiolabelling expensive and time-consuming, but also compromises the safety of the worker by repeated exposure to ionising radiation.

(ii) Radiolabelled probes also vary in detection sensitivity with time and batch and this may introduce uncertainty in subsequent experimental results. This is not a problem with non-radioactive labelling, as labelled probes may be synthesised in a very controlled manner and stored indefinitely with no change detection sensitivity.

(iii) The expense involved in maintaining statutory levels of safety may in itself be prohibitive. This not only includes adequate staff training, shielding and monitoring, but also containment and the disposal of low-level radioactive waste.
It is clear that radiolabelling, despite being a very sensitive technique, does have many disadvantages. Hence the demand for alternative labelling strategies has fuelled the development of a wide range of alternative, non-radioactive labelling systems.

1.1 Principles of Non-radioactive Labelling

1.1.1 Directly detectable labelling

This involves the attachment of a label such as an enzyme or a fluorescent dye to an oligonucleotide via a covalent bond. When the label is an enzyme (e.g. alkaline phosphatase), detection may be carried out by incubation of the labelled probe with an appropriate chemical substrate. Depending upon substrate choice, this will result in either the release of a coloured product (detectable by eye or quantifiable by densitometry) or the production of chemiluminescence (detectable by charge coupled device (CCD) or photographic film). If the label is a fluorescent dye (e.g. Texas Red) irradiation of the probe with visible radiation of the appropriate wavelength will result in fluorescence. This is detectable by fluorimetry, or in the case of \textit{in situ} hybridisation experiments by fluorescence microscopy (Figure 1).

1.1.2 Indirectly detectable labelling

This involves the covalent attachment of an antigenic molecule to an oligonucleotide and requires subsequent antibody mediated detection of the hapten. In its simplest form, the use of an antibody-enzyme conjugate allows, through the hydrogen bonding interaction of antibody and hapten, the indirect attachment of an enzyme to the labelled oligonucleotide. Treatment of the associated enzyme with an
Immobilise target DNA/RNA on nylon/nitrocellulose membrane or prepare tissue thin section.

- Incubate with directly labelled oligonucleotide probe

(2) Hybridisation of sequence specific probe

- Wash away non-specifically bound oligonucleotide probes

(3) Detect label
  - If label is fluorescent, irradiate and observe emission of light
  - If label is an enzyme, incubate with appropriate chemical substrate to produce coloured precipitate or chemiluminescence

Figure 1: Detection of target DNA/RNA with a directly detectable probe
appropriate chemical substrate will, as in the case of direct labelling, give rise to either a colour reaction or the production of chemiluminescence.

To increase the efficiency of the hapten-antibody interaction, the hapten may be located at the end of a linker arm, distancing the label from the oligonucleotide and minimizing steric hindrance between the large antibody and oligonucleotide molecules (9,10) (Figure 2). The detection sensitivity may also be improved by increasing the number of enzyme molecules associated with each oligonucleotide probe. This can be achieved by employing a secondary antibody-enzyme conjugate system, in which the primary antibody remains unlabelled, but provides multiple antigenic sites for capture by a secondary antibody-enzyme conjugate (Figure 3). This will result in the association of several enzymes per oligonucleotide, thereby increasing the strength of the detection signal.

Although the antibodies used for hapten detection are commonly monoclonal or polyclonal whole IgG molecules, antibody fragments such as Fab or F(ab')2 may be used in applications such as in situ hybridisation where efficient cell membrane penetration is important (11).

An alternative to the system described above has been made possible by exploiting the exceptionally high affinity of avidin and streptavidin for the naturally occurring biotin molecule. This essentially irreversible interaction has been used extensively for indirect labelling in a manner analogous to that of antibody-hapten methodology, where oligonucleotide probes are labelled with biotin and detected using avidin-enzyme conjugates.
Figure 2: Relative sizes of a 25-mer oligonucleotide probe, an IgG antibody and an IgG-enzyme conjugate
Indirectly labelled probe hybridised to target DNA/RNA

- Incubate with primary antibody

Primary antibody recognition and capture of hapten label

- Incubate with secondary antibody-enzyme conjugate

(3) Recognition and capture of primary antibody, followed by addition of substrate and signal detection.

Figure 3: Detection and signal enhancement with indirectly detectable probes
2.0 Types of Labels and Methods of Detection

2.1 Directly Detectable Labels

2.1.1 Enzymes

The covalent attachment of enzymes such as alkaline phosphatase (AP) and horseradish peroxidase (HRP) to oligonucleotides has been achieved by several methods (12-15). The detection of these enzyme labelled probes can be carried out by the following methods:

(a) Colorimetric detection.

This involves a reaction in which the enzyme converts a colourless chemical substrate into a highly coloured product, the choice of enzyme/substrate combination being selected to suit the particular application. For in-situ hybridisation or immunoblot experiments where permanent staining is required, a substrate resulting in an insoluble end product is used, e.g. 3-3'diaminobenzidine with horseradish peroxidase or a 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium mixture with alkaline phosphatase (16-20). These produce dark brown and dark blue/purple precipitates respectively (Figure 4). Alternatively, for microtitre experiments on transparent plates, p-nitrophenyl phosphate used in conjunction with alkaline phosphatase gives a soluble end product, measurable spectrophotometrically.

(b) Chemiluminescent detection.

Substrates are also known which, in the presence of the appropriate enzyme, react to produce chemiluminescence (Figure 4a)

For example, incubation of horseradish peroxidase in the presence of luminol (21-24), or the action of alkaline phosphatase on AMPPD (25) or commercially available derivatives such as Lumiphos (26), result in the production of chemiluminescence. This approach is not only very
Possible mechanism for chemiluminescence from HRP/luminol

Mechanism of chemiluminescence from alkaline phosphatase/AMPPD

Figure 4: Chemiluminescence from HRP and Alkaline phosphatase
(a) Coloured precipitate generation using diaminobenzidine/HRP

(b) Coloured precipitate generation using BCIP/NBT/Alkaline phosphatase

Figure 4(a): Production of insoluble, coloured precipitates using horseradish peroxidase and alkaline phosphatase
sensitive (27), but also has several advantages over colorimetric
detection:

(i) The detection of the chemiluminescent signal is very rapid. The
use of a CCD camera or photographic film typically requires only a 20
second exposure time (colorimetric detection normally requires 30 min.-
24 hrs.)

(ii) The use of a CCD allows simple quantification of the light
signal. Accurate quantification is not possible for colorimetric detection as
conventional optical densitometers are unreliable.

(iii) Unlike insoluble precipitates which permanently mark blots or
tissue samples, several hybridisation experiments with different sequence
specific probes may be carried out on the same sample.

(iv) The background is theoretically zero.

However, the covalently linked enzyme-oligonucleotide based
detection system does suffer from several disadvantages:

(i) The synthesis and purification of an enzyme-oligonucleotide
conjugate is very expensive and time consuming. A typical custom made
25mer oligonucleotide-alkaline phosphatase conjugate would cost at
current 1994 prices £85,000/μmol (28). In comparison, the corresponding
biotinylated oligonucleotide, would cost £220/μmol (29).

(ii) Only one enzyme may be attached to each oligonucleotide
during conjugate synthesis. This system does not allow, as does indirect
detection, the post hybridisation enhancement of signal sensitivity through
the association of more than one enzyme per oligonucleotide.

(iii) The activity of the enzyme itself may be severely impaired,
possibly during the conjugation process itself, or through the masking of
an active site by an attached oligonucleotide.
(iv) The attachment of a relatively large molecule such as an enzyme may adversely affect the hybridisation properties of the oligonucleotide probe. This effect has been observed by Jablonski (12), where a $10^\circ C$ decrease in the melting temperature of an alkaline phosphatase labelled probe was noted in comparison to that of the unmodified oligonucleotide.

(v) To ensure specificity, some hybridisation experiments require relatively high hybridisation temperatures ($>45^\circ C$). Alkaline phosphatase and horseradish peroxidase cannot withstand prolonged exposure to these temperatures. This is especially true of horseradish peroxidase which rapidly loses activity above $42^\circ C$. (21)

### 2.1.2 Fluorescent dyes

Oligonucleotides have been covalently labelled with a wide range of fluorescent molecules such as fluorescein, Texas red (30), FAM (31) and BODIPY 530IA (32) (Figure 5). These molecules are commonly attached by reaction of their active ester or isothiocyanate derivatives with amino modified and thiol modified oligonucleotides respectively. This type of label has been used successfully in applications such as \textit{in situ} hybridisation (33) and DNA sequencing (34). As each label has a characteristic pattern of absorption and fluorescence maxima (Figure 6), this allows the simultaneous irradiation and detection of oligonucleotides labelled with different, carefully selected fluorophores.

This multiple labelling technique has seen applications in automated DNA sequencing (35-39), fluorescence activated cytometry (40,41) and videomicroscopy (42).

Although this method of detection has advantages similar to that of chemiluminescent detection, such as rapid yield of signal and reusable
Figure 5: Fluorescent labelling groups
Figure 6: Absorption and fluorescence ranges of selected dyes
target samples, this technique does suffer from several disadvantages:

(i) Although low cost optical sources and detectors are becoming more widely available, specialised equipment such as fluorescence microscopes remain very expensive.

(ii) The absorption and fluorescence properties of many labels are often highly dependent upon pH and solvent polarity within cells, membranes, proteins and other structures.

(iii) Signal distortion can be caused by autofluorescence of cells, tissues and biological fluids.

(iv) In low level detection experiments, Raman scattering from water molecules seriously minimises the detection signal.

2.2 Indirectly Detectable Labels

2.2.1 Biotin

An interaction which has been widely exploited in molecular biology involves the specific binding of the water soluble vitamin biotin to the egg white glycoprotein avidin. This protein and a non-glycosylated, bacterially derived analogue streptavidin, both contain four identical subunits, each with a high affinity binding site for biotin (43) (Figure 7). This interaction with a dissociation constant, $K_D \approx 10^{-15} M$, remains unaffected by extremes of pH, buffer concentration and denaturing agents such as 3M guanidine hydrochloride. Indeed, the first reported use of an efficient non-radioactive labelling system can be attributed to the biotin/avidin interaction. In this system, *Drosophila* ribosomal RNA was coupled to biotin, hybridised *in situ* to salivary gland chromosomes and detected by scanning electron microscopy with avidin coupled to polymethacrylate microspheres (44).
Figure 7: The three most common labels used for hapten based detection of oligonucleotides
Today however, in a widely used method analogous to that of antibody/hapten detection systems, incubation of a biotinylated oligonucleotide with an avidin-enzyme conjugate results in the formation of an oligonucleotide-biotin-avidin-enzyme complex. Detection is then simply carried out by treatment of the complex with an appropriate chemical substrate.

Alternatively, biotin may be detected using anti-biotin antibodies (9,11,45). However, as dissociation constants for antibody-hapten interactions are on average \( \sim 10^{-12} \text{M} \), loss in detection sensitivity may occur, although signal enhancement through secondary antibody systems may be possible.

As with many other detection methods the biotin-avidin system suffers several disadvantages. As well as being very expensive, biotin is insoluble in most solvents making the synthesis of derivatives difficult. In addition, as high levels of endogenous biotin are found in many biological samples, some applications such as \textit{in situ} hybridisation may be severely restricted.

\textbf{2.2.2 Digoxigenin}

The leading alternative to biotin for non-radioactive labelling is currently digoxigenin, an alkaloid occurring exclusively in \textit{Digitalis} plants (46,47). For this reason, in-situ hybridisation experiments may be carried out on biological samples which would otherwise be impossible with biotin. The detection of oligonucleotide probes containing multiple digoxigenin labels (introduced enzymically by deoxynucleotidyl terminal transferase) is carried out immunogenically using anti-digoxigenin antibodies conjugated to alkaline phosphatase. As with biotin however, digoxigenin is expensive and synthesis of derivatives difficult due to multifunctionality of the
molecule. In addition, digoxigenin is not suitable for applications involving PCR, where the steric hindrance of the large terpenoid structure inhibits the polymerase enzyme (48).

2.2.3 The 2,4-Dinitrophenyl group (DNP)

The highly antigenic and strongly coloured DNP group has been used as a standard hapten in immunology for many years, although its use has been restricted almost exclusively to that of a protein marker. However, the potential of this molecule for use as an oligonucleotide label has been recognised in recent years (49-53).

Unlike biotin and digoxigenin, the DNP group is particularly attractive as a non-radioactive label for several reasons:

(i) It is inexpensive.
(ii) It is chemically simple and relatively inert.
(iii) It is small, minimising the possibility of interference with oligonucleotide hybridisation.
(iv) It is extremely immunogenic and high affinity anti-DNP antibodies are commercially available from many sources.
(v) It is not synthesised naturally, and therefore not found in vivo.

2.2.4 Phosphotyrosine

This group has been reported once as an oligonucleotide label (45), being detected immunogenically using an anti-phosphotyrosine antibody and secondary antibody-horseradish peroxidase conjugate system.

2.2.5 N-acetoxy-N²-acetylamino-fluorene (AAAФ)

Incorporated by direct reaction with the 8-position of purines and detected using an anti-AAAФ antibody and secondary antibody alkaline
phosphatase conjugate (54-57). However the use of this group as a label is severely limited due the carcinogenity of AAAF and its derivatives.

2.2.6 Fluorescein

Previously described as a fluorescent labelling group, fluorescein has also been incorporated into oligonucleotides and detected using anti-fluorescein antibody-enzyme conjugates (58).

2.2.7 Modified bases

Modified bases such as 5-bromo-2'-deoxyuridine (59,60) and N-4-methoxy-5,6-dihydrocytosine-6-sulphonate (61) have also been used as immunochemically detectable haptens.
3.0 The Introduction of Labelling Groups Into Oligonucleotides

3.1 Post-Synthetic Labelling

The post-synthetic labelling of oligonucleotides with both radio- and non-radioactive reporter groups remains a common procedure in many molecular biology and biomedical laboratories. This method requires the synthesis and purification of the oligonucleotide to be labelled, the chemical or enzymatic attachment of the label, and finally purification of the resultant labelled probe. Due to the nature of the labelling reactions, these processes are not readily automated. Consequently, post-synthetic synthesis of labelled oligonucleotides is not only expensive, but remains extremely time-consuming.

3.1.1 Enzymatic labelling of oligonucleotides

The enzymatic modification of DNA to form non-radioactive probes has remained popular since its introduction by Langer et al in 1981 (62). This first system employed biotinylated deoxyribonucleotide triphosphates as substrates for the Klenow fragment of DNA polymerase I obtained from *Escherichia coli*. This enzyme, like most polymerases, catalyses the addition of nucleoside triphosphates to a growing strand of DNA, but requires the presence of a complementary strand of DNA to act as a template. Use of this type of enzyme allows the introduction of modified bases along the full length of the growing strand of DNA, position being dependant upon the sequence, and hence the choice of modified nucleoside triphosphate.

Alternatively, labels may be added through the action of deoxynucleotidyl terminal transferase, an enzyme which which adds bases to free 3'-ends of DNA. This enzyme does not require a complementary
template strand, and allows the attachment of a string of labelled bases onto the ends of single stranded DNA, and for this reason is especially suited for work with synthetic DNA.

Today, the most commonly used modified nucleoside triphosphates are derivatives of 5-(3-aminoallyl) 2'-deoxyuridin 5'-triphosphate (amino-4-dUTP), 5-(N-(ε-aminocaproyl)-3-aminoallyl) 2'-deoxyuridine 5'-triphosphate (amino-11-dUTP) and 5-(N-(ε-aminocaproyl-γ-aminobutyryl)-3-aminoallyl) 2'-deoxyuridine 5'-triphosphate (amino-16-dUTP). These are all commercially available pre-labelled with biotin (40,62), digoxigenin (63) and fluorescein (64,65) attached to the linker arms (Figure 8).

All of these compounds, as well as the less commonly used 2'-deoxycytidine and 2'-deoxythymidine analogues, have been used extensively as substrates for DNA polymerase and terminal transferase.

Purine triphosphates (dATP, dGTP) modified at the N-6 position may also be used in enzymatic labelling experiments (66). However, purines modified at the C-8 position are not efficient substrates for DNA polymerase, but become excellent substrates if modified with a long linker arm and incorporated with terminal transferase. This method has been used to label synthetic oligonucleotides with DNP labels (52) (Figure 9).

Enzymatic labelling has also been carried out with bacteriophage T4 RNA ligase. In the absence of ATP, this enzyme transfers the non-adenylyl portion of an appropriate substrate to the 3'-hydroxyl end of an RNA acceptor to form a phosphodiester bond and the AMP portion is released. This enzyme has been used to incorporate labels such as bimane derivatised 2'-deoxycytidine-3'-thiophosphate-5'-phosphate and
Figure 8: Amino modified nucleoside triphosphates and an example of a commercially available hapten labelled derivative, digoxigenin-16-dUTP.
Figure 9: Modified nucleoside triphosphates offering alternatives to the widely used dUTP substrates
biotin, fluorescein and tetramethylrhodamine derivatives of P₁-(6-aminohex-1-yl)-P₂-(5'-deoxyadenosine) into RNA (67,68).

More recently, AEDANS-S-dUTP was used as a substrate for the Klenow fragment of DNA polymerase I, and the resultant fluorescent probe used to study DNA binding proteins (69).

Other enzymatic methods for the introduction of labels into DNA and RNA, such as nick translation and random priming are described in detail by Keller and Manak (8). These methods are not generally applicable to synthetic oligonucleotides.

3.1.2 Chemical labelling of oligonucleotides

The post synthetic chemical labelling of oligonucleotides was until recently the method of choice for the attachment of reporter groups to oligonucleotides. Indeed, some labels such as enzymes and digoxigenin still rely on this technique (Figure 10). Labelling by this method primarily involves the introduction of reactive groups, commonly nucleophiles such as amines or thiols, into an oligonucleotide. Subsequent reaction of these groups with appropriately activated reporter groups, commonly active esters, allows the formation of a covalent bond between label and oligonucleotide (70).

3.1.2.1 Amino-modification of oligonucleotides

The most common method of post-synthetic chemical labelling involves the attachment of a linker arm, functionalised with an amine, onto an oligonucleotide. This may be carried out in a variety of ways, but today, the standard method of introducing primary amino groups onto the 5'-ends of oligonucleotides is via the commercially available 'Aminolink 2' phosphoramidite (71). Consisting of N-trifluoroacetylated
Figure 10: Post synthetic labelling, biotinylation via 'Aminolink 2'
aminohexanol which has been phosphitylated on the free hydroxyl group (Figure 10), this molecule may be used during solid phase phosphoramidite synthesis to incorporate a protected amine. The free amine is released once the oligonucleotide undergoes normal base deprotection, and may then be reacted with a large range of activated labels. These labels are normally contain reactive moieties such as isothiocyanates or active esters (eg. N-hydroxysuccinimide).

This is the standard approach for labelling with reporter groups such as the popular digoxigenin hapten (72), which cannot be incorporated during automated synthesis.

The linker arm-protected amine methodology has been used extensively to produce a large variety of non-radioactive oligonucleotide probes. Many of these have been similar in form to the 'Aminolink 2' molecule, being based upon the H₂N-(CH₂)ₙ-O-Oligo system. Coull et al synthesized a 2-cyanoethyl phosphoramidite monomer of N-trifluoroacetyl-2-aminoethanol. The subsequent free amine was reacted with biotin-N-hydroxysuccinimide active ester to give a biotinylated probe (73). Similarly, Agrawal et al synthesized the analogous 2-cyanoethyl phosphoramidite monomer of N-Fmoc-2-aminoethanol, again using this for biotin attachment (74). Tanaka et al utilised the short 2-aminoethanol based linker arm to attach fluorescein isothiocyanate to an oligonucleotide probe. In this case N-(monomethoxytrityl)-2-aminoethanol was synthesized and used in phosphotriester type oligonucleotide synthesis (75). Connolly synthesized the methyl phosphoramidite monomer of N-monomethoxytrityl-3-aminopropan-1-ol. This was used to introduce biotin and dansyl groups onto the 5'-end of oligonucleotides (76). Similarly, Bannwarth et al synthesized the 2-cyanoethyl phosphoramidite monomer of N-monomethoxytrityl-3-
aminopropan-1-ol. This was reacted with the N-hydroxysuccinimide active ester of a fluorescent bathophenanthroline-ruthenium (II) complex to give an oligonucleotide probe detectable by electron microscopy (77). Sinha and Cook synthesized monomers analogous to the 'Aminolink 2' molecule, using 6-aminohexanol as a basis for N-tritylated, phosphoramidite and hydrogen phosphate monomers. These were used to introduce fluorescein, AEDANS and biotin labels (78). Kaiser et al used a 2-cyanoethyl phosphoramidite N-Fmoc-6-aminohexanol monomer to introduce fluorescent dyes into oligonucleotides to be used in DNA sequencing (38). Methyl phosphonate N-Fmoc-6-aminohexanol has also been synthesized by Agrawal to attach fluorescent groups to oligonucleotides for cell uptake studies (79).

The simple 3-amino-1,2-propanediol backbone has also been used to incorporate multiple amino groups onto the 5'-end of oligonucleotides (Figure 11). Nelson et al (80) used this approach to synthesise a non-radioactively labelled probe containing five biotin moieties. Although this was carried out with the aim of improving detection sensitivity, no comparison between this and a singly labelled biotin oligonucleotide was reported. This backbone has also been used to introduce 3'-end amino functionality by the synthesis of 3-amino-1,2-propanediol derivatised controlled pore glass (81).

A different approach to amino modification may be taken by employing nucleosides themselves as carriers for amino functionality. Although more expensive than the non nucleosidic approach, this route does allow the site specific incorporation of labels along the full length of an oligonucleotide probe. This facility is particularly important in applications such as DNA-protein binding studies which may require fluorescent labels at precise locations.
Non-nucleosidic multiple addition aminomodifier (Nelson et al. 1989)

5'-Amino-2',5'-dideoxynucleoside phosphoramidites (Sproat et al. 1987)

2'-O-alkylaminoribonucleoside phosphoramidite (Manoharan et al. 1991)

Figure 11: Aminomodifier phosphoramidites
Nucleosidic phosphoramidites have been reported in which amino modification has been made on the ribose moiety (Figure 11). This approach was first reported by Smith et al (35) using a N-trifluoroacetyl-5'-amino-5'-deoxythymidine phosphoramidite to introduce a single 5'-amino group onto oligonucleotide probes. These probes were subsequently labelled with four different fluorophores and used as sequencing primers. A similar approach was carried out by Sproat et al (82) using N-trifluoroacetyl-5'-amino-2',5'-dideoxynucleoside phosphoramidites. All four bases were synthesized and the resultant oligonucleotides labelled with iridium complexes and other labels, the former being used in electron microscopy experiments. In a slightly different approach Manoharan et al (83) recently synthesized a phosphoramidite monomer capable of introducing amino functionalities at multiple sites within an oligonucleotide. This was achieved by synthesizing a 2'-alkylaminoribonucleoside, a phosphoramidite which retains the 3'- and 5'- hydroxyls necessary for normal oligonucleotide synthesis (Figure 11).

Nucleoside phosphoramidites have also been prepared in which an amino modification has been made to the base. This approach is somewhat limited as modifications may only be made at certain positions on individual bases to minimise potential interference with hybridisation. Indeed, studies have shown that oligonucleotides containing N-4 aminoalkyl 2'-deoxycytidine (84) and C-5 aminoalkyl 2'-deoxyuridine (85) can destabilise hybrid formation, but despite this these amino modified nucleosides have been used frequently to introduce non-radioactive labels into oligonucleotides (14,86-91) (Figure 12). In addition N-6 aminoalkyl 2'-deoxyadenosine (59) and C-8 aminoalkyl 2'-deoxy-
Figure 12: Aminoalkyl modified nucleoside phosphoramidites
guanosine and -adenosine (52,59) have also been used in post-synthetic labelling experiments.

A recent development has been the synthesis of alkynylamino nucleosides (92-96) (Figure 13), modification normally taking place at C-5 in 2'-deoxyuridine, although C-5 modified 2'-deoxycytidine and C-7 modified 7-deazapurines have also been reported (97). The incorporation of a rigid, sterically undemanding alkyne moiety at these positions decreases the likelihood of affecting hybridisation, as subsequent labels are directed away from sites of inter-nucleotide hydrogen bonding.

Alternative strategies for the introduction of amino functionality include direct chemical modification of the terminal 5'-hydroxyl or modification of internucleoside phosphate linkages after oligonucleotide synthesis.

(1) Chu et al enzymically introduced a monophosphate moiety at this position, followed by formation of a 5'-phosphoroimidazolide and reaction with excess alkyl diamine. This produced a stable 5'-aminoalkylphosphoramidate (98). Similarly, Chollet et al produced 5'-aminoethyl- and 5'-aminohexylphosphoramidates via a single step reactions with N-methylimidazole, water soluble carbodiimide and excess alkylamine (43). Kansal et al synthesized aminoalkylphosphonic acids and conjugated these to oligonucleotide 5'-hydroxyl groups by simple carbodiimide coupling (99). Wachter et al employed the bifunctional coupling reagent 1,1'-carbonyldiimidazole to attach hexanediame via an amide bond to the free 5'-hydroxyl group of oligonucleotides (100). Agrawal et al has reported the oxidative amination of hydrogen phosphonate internucleoside linkages with N-trifluoroacetyl protected alkyl diamines. As H-phosphonate linkages can be made at any time during automated phosphoramidite oligonucleotide synthesis, albeit
DMTrO-1 H_{3}C-5 Propynyl modified 2'-deoxyuridine phosphoramidite (Haralambidis et al. 1987)

DMTrO-1 CPG

C-5 Propynyl modified 2'-deoxyuridine CPG used as basis for oligonucleotide-peptide-biotin conjugate. (Tong et al. 1992)

Figure 13: Alkynyl modified nucleosides
with changes to synthesis cycle and reagents, this method allowed the site specific incorporation of amino functionality into oligonucleotides (101-103). Haralambidis et al adopted a more complex strategy in synthesizing oligonucleotide-peptide conjugates. The lysine residues contained in the peptide portion of the molecule were subsequently labelled with biotin and fluorescent groups (104,105). More recently, Tong et al employed similar oligonucleotide-peptide methodology, combining this with alkynyl modified 2'-deoxyuridine controlled pore glass to give biotin labelled primers for PCR. (96) (Figure 13)

3.1.2.2 Thiol-modification of oligonucleotides

In addition to amino modification, another popular strategy for the post synthetic labelling of oligonucleotides is via thiol modification. In a method analogous to that for amines, free thiol groups may be reacted with reporter groups containing moieties such as maleimides, bromo or iodo acetamides, aziridinylsulphonamides or γ-bromo-α,β-unsaturated carbonyls (106). On reaction with thiols, these groups form stable covalent bonds and have been used successfully to conjugate fluorophores, haptens and enzymes to oligonucleotides (Figure 14). The introduction of thiols can be carried out by the reaction of a protected thiol containing an active ester with a previously amino functionalized oligonucleotide. This approach was taken by Gaur et al (107) by reacting N-succinimydyl-3-(2-pyridyldithio) propionate (SPDP) with an amino modified oligonucleotide. Reduction of the resultant disulphide with dithiothreitol (DTT) produced an oligonucleotide containing a free thiol group. More simply, Kumar et al (108) reacted N-acetyl-DL-homocysteine thiolactone with amino modified oligonucleotides. After treatment with DTT, the subsequent thiols were reacted with N-(4-
Figure 14: Post synthetic labelling, fluoresceination via thiol modification
dimethylaminoazobenzene)-4'-idoacetamide and N-(3-pyrenyl) maleimide to give fluorescently labelled oligonucleotide probes. Li et al (109) treated an amino modified oligonucleotide with the bifunctional coupling reagent dithiobis-(succinimidylopionate) (DSP). This resulted in the conjugation, via a disulphide bridge, of two identical oligonucleotides by their respective 3'-terminii. Treatment with DTT released two thiol modified oligonucleotides which were reacted with bromoacetyl activated alkaline phosphatase. Dithiobis-(succinimidylopionate) has also been employed by Gupta et al (110) to produce a 3'-thiol modified solid support. Reaction with amino-alkyl CPG resulted in a solid support containing a disulphide bridge easily cleaved to the free thiol after oligonucleotide synthesis. Similarly, Bonfils and Thuong (111) coupled a mono-(dimethoxytrityl) protected diol containing a disulphide bridge to amino alkyl CPG via succinic anhydride. Treatment with DTT after normal phosphoramidite synthesis released a 3'-thiol modified oligonucleotide subsequently labelled with fluorescein iodoacetamide. The introduction of a sulphydryl group into a nucleoside solid support has been carried out by Zuckermann et al (112). Phosphorylation of 5'-DMTr-2'-deoxythymidine at the 3'-hydroxyl with 2-chlorophenyl-O,O-bis-(1-benzotriazolyl) phosphate, was followed by coupling to 3,3'-dithiodipropanol. The resulting 3'-(propyldithio)propanol thymidine derivative was coupled via succinic anhydride to amino alkyl CPG.

Alternatives to the relatively complex, 'thiol modification via amino modification' procedures have also been reported. Connolly (113) synthesized S-trityl-O-methoxymorpholinophosphite derivatives of mercaptoalkanols. These were used in phosphoramidite synthesis to directly attach thiol groups to the 5'-ends of oligonucleotides. Similarly,
Saiki et al (15) modified the above procedure to synthesize an S-trityl-O-
(2-cyanoethyl phosphoramidite) derivative of tetraethylene glycol. The
resultant thiolated oligonucleotides were then labelled by reaction with
maleimido activated horseradish peroxidase. H-phosphonate analogues of
these molecules have also been synthesized by Sinha and Cook (78). The
resultant thiol modified oligonucleotides were coupled to maleimido
eosin, maleimido coumarin, iodo-AEDANS, monobromobimane and
maleimido biotin.

A relatively simple method of attaching thiol reactive compounds to
oligonucleotides is through the synthesis of phosphorothioate linkages at
specific sites along the oligonucleotide phosphate backbone. These
phosphorothioate bonds may be formed during phosphoramidite
oligonucleotide synthesis by the inclusion of an additional sulphurisation
step immediately after the formation of an internucleotide phosphate bond
(114,115). Although this method produces relatively sterically hindered
thiol moieties, these can still be derivatised with thiol reactive groups,
preferably attached to the label through linker arms. This method of site
specific attachment of reporter groups has attracted much attention in
recent years. Thuong and Chassignol (116) reacted iodoacetamide
derivatised acridines with oligonucleotides containing a phosphorothioate
group. Hodges et al (117) used 2'-deoxynucleoside 5'-O-(1-
thiotriphosphates) as substrates for DNA polymerase reactions. These
introduced specific phosphorothioate bonds into enzymically synthesized
DNA at sites corresponding to the particular thiotriphosphate nucleoside
used. DNA synthesized in this way was subsequently labelled with
monobromobimane. Cosstick et al (67) also used bimane as a label,
reacting monobromobimane with 2'-deoxycytidine-5'-phosphate-3'-O-
phosphorothioates and ligating these to RNA by T4 RNA ligase. Fidanza
et al (118) attached a variety of labels including the PROXYL electron spin resonance label onto phosphorothioate oligonucleotides by reaction with iodoacetamide derivatives. Similar work has been carried out by Conway et al (106). Agrawal and Zamecnik (102) have synthesized aminomodified phosphorothioate oligonucleotides. This allowed the attachment of two different fluorescent labels at different sites on the same oligonucleotide, by reacting the modified oligonucleotide with monobromobimane and then fluorescein isothiocyanate. Conditions were chosen such that the amino group remained unreactive during the phosphorothioate reaction (Figure 15).

Various other methods have also been used to thiolate and then label oligonucleotides. Sproat et al (119) synthesized the four base protected 5'-(S-trityl)-mercapto-2',5'-dideoxyribonucleoside 2-cyanoethyl phosphoramidites (Figure 15). These were subsequently labelled with fluorescent labels and heavy metals for use in DNA sequencing and X-ray crystallographic studies (Figure 15). Eshagapour et al (120) introduced 4-thiouridine enzymatically into large DNA fragments, followed by reaction with several haloacetamide derivatised fluorescent and cross-linking labels. Murakami et al (121) synthesized alkaline phosphatase-oligonucleotide conjugates by the carbodiimide coupling of a 5'-phosphate oligonucleotide to cystamine. Cleavage of the cystamine disulphide with DTT and reaction with disulphide modified alkaline phosphatase gave an enzyme labelled oligonucleotide probe. A similar approach was taken by Chu and Orgel (98) to conjugate oligonucleotides to a range of other proteins including horseradish peroxidase.
Attachment of two different labels to an oligonucleotide:
(1) Reaction of monobromobimane with a phosphorothioate
(2) Fluorescein isothiocyanate and aminoalkyl phosphoramidate
(Agrawal and Zamecnik, 1990)

Figure 15: Thiol modified phosphoramidites and labelling via phosphorothioates and aminoalkyl phosphoramidates
3.1.2.3 Labelling via 2',3' oxidation in 5'-linked ribonucleotides

In a bid to simplify existing synthetic DNA labelling procedures, Agrawal et al (74) combined phosphoramidite methodology with a well established procedure for the labelling of RNA. The synthesis of 2',3'-di-O-pixyluridine-5'-O-(methyl N,N-diisopropylamino) phosphite (Figure 16) allowed the 5'-5' coupling of the above ribonucleotide to an oligonucleotide during solid phase phosphoramidite synthesis.

Figure 16: Ribonucleotide 5'-phosphoramidite (Agrawal et al. 1986)

Subsequent sodium periodate treatment of the oligonucleotide resulted in the oxidation of the terminal ribonucleoside residue to give a 2',3'-dialdehyde derivative. The dialdehyde was then reacted with a variety of labelling reagents containing primary amino groups (Figure 17).

3.1.2.4 Photochemical labelling of oligonucleotides

The UV irradiation of labels containing photoactivatable moieties has also been used to attach reporter groups to oligonucleotides. The most popular reagents for this purpose are aryl azide derivatised compounds,
Figure 17: Biotinylation via 5' ribonucleotide coupling (Agrawal et al. 1986)
yielding nitrenes on irradiation, which then react readily with the heterocyclic bases in DNA. This results in the covalent linking of labels to oligonucleotides, albeit in a completely random manner. This is a major disadvantage which seriously restricts photochemical labelling, as labels attached in this way are liable to inhibit hybridisation. Nevertheless, this method has been used to attach biotin (122), digoxigenin (10) and 2,4-dinitrophenyl (8) labels to oligonucleotides (Figure 18).

3.2 Introduction of Labels during Solid Phase Synthesis

The incorporation of non-radioactive labels into oligonucleotides is carried out most simply during normal solid phase synthesis. In many cases, no alteration to normal DNA synthesizer cycles or reagents are required. This ensures that labelling is carried out with the minimum amount of additional time and expense. Similarly, cleavage of the oligonucleotide from the solid support, deprotection and purification may be carried out following the conventional work up procedures for unmodified DNA. Indeed, the attachment of (usually lipophilic) labels at the synthesis stage, often simplifies the purification procedure by significantly altering the electrophoretic properties of an oligonucleotide. This allows the efficient separation of labelled oligonucleotide and unlabelled failure sequences through readily available techniques such as reverse phase HPLC. However, the greatest advantage to be gained by attaching labels during solid phase synthesis is the ability to introduce reporter groups at specific positions within an oligonucleotide. Unlike many post-synthetic labelling systems, this can be carried out in a completely controlled manner, allowing not only the attachment of multiple labels, but also the combination of different labels in a single oligonucleotide. The potential of multiple labelling has been realised as a
Figure 18: Photoactivatable reporter groups
means of increasing the sensitivity of detection in diagnostic applications, whilst the combination of different labels allows more sophisticated applications.

Unfortunately, phosphoramidite monomers are required to be compatible with conventional DNA synthesis chemistry, something which is not always possible. Labels such as digoxigenin are insoluble in anhydrous acetonitrile (the standard monomer solvent), and instead must rely on post-synthetic labelling methodology. In addition digoxigenin has sensitive functional groups which could not be exposed to oligonucleotide synthesis conditions without complex protection procedures.

3.2.1 Single label addition.

Single addition phosphoramidite monomers are commonly derivatives of long chain aliphatic amino alcohols, labelled at the amine group and prepared for use on DNA synthesizers by phosphitylation of the hydroxyl group. This approach has been used for many applications, none more so than in the synthesis of single addition biotin monomers (Figure 19).

The first biotin phosphoramidite was produced by Alves et al (123) who overcame the inherent insolubility of biotin in most organic solvents by the N-1 tritylation of biotin methyl ester. Hydride reduction of this ester gave the corresponding alcohol from which the biotin phosphoramidite was produced. Unfortunately, this compound and also its N-1 tetrahydropyranyl analogue were insoluble in acetonitrile, necessitating the use of a dichloromethane/acetonitrile mixture as phosphoramidite solvent. This resulted in poor coupling efficiencies (4-11%).
Figure 19: Single addition biotin phosphoramidites
A different approach was adopted by Cocuzza (124) who reacted
*p*-aminophenethyl alcohol with biotin N-hydroxysuccinimide active ester,
followed by phosphitylation of the product. This yielded a novel biotin
monomer containing an aromatic amide spacer arm. However, this biotin
phosphoramidite was insoluble in acetonitrile and required a
DMF/dichloromethane solvent mixture for "optimum coupling efficiency",
although exact figures for this are not quoted.

A more involved monomer synthesis was carried out by Pon (125).
Biotin was coupled using a carbodiimide to 6-amino-*tert-*
butyldimethylsilylhexan-1-ol, followed by N-1 tritylation of the product.
Removal of the TBDMS protecting group and subsequent phosphitylation
gave a biotin phosphoramidite which was soluble in acetonitrile. The
monomer was used under standard synthesis conditions, giving coupling
efficiencies of 93%.

In an attempt to improve upon existing, very expensive biotin
monomers, David Will of this group (126), synthesized two N-1 benzoyl
protected biotin phosphoramidites with differing spacer arm lengths.
These monomers, both 3-oxapentyl and octamethylene spaced, dissolved
in acetonitrile and coupled at >95%.

Other single addition phosphoramidite monomers include those
used for fluorescent labelling (Figure 20). A simple fluorescein
phosphoramidite was synthesised by Schubert et al (127) by the alkylation
of fluorescein methyl ester with 4-chloro-(4,4'-dimethoxytrityl)-butan-1-
ol, followed by detritylation and phosphitylation. The resulting monomer
was soluble in acetonitrile and gave coupling efficiencies "similar to those
of normal phosphoramidites".

Bannwarth and Schmidt (128) have successfully used a
bathophenanthroline-ruthenium (II) complex to fluorescently label
Figure 20: Single addition fluorescent and hapten monomers
oligonucleotides. The complex was derivatised with a hydroxypentyl function on one of three bathophenenthroline ligands. Phosphitylation gave a monomer soluble in acetonitrile and which coupled at ~50%.

Fluorescent 2-methoxyacridine single addition phosphoramidites (129) are commercially available although not reported in the literature (Cambridge Research Biosystems).

Single addition phosphoramidites for the attachment of hapten labels have also been synthesized. Clare Pritchard of this group (130) synthesized a DNP monomer based on 6-aminohexan-1-ol which was soluble in acetonitrile and coupled at >97% as determined by HPLC.

### 3.2.2 Multiple labelling

The multiple labelling of oligonucleotides using phosphoramidite methodology was first achieved by Roget et al (131) using a modified nucleoside approach. Four labels; biotinyl, dinitrophenyl, pyrenyl and dansyl were each attached separately to the amino group in N-4 aminohexyl derivatised 2'-deoxycytidine (Figure 21). Subsequent tritylation and phosphitylation gave four monomers, all of which were soluble in acetonitrile and gave coupling efficiencies of >97%. However, under normal base deprotection conditions (55°C, conc. NH₄OH, 16hrs), oligonucleotides bearing DNP groups showed severe degradation accompanied by the release of dinitrophenol. This necessitated the use of DNA monomers containing base protecting groups which were more base labile (132).

Similarly, Pieles et al (133) synthesized biotinylated 2'-O-methyl and 2'-O-allylribonucleotide phosphoramidite monomers for the synthesis and study of oligoribonucleotide-protein complexes. In this case, as extended coupling times and stronger activators were being used
Figure 21: Nucleoside based multiple addition phosphoramidites
for the synthesis of modified RNA in contrast to normal DNA, the biotin moiety was first N-1 protected with 4-tert-butyl benzoyl to prevent side reactions.

Although both of the above methods resulted in the successful incorporation of labels into DNA and RNA, these both suffer from the disadvantages of having expensive starting materials and relatively long syntheses. Alternatively, non-nucleosidic phosphoramidites may be used to simplify synthetic pathways and reduce cost.

Misiura et al (45) incorporated multiple biotin and phosphotyrosine labels into DNA by the use of a non-nucleosidic, glycerol type backbone (Figure 22). This was produced by the Michael type addition reaction between the inexpensive solketal and acrylonitrile in the presence of sodium hydride. Reduction of the resultant 2-cyanoethyl solketal with sodium borohydride/cobalt (II) chloride gave 3-aminopropyl solketal, a compound containing protected primary and secondary hydroxyl functions and also a free amino group onto which labels could be attached. Both biotin and phosphotyrosine monomers dissolved in acetonitrile and coupled with efficiencies of >99%. Recently, Theisen et al (134) synthesized a multiple addition fluorescein monomer also based upon the above backbone. Although, this phosphoramidite was soluble in acetonitrile, coupling efficiency was reported to be only ~70%.

Nelson et al (135) recently synthesized a 2-aminobutyl-1,3-propanediol backbone. This is now commercially available pre-labelled with biotin, DNP, fluorescein dipivaloate and 6-chloro-2-methoxyacridine (Clontech Laboratories Inc.) (136).

Finally, David Will of this group (137) synthesized a 3,6-diazaoctane-1,8-diol backbone which contained two DNP groups per
6-Chloro-2-methoxyacridine on a 2-aminobutyl-1,3-propanediol based phosphoramidite (Nelson 1992)

Multiple addition DNP phosphoramidite based on 3,6-diazaoctane-1,8-diol (Will et al. 1991)

Multiple addition phosphotyrosinyl phosphoramidite based upon an aminohexane-6,7-diol backbone (Misiura et al. 1990)

Figure 22: Non-nucleosidic multiple addition phosphoramidites
monomer. Unfortunately, this was not soluble in acetonitrile and labelled oligonucleotides degraded under base deprotection conditions
4.0 Aims

It is clear from the disadvantaged non-radioactive labelling systems of biotin and digoxigenin that an alternative system which would offer the controlled attachment of inexpensive labels to oligonucleotide probes is desirable. This should be possible by the development of a phosphoramidite based labelling system which would allow the multiple attachment of highly sensitive, hapten-based labels to oligonucleotides during routine solid phase synthesis.

The aim of the work described in this thesis was to develop such a system, based primarily on the highly antigenic molecule 2,4-dinitrophenyl and using a highly specific and sensitive antibody to facilitate detection. The long term goal would be the use and commercialisation of such a system in kit form for biomedical research.
5.0 Synthesis of 2,4-Dinitrophenyl Containing Phosphoramidite Monomers and DNP-labelled Oligonucleotides

The first step in the synthesis of a 2,4-dinitrophenyl multiple addition phosphoramidite is the construction of the backbone onto which the DNP label, a dimethoxytrityl group and a phosphoramidite group are attached. There are three basic requirements for this backbone:

(i) Two hydroxyl functions, preferably one primary and one secondary. This gives the necessary specificity for the sequential addition of the dimethoxytrityl and phosphoramidite moieties.

(ii) An amino group separated from the backbone via a linker unit. This allows attachment of a DNP group, or any other suitably functionalised label.

(iii) The starting materials must be inexpensive. Although nucleoside based monomers allow the placement of labels anywhere within a given sequence, compounds of this type can be very expensive. Fortunately, most applications do not require internal labelling and simple aliphatic compounds may be used as alternative, inexpensive backbones for multiple 5'- or 3'- end labelling.

One compound which satisfies the above requirements is the commercially available bis-(2-hydroxyethyl)-ethylene diamine. This was used in a previous attempt by this group to synthesize a multiple addition DNP monomer (Figure 22). Unfortunately the monomer failed through product instability during base deprotection. The reason for this remains unclear, although it has been proposed that this particular type of monomer may undergo base catalysed β-elimination during base deprotection with c.NH₄OH (Figure 23). In contrast, previous work on a single addition DNP monomer showed the DNP group to be stable.
Figure 23: Possible mechanism of degradation for the bis-(2-hydroxyethyl)-ethylene diamine based DNP monomer
during both oligonucleotide synthesis and deprotection (Figure 20). To further investigate the general properties and stability of multiple addition DNP monomers, we decided to first synthesize a simple, short chain, multiple addition DNP phosphoramidite.

5.1 3-Aminopropane-1,2-diol Based DNP Monomers

Nelson et al (80) have reported the use of 3-aminopropane-1,2-diol as the basis for a trifluoroacetylated multiple addition aminolink phosphoramidite. This glycerol analogue is inexpensive, and is in effect a preformed backbone, allowing the construction of multiple addition monomers in the minimum number of steps. Indeed, attachment of any label to the amino function of 3-aminopropane-1,2-diol can be followed by tritylation and phosphitylation to give a multiple addition monomer. We therefore decided to use this method as basis for a simple, multiple addition DNP monomer.

5.1.1 Synthesis of multiple addition DNP monomer [3] (Scheme 1)

3-Aminopropane-1,2-diol was dissolved in methanol containing 3 equivalents of triethylamine, followed by the addition of 2,4-dinitrofluorobenzene (Sanger's reagent). Excess triethylamine was used to remove hydrogen fluoride released during the substitution reaction as triethylamine hydrofluoride. Maintaining a high pH ensured that any unreacted 3-aminopropane-1,2-diol remained unprotonated and nucleophilic. Once the reaction was complete, purification was carried out by wet flash column chromatography, giving the pure product [1] in 82% yield. The product [1] was then tritylated using 4,4'-dimethoxytrityl chloride in anhydrous pyridine at room temperature, purification being carried out by silica gel chromatography. As hydroxyl moieties
Scheme 1: Synthesis of DNP phosphoramidite [3]
on silica gel can be acidic enough to cleave acid labile dimethoxytrityl
protecting groups, the silica gel used for the column was pre-equilibrated
with 2% triethylamine in dichloromethane. Pretreatment with
triethylamine ensured that the column remained basic and prevented
product degradation. The purified oil obtained was dissolved in the
minimum amount of dichloromethane, and precipitated from hexane at -
78°C. This gave the product [2] as a yellow powder. It was noted that
thorough drying of the product [2] by coevaporation with anhydrous
pyridine (3x10ml), and the use of freshly recrystallised DMTrCl, was
necessary to ensure good yields for this reaction (>90%). The
phosphitylation of product [2] was carried out using 2-cyanoethyl N,N'-
diisopropylchlorophosphoramidite in THF containing
diisopropylethylamine at room temperature, the reaction being complete
in 3 hours. Purification was again carried out by column chromatography,
eluting with ethyl acetate in dichloromethane. The use of ethyl acetate
instead of methanol prevents attack by the latter on the product phosphite
group, a side reaction which may occur at areas of localised acidity on
silica gel. The resulting oil was dissolved in the minimum volume of
dichloromethane, and precipitated twice from hexane at -78°C, to give the
product [3] as a yellow oil in 47% yield. Double precipitation served to
remove traces of organic base which would otherwise interfere with the
tetrazole activation step during oligonucleotide synthesis.

5.1.2 Oligonucleotide synthesis using [3]

The short chain multiple addition DNP phosphoramidite monomer
[3] was very soluble in anhydrous acetonitrile, the standard solvent for
DNA synthesis monomers. Used as a 0.15M solution in anhydrous
acetonitrile during standard oligonucleotide synthesis, a series of short
test sequences were synthesized to assess both coupling efficiency and stability of [3] to standard deprotection conditions. The synthesis cycle used was a standard 0.2μmol ABI cycle with an extended monomer coupling time of 4 minutes. This resulted in coupling efficiencies, measured by the release of dimethoxytrityl cations of >98%. After cleavage from the solid support, oligonucleotides labelled in this way were bright yellow, the colour becoming stronger as the number of labels per oligonucleotide was increased.

5.1.3 HPLC analysis of oligonucleotides labelled with [3]

The analysis and purification of DNP oligonucleotides can be carried out easily using reversed-phase HPLC. The attachment of lipophilic labels causes labelled oligonucleotides to elute much later than unlabelled failure sequences. This effect can be particularly marked, with oligonucleotides containing 5 or more DNP labels eluting >15 minutes later under normal HPLC purification conditions (a gradient of 0-20% acetonitrile in 0.1M ammonium acetate). In these cases it was necessary to increase the final acetonitrile concentration to 50%.

5.1.4 Stability of the DNP label to synthesis and deprotection conditions

An HPLC chromatogram of crude ([3]p)5(Tp)11T before subjection to base deprotection conditions is shown in Figure 24(a). Clearly this trace is not consistent with the recorded coupling efficiency for monomer [3] of >98%. This suggests that substantial product degradation has taken place, not during synthesis, but during the end procedure in which oligonucleotides are cleaved from the solid support
Figure 24(a): Reversed phase HPLC chromatogram of crude ([3]p)5(Tp)11T.

Figure 24(b): Reversed phase HPLC chromatogram of (1) purified ([3]p)5(Tp)11T and (2) after treatment with c.NH4OH at 55°C for 12hrs.
by treatment with ammonia. Nevertheless, peak (I) in Figure 24(a) was isolated using reversed phase HPLC as an intensely yellow oligonucleotide product. Assuming that complete label degradation had not taken place, both the long retention time and colour suggested that this product was pure ([3]p)_5(Tp)_{11}T.

Surprisingly, subjection of the DNP labelled oligonucleotide to prolonged base deprotection conditions (c.NH4OH @ 55°C for 12 hours) resulted in only a small amount of further degradation (Figure 24(b)). Contrary to initial observations, this suggests that multiple DNP labels are stable to ammonia treatment. This apparent discrepancy will be discussed more fully in conjunction with monomer [12].

5.1.5 Synthesis of multiple addition DNP monomer [9]. (Scheme 2)

In addition to stability problems, the usefulness of monomer [3] is also restricted for the following reasons:

(a) The distance between the DNP label and the oligonucleotide phosphate backbone is relatively small, in the above case this was a two atom ethylene spacer. As both antibody and labelled oligonucleotide are large molecules, subsequent steric hindrance may result in a decrease of anti-DNP antibody association constant. This would reduce signal sensitivity in detection experiments.

(b) The monoclonal antibody to be used in subsequent detection experiments was raised against inactivated *E. coli.* treated with an excess of 2,4-dinitrofluorobenzene. This compound reacts with accessible cysteine and lysine residues, converting these groups into their DNP derivatives. Antibody sensitivity should therefore be greater towards DNP groups located at the ends of slightly longer alkylamino chains.
Scheme 2: Synthesis of DNP phosphoramidite [9]
We therefore decided to synthesize another multiple addition DNP monomer, retaining the 3-aminopropane-1,2-diol moiety, but distancing the DNP group from this by means of an aminohexanoic acid spacer arm. This would produce a more suitable DNP label and also allow further investigation into the stability of 3-aminopropane-1,2-diol based monomers.

6-Aminohexanoic acid was treated with di-t-butyldicarbonate in dioxane under basic conditions, to give after acidic work-up, the t-butyloxycarbonyl (BOC) protected amino acid [4] in 90% yield. This was converted into the pentachlorophenyl active ester [5] in 95% yield by reaction with pentachlorophenol and dicyclohexylcarbodiimide in anhydrous dichloromethane. Subsequent treatment of the active ester [5] with 3-amino-1,2-propanediol in DMF in the presence of triethylamine, gave after wet flash column chromatography the BOC-diol [6] in 91% yield. The BOC group was then removed by treatment with trifluoroacetic acid in dichloromethane. On basification with triethylamine prior to addition of the DNP group, a dramatic change in the colour of the solution was observed, from deep blue in acidic solution, to a bright yellow in excess base. Addition of 2,4-dinitrofluorobenzene converted the crude free amine into the DNP-diol [7], which gave, after an extensive work-up, a yield of 69%. This compound was then tritylated and phosphitylated following the standard procedure outlined above for the previous phosphoramidite, to give the DNP monomer [8] as a yellow solid in an overall yield of 67%.

5.1.6 Oligonucleotide synthesis using [9]

Unfortunately the DNP phosphoramidite [9] proved to be insoluble in anhydrous acetonitrile, necessitating the use of dichloromethane as
solvent. To compensate for the higher density of dichloromethane relative to acetonitrile, the standard 0.2µmol synthesis cycle for this monomer was altered to ensure delivery of the correct volume of monomer solution to the column. Unfortunately this phosphoramide repeatedly gave coupling efficiencies of <85% and increases to both the original monomer concentration of 0.15M and monomer to column wait time had little effect in raising coupling efficiency. This result remains unexplained, although the low coupling may arise from interactions between the phosphoramide moiety and the adjacent amide bond during oligonucleotide synthesis. Thus phosphoramide [9] is unsuitable for use as a multiple addition DNP monomer.

5.1.7 HPLC analysis of oligonucleotides labelled with [9]

Although DNP phosphoramide [9] failed as an efficient multiple addition monomer, oligonucleotides containing multiple DNP labels using this compound were obtained, albeit in very low yields. These were then subject to the same base deprotection stability tests as oligonucleotides labelled with DNP monomer [3]. HPLC analysis did show that oligonucleotides labelled with [9] are reasonably stable to base deprotection conditions. (Figure 25)

5.2 3-Aminopropyl solketal based DNP monomers

Misiura et al (45) have recently reported the use of 3-aminopropyl solketal as the basis for both biotin and phosphotyrosine multiple addition monomers. This compound contains an acetonide protected 1,2-diol moiety and an amine group located at the end of a 5-atom spacer
Figure 25: Reversed phase HPLC chromatogram of (1) purified ([9]p)$_3$(Tp)$_{11}$T and (2) after treatment with c.NH$_4$OH at 55°C for 12 hrs.
arm. We decided to investigate this system for the synthesis of DNP multiple addition monomers.

5.2.1 Synthesis of multiple addition DNP monomer [12] (Scheme 3)

No alterations were made to the original experimental procedure as described by Misiura et al, and 3-aminopropyl solketal was obtained in similar yield. 3-Aminopropyl solketal was treated with 2,4-dinitrofluorobenzene in methanol in the presence of triethylamine to give, after wet flash column chromatography the DNP derivative [10] in 94% yield. The isopropylidene group was removed by treatment with aqueous HCl in tetrahydrofuran, residual HCl and water were removed by coevaporation with anhydrous pyridine, and the resulting diol reacted with 4,4′-dimethoxytrityl chloride in anhydrous pyridine to give the DNP monomer precursor [11], after wet flash column chromatography, in 79% yield. Reaction of this compound with 2-cyanoethyl-N,N-diisopropylphosphoramidochloridite in anhydrous tetrahydrofuran in the presence of N,N-diisopropylethylamine, gave the DNP phosphoramidite [12] in 83% yield. Unlike the previous monomer syntheses this phosphoramidite was not subject to a hexane precipitation procedure, the monomer was simply dried under oil pump vacuum overnight.

5.2.2 Oligonucleotide synthesis using [12]

The aminopropylsolketal based DNP phosphoramidite [12] was very soluble in anhydrous acetonitrile and was used as a 0.15M solution during standard DNA synthesis cycles. The routine coupling efficiency for monomer [12] was 98%.
Scheme 3: Synthesis of DNP phosphoramidite [12]
5.2.3 Stability of DNP monomer [12] to DNA synthesis and deprotection conditions

Misiura et al reported that labelled oligonucleotides synthesized with 3-aminopropyl solketal based monomers had to be synthesized "trityl-on", i.e. the 5'-terminal dimethoxytrityl protecting group was not removed prior to oligonucleotide cleavage from the resin with c.NH₄OH. This was found necessary to prevent the attack of the free primary hydroxyl moiety on the adjacent phosphodiester linkage under strongly basic conditions. Confirmation of this was obtained by the synthesis of [12]p(Tp)₁₄T using the standard "trityl-off" procedure. It is clear from Figure 26(a) that despite monomer [12] having a coupling efficiency of 98%, substantial product degradation has taken place before subjecting the labelled oligonucleotide to base deprotection conditions. The peak marked (I) corresponds to the bright yellow, DNP labelled product, while Figure 26(b) confirms that peak (II) is (Tp)₁₄T. The probable mechanism of degradation is shown in Figure (27). This also accounts for the degradation observed after synthesis of oligonucleotides labelled with monomer [3]. The same vicinal diol arrangement is present in both the 3-aminopropane-1,2-diol and 3-aminopropyl solketal based monomers. Fortunately multiple DNP labelled oligonucleotides synthesized "trityl-on" are completely stable to normal deprotection conditions as shown in Figure 28(a). Indeed, an additional advantage is gained by "trityl-on" syntheses in that the extra lipophilicity makes separation of DNP labelled from unlabelled failure sequences easier. Subsequent treatment of "trityl-on" oligonucleotides with acetic acid removes the terminal dimethoxytrityl group quickly and cleanly. The effect on the HPLC behaviour of oligonucleotides labelled with increasing numbers of DNP groups is shown in Figure 28(b).
Figure 26(a): Reversed phase HPLC chromatogram of crude [12]p(Tp)_{14}T synthesized "trityl-off". (analytical injection before base deprotection)

Figure 26(b): Reversed phase HPLC chromatogram of crude [12]p(Tp)_{14}T synthesized "trityl-off" with (Tp)_{14}T peak enhancement
Figure 27: Possible mechanism of degradation for labelled oligonucleotides containing a 1,2-diol backbone.
Figure 28(a): Reversed phase HPLC chromatogram of crude ([12]p)₄(Tp)₉T synthesized "trityl-on" and after treatment with c.NH₄OH @ 55°C for 12hrs.

Figure 28(b): Reversed phase HPLC chromatogram of a mixed injection of purified detritylated multiple DNP labelled 24mers and unlabelled reference sequence: From left to right: unmodified 24mer, (DNP)₃-24mer and (DNP)₅-24mer. DNP labels were introduced via phosphoramidite [12].
5.2.4 Synthesis of multiple addition DNP monomer [18] (Scheme 4)

In order to establish the length of spacer arm most suitable for optimum antibody binding we decided to synthesize an additional DNP monomer containing a longer alkyl spacer. This was achieved by extending the existing 5-atom spacer to 12 atoms by the addition of 6-aminohexanoic acid to 3-aminopropyl solketal.

6-Aminohexanoic acid was reacted with ethyl trifluoroacetate to give a near quantitative yield of trifluoroacetyl (TFA) protected amino acid [13]. This was converted to the 2-nitrophenol active ester [14] which was reacted with 3-aminopropyl solketal to give the TFA-acetonide [15] in quantitative yield. Heating this compound in a sealed pressure tube containing conc. aqueous ammonia at 70°C cleanly removed the TFA protecting group and subsequent reaction of the crude free amine with 2,4-dinitrofluorobenzene in methanol and triethylamine gave the DNP-acetonide [16] in 93% yield. Removal of the isopropyldine group by acidic treatment followed by standard tritylation and phosphitylation procedures, gave the DNP phosphoramidite [18] in an overall yield of 78%.

5.2.5 Oligonucleotide synthesis using [18]

The long chain DNP phosphoramidite [18] was found to be very soluble in anhydrous acetonitrile and was used as a 0.15M solution during standard DNA synthesis cycles. The routine coupling efficiency for monomer [18] was 98%.
Scheme 4: Synthesis of DNP phosphoramidite [18]
5.2.6 Stability of DNP monomer [18] to DNA synthesis and deprotection conditions

As monomer [18] is essentially an extended analogue of 3-aminopropyl solketal based monomer [12] syntheses were carried out "trityl-on". Labelled oligonucleotides synthesized in this way were stable even after prolonged c.NH₄OH treatment as shown in Figure 29(a). The effect on the HPLC behaviour of oligonucleotides labelled with increasing numbers of DNP groups from monomer [18] is shown in Figure 29(b).

5.3 Conclusions

The following conclusions may be drawn from work carried out on the synthesis and use of multiple addition DNP monomers [3], [9], [12] and [18]

(1) All of the DNP monomers were synthesized in good yield from inexpensive starting materials.

(2) Monomers [3], [12], [18] coupled with high yield (>98%) during normal solid phase synthesis with only a minor modification required to the standard synthesis cycle for [3]. Monomer [9] was insoluble in acetonitrile and continued to give low coupling efficiencies even after modification to solvent and synthesis cycles.

(3) Substantial degradation of oligonucleotides labelled with monomers [3], [12] and [18] occurred during cleavage of the oligonucleotide from the solid support with ammonia. This is an inherent problem with monomers containing 1,2-diol backbones through the attack of the free primary hydroxyl moiety on adjacent phosphodiester linkages under strongly basic conditions. However, this could be prevented by carrying out syntheses "trityl-on"
Figure 29(a): Reversed phase HPLC chromatogram of crude \([(17)p]_4(Tp)_{11}T\) synthesized "trityl on" and after treatment with c.NH_4OH @ 55°C for 12hrs.

Figure 29(b): Reversed phase HPLC chromatogram of a mixed injection of purified detritylated multiple long chain DNP labelled 24mers and unlabelled reference sequence. From left to right: unmodified 24mer, (DNP)\_1\-24mer, (DNP)\_3\-24mer and (DNP)\_5\-24mer. Labels were introduced via phosphoramidite [17]
(4) Subjection of "trityl-on" oligonucleotides to even prolonged base deprotection conditions (c.NH₄OH @ 55°C for 12hrs) results in little or no additional product degradation.

(5) After base deprotection, the dimethoxytrityl group may be removed by a simple two-step procedure with no additional HPLC purification required.
6.0 Antibody Mediated Detection of DNP Labelled Oligonucleotides

6.1 Synthesis of DNP Labelled Oligonucleotides

The following colorimetric detection experiments were carried out using oligonucleotides labelled with DNP monomer [12] unless otherwise stated. All syntheses were carried out "trityl-on" and the trityl group removed after base deprotection with acetic acid. Purification was carried out by preparative reversed phase HPLC as described in the preceding chapter. The oligonucleotide sequence in the following experiments was originally designed by Dr. Rick Randall of St. Andrews University as a PCR primer for HIV detection. Although chosen mainly for convenience, this sequence contains a number of thymines which enhance the crosslinking ability of oligonucleotides to nylon membrane during UV irradiation.

6.2 DNP Label Detection.

The detection of DNP labelled oligonucleotides (Figure 30) was carried out simply and conveniently using the following procedure:

(i) Known amounts of DNP labelled 24-mer probes were fixed to a nylon membrane by exposure to 300 nm UV irradiation for 5 mins (The DNP labels were stable to prolonged irradiation).

(ii) After blocking with non-fat skimmed milk the membrane was incubated with a mouse IgG anti-DNP monoclonal antibody, followed by incubation with a goat anti-mouse IgG horseradish peroxidase conjugate.

(iii) Subsequent incubation with diaminobenzidine/H2O2 allowed colorimetric visualisation of the fixed oligonucleotides.

The detection of biotin labels was carried out similarly, substituting step (ii) for an incubation with an avidin-horseradish peroxidase conjugate.
Figure 30: Colorimetric detection of DNP labelled probes using an indirect antibody ELISA system
It is clear from Figure 31(a) that three DNP labels give better sensitivity than a single DNP group. However increasing the number of DNP labels from three to five did not give any further improvement (Figures 31(a), 33(a) and 34). Why do three DNP groups give optimum sensitivity? Clearly, it is unlikely that more than one antibody molecule can bind simultaneously to a short string of DNP groups due to the huge size of an IgG molecule relative to the DNP monomer units. In addition, the space between the two FAB regions of an IgG molecule is very large so it is not possible for both arms of the antibody to bind to a single oligonucleotide.

Does the steric bulk of the oligonucleotide hinder the interaction between some of the DNP groups and the antibody, with the first two labels acting merely as spacers? To investigate this further we decided to incorporate an additional spacer to distance the DNP groups from the oligonucleotide backbone. The lengths of different spacer arms were compared using a molecular structure program (Alchemy II, Tripos Associates) and tetraethylene glycol chosen as the one corresponding most closely to two fully extended DNP monomers. The distance of a terminal DNP group from the oligonucleotide in a triply labelled probe could therefore be mimicked by the addition of one DNP group onto the end of a tetraethylene glycol spacer (Figure 32). If the increase in sensitivity on going from one to three DNP groups is due only to a reduction in steric constraints, the spaced system should give the same sensitivity during antibody detection as a triply labelled probe.

Tetraethylene glycol was tritylated with 4,4'-dimethoxytrityl chloride in pyridine to give the monotrityl compound [20] in 60% yield. Standard phosphitylation procedure gave the tetraethylene glycol monomer [21] in 96% yield (Scheme 5). The phosphoramidite was used
Figure 31(a): Colorimetric detection of DNP labelled 24-mer probes on nylon membrane. DNP labels introduced via DNP monomer [12].

Figure 31(b): Colorimetric detection of DNP labels attached to 24-mer probes by a tetraethyleneglycol linker arm. DNP labels were introduced via DNP monomer [12].
as a 0.15M solution in anhydrous dichloromethane due to insolubility in acetonitrile. A modified 0.2μmol synthesis cycle was used which included an extended coupling time of 4 mins and additional dichloromethane and acetonitrile washes. This gave a monomer coupling efficiency of 90%. Oligonucleotides were synthesized incorporating this spacer followed by additions of one or three DNP groups.

Although the tetraethylene glycol linker arm separates the DNP groups from the oligonucleotide it does not produce any enhancement in colorimetric detection (Figure 31(b)). As tetraethylene glycol is similar in length to two DNP monomer units and is sterically undemanding, the lack of increased signal with this spacer suggests that the steric bulk of the oligonucleotide does not hinder the antibody-hapten interaction. It is possible that the increased sensitivity achieved by the three DNP groups relative to one arises from co-operativity between DNP groups. When one DNP group occupies the antibody binding site, its neighbours all lie very close to the site. Thus there is a high local concentration of haptens around the antibody binding site. Under these circumstances it is reasonable to assume that three DNP groups may be as effective as five.

Significantly, labelling with three or more DNP groups gave detection limits comparable to those of biotin labelled oligonucleotides (Figure 33(a) and 33(b)).

DNP monomers [12] and [18] were also used in conjunction with a DNP monomer (125) developed by David Will (formerly of this research group) to investigate the relationship between the sensitivity of detection and spacer arm length. Figure (34) shows that there is no significant difference in detection sensitivity between any of the monomers. This suggests that the optimum DNP labelling system will
Scheme 5: Synthesis of the tetraethyleneglycol monomer [20]

Figure 32: Comparison of linker arm lengths using Alchemy II
Figure 33(a): Colorimetric detection of the DNP labels in 3'-DNP/5'-biotin labelled 24-mer probes. DNP labels were introduced via phosphoramidite [12].

Figure 33(b): Colorimetric detection of the biotin labels in 3'-DNP/5'-biotin labelled 24-mer probes. DNP labels were introduced via phosphoramidite [12].
Figure 34: Colorimetric detection of DNP labels of varying spacer arm length in 24-mer probes. The labels denoted SDNP, MDNP and LDNP were introduced using DNP monomers [12], [18] and [DW].

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DNP monomer [DW]
consist of three DNP labels attached to an oligonucleotide probe via a spacer arm of any reasonable length.

6.3 DNP Label Detection via anti-DNP Antibody-Enzyme Conjugates

Although DNP labels may be detected with high sensitivity using the indirect antibody ELISA system described above, it may be possible to improve both results and procedure by the use of a single anti-DNP antibody-enzyme conjugate. This system has the following advantages:

(1) Cost is lowered by reducing the number of reagents required for detection.

(2) Results can be obtained more rapidly by reducing the number of steps in the detection procedure.

(3) Reducing the number of antibodies to which membranes are exposed can decrease background signal caused by the non-specific binding of proteins.

(4) Anti-DNP antibody-horseradish peroxidase conjugates are commercially available from many sources.

However, the use of antibody-enzyme conjugates may suffer from the following disadvantages:

(1) It is possible for only one conjugate molecule to bind to an oligonucleotide probe at any one time, unless the latter is very long and is labelled at both ends. Therefore, unlike the indirect antibody ELISA system, signal amplification through multiple secondary to primary antibody binding cannot take place. This will result in a lowering of detection sensitivity

(2) Commercially available anti-DNP antibodies may be less sensitive to DNP groups located on the end of alkylamino chains than the
specific monoclonal antibody supplied by our collaborator. Again, this may result in a reduction of detection signal.

We therefore decided to conjugate our own anti-DNP antibody to horseradish peroxidase and compare this to both a commercially available conjugate and to the original indirect antibody ELISA system discussed previously.

6.3.1 Purification of mouse monoclonal IgG anti-DNP antibody:

The anti-DNP antibody (designated K3) used in the above detection experiments was supplied as crude murine ascitic fluid. This necessitated purification of the antibody before conjugation to horseradish peroxidase.

Preliminary purification consisted of antibody precipitation from 45% ammonium sulphate solution followed by centrifugation to yield an off-white pellet of crude antibody. This was purified further by FPLC using a Protein A superose column and eluting with 0.1M citric acid and a gradient of pH=8 to pH=3. Antibody purified in this way was desalted on PD-10 columns followed by concentration with nitrocellulose centrifuge filters (30,000 Mwt. cut off, Amicon). Alternatively, purification was carried out on Protein A gravity elution columns (Pierce). Yields of up to 5mg of antibody/ml of ascitic fluid were obtained using the above procedures. Samples not required for immediate use were then lyophilised or stored frozen at -20°C.

6.3.2 Conjugation of anti-DNP antibody to horseradish peroxidase.

Horseradish peroxidase was first activated by treatment with sodium periodate which converts cis 1,2-diols (as found in glycosylated regions of horseradish peroxidase) into dialdehydic moieties. These are
Figure 34(a): Pure anti-DNP antibody (K3) analysed by polyacrylamide gel electrophoresis. Antibody samples were denatured with β-mercaptoethanol prior to electrophoresis. Lanes 1 and 10, molecular weight markers. Lanes 2 to 9, samples of pure antibody, 8μg to 1μg respectively.
very reactive to primary amines and addition of pure antibody under basic conditions results in a crosslinking of antibody lysine residues to the enzyme.

Initial purification of the conjugate involved precipitation from saturated ammonium sulphate solution and centrifugation to give a faintly brown pellet. Passage through a Sephacryl-200 gel filtration column gave after concentration and desalting, 3ml of conjugate solution at ~0.25mg/ml.

6.3.3 Detection of DNP labelled oligonucleotides with anti-DNP-horseradish peroxidase conjugates

The detection of DNP labelled oligonucleotides was carried out as described previously with the following modifications:

(1) DNP labelled probes were hybridised to complementary target oligonucleotides bound to the membrane by UV crosslinking. The latter were synthesized with additional 5'-poly-T tails. Theoretically, this would allow efficient crosslinking whilst maintaining the integrity of the duplex forming section of oligonucleotide.

(2) DNP labels were detected using the K3 anti-DNP antibody-peroxidase conjugate described above and a commercially available rabbit anti-DNP polyclonal antibody-horseradish peroxidase conjugate (Dakopats). Detection using the indirect antibody ELISA system was carried out under identical conditions to give a direct comparison of sensitivity and background levels.

It is clear from Figure (35) that both the K3 and the commercial anti-DNP antibody conjugates give detection sensitivities slightly lower than that of the indirect antibody ELISA system. This decrease in sensitivity may be a result of any of the following:
Figure 35: Colorimetric detection of a hybridised triple DNP labelled 24-mer probe using K3 anti-DNP-HRP conjugate, commercial anti-DNP-HRP conjugate (DAKO) and the anti-DNP/anti-IgG-HRP system.
(1) The absence of signal amplification gained through multiple secondary to primary antibody binding.

(2) A reduction in antibody association constant through the attachment of a very large, sterically demanding horseradish peroxidase molecule. This is essentially an uncontrolled reaction with crosslinking taking place at any accessible nucleophile, usually lysine, on the surface of the antibody. Crosslinking close to an antibody binding site may therefore hinder interaction with the antigen.

(3) For reasons similar to (2), the conjugation procedure may have decreased the activity of the horseradish peroxidase.

Figure (35) also shows a substantial decrease in background signal by using the K3 and commercial conjugate instead of the indirect antibody ELISA system. Unfortunately, the use of the K3 conjugate did not result in improved sensitivity over the commercial conjugate, but rather, gave almost identical results. It is likely then that the antibody used for the commercial conjugate is itself very compatible with this system, and that what we are observing is a limit imposed on detection sensitivity by the efficiency of the enzyme in a one hapten-one enzyme detection system.

6.4 DNP Label Detection via anti-DNP-Alkaline Phosphatase Conjugates

Of the detection systems discussed previously, all have made use of horseradish peroxidase, an enzyme made popular through high activity, low cost and easy handling. However, many applications use conditions which are not ideally suited to this enzyme. Indeed, horseradish peroxidase denatures and rapidly loses activity at temperatures above 42°C (21). This has prompted the use of the more thermally stable
enzyme alkaline phosphatase. This enzyme has an optimum operating temperature of 45°C and can remain active for more than 48hrs.

It is interesting to note that Boehringer Mannheim, patent holder for the digoxigenin detection system, supply only anti-digoxigenin antibody-alkaline phosphatase conjugates for digoxigenin detection. As this system has effectively set the standards for non-radioactive labelling, we decided to directly compare the DNP and digoxigenin systems by detection with antibody-alkaline phosphatase conjugates.

Unfortunately, anti-DNP-alkaline phosphatase conjugates are not commercially available and therefore the synthesis of our own K3 antibody-alkaline phosphatase conjugate was attempted. The following procedure was modified from a method currently in use by BioScot Ltd. to produce commercial mouse IgG antibody-alkaline phosphatase conjugates.

6.4.1 Synthesis of K3 anti-DNP antibody-alkaline phosphatase conjugate

Alkaline phosphatase (2mg) was activated by the addition of glutaric dialdehyde in phosphate buffer (50mM, pH=7.2). After incubation at room temperature for 1hr, one equivalent of purified K3 anti-DNP antibody (1mg) was added and the resulting mixture incubated for a further 2hrs. The crude product was then purified by passage through a column of Sephacryl-200, eluting with PBS (50mM, pH=7.4). The resulting product was concentrated using Microcon centrifuge filters (30,000 M.Wt.) to give ~1mg of purified conjugate.

6.4.2 Detection of DNP labelled probes with an anti-DNP antibody-alkaline phosphatase conjugate
Similar to the detection of DNP labelled probes using anti-DNP antibody-horseradish peroxidase conjugates, a complementary oligonucleotide with an additional 5'-poly-T tail was synthesised and attached to nylon membrane by UV irradiation. Hybridisation experiments were then carried out using a triply labelled DNP probe and a singly labelled digoxigenin probe. Identical blocking, washing and development protocols were used in each case.

Unfortunately, the detection of DNP labelled probes using the K3 anti-DNP antibody-alkaline phosphatase conjugate gave very poor detection sensitivities (results not shown) when compared to the digoxigenin system. Detection of DNP probes directly bound to the membrane (ie. no hybridisation step) also gave very poor results. Despite more stringent purification of the conjugate and modification to the conjugation procedure itself no improvement in conjugate performance was obtained. However, after discussions with the Biological Research and Development section of Dakopats Ltd., a small amount of recently developed, commercially unavailable, anti-DNP-alkaline phosphatase conjugate was provided as a gift. The hybridisation experiments were repeated and detection of DNP labelled probes carried out with this conjugate. In addition, the opportunity was taken to compare both the DNP and digoxigenin systems to a fluorescein detection system recently developed by Dakopats. This also employs an anti-fluorescein antibody-alkaline phosphatase conjugte. Singly labelled fluorescein probes were produced by the treatment of an aminolink oligonucleotide with fluorescein isothiocyanate, and used directly after purification in identical hybridisation experiments.
It is clear from Figure (36) that all three detection systems give very high, almost identical detection sensitivities, with a slightly higher background evident for the fluorescein system.

It is interesting to note that detection of DNP labelled probes with alkaline phosphatase gave a 10-fold increase in sensitivity over the indirect antibody horseradish peroxidase system.

6.5 Conclusions

The above results have shown that:

(i) Oligonucleotides labelled with 3 DNP groups give better sensitivity than a single DNP label, however increasing the number of labels to 5 did not give any further improvement.

(ii) The optimum DNP labelling system would consist of 3 DNP groups attached to a linker arm of any reasonable length.

(iii) The detection of triply labelled DNP oligonucleotides using antibody-horseradish peroxidase conjugates, either directly or indirectly, give detection sensitivities comparable to those obtained by biotin labelled systems. In addition, the use of anti-DNP antibody-enzyme conjugates gives a lower background signal than that of indirect ELISA systems.

(iv) The detection of triply labelled DNP oligonucleotides using an anti-DNP antibody-alkaline phosphatase conjugate gives detection sensitivities comparable to those obtained from digoxigenin labelled systems.

(v) The use of alkaline phosphatase conjugates gives a 10-fold increase in detection sensitivity over similar horseradish peroxidase conjugate systems.
It is clear that the DNP system has all of the advantages, but none of the disadvantages of currently available oligonucleotide labelling systems. This augurs well for the establishment of DNP labelling as a viable alternative to both biotin and digoxigenin labelling systems.
Figure 36: Colorimetric detection of hybridized digoxigenin (DIG), 2,4-dinitrophenyl (DNP) and fluorescein (FLU) labelled 24-mer probes using the corresponding antibody alkaline phosphatase conjugates.
6.6 Applications

Having demonstrated that the DNP labelling system works well in simple dot-blot and hybridisation experiments we decided to synthesize DNP labelled oligonucleotide probes for use in "real" applications. Over 160 DNP labelled oligonucleotides were synthesized for a wide variety of applications. Examples of some of these applications and those responsible for the work are shown below:

(1) Dr. A. Tabor, Department of Chemistry, Edinburgh University.

DNP labelled probes were synthesized for the detection of cells producing specific DNA binding proteins. Protein producing plaques can be incubated with DNP labelled probes containing a target base sequence. Subsequent adsorption of protein-DNA complexes onto nitrocellulose and detection by anti-DNP antibody-enzyme conjugates film allows the identification of cell lines of interest.

(2) Dr. P. Simmonds, Department of Pharmacology, Edinburgh University.

Oligonucleotides containing 5'-phosphate and 3'-DNP groups were synthesized to enable the detection of ligase chain reaction products. Ligation of these to biotinylated probes produced 5'-biotin/3'-DNP labelled oligonucleotides. These were detectable using simple capture and detect experiments.

(3) Paul Turner, Department of Immunology, Shell Research, Sittingbourne.

DNP labelled oligonucleotides were synthesized to assist the development of a hybridisation assay for use in DNA mismatch detection.
This enabled a successful evaluation of the microtitre ELISA based system before mismatch detection with anti-DNA mismatch antibodies.

(4) Dr. P. Balfe. Department of Medical Microbiology, UCMSM, London.

Work was carried out to develop a microtitre format ELISA system for the detection of PCR products using biotinylated and DNP labelled PCR primers.

(5) Dr. K. Stevenson, Moredun Research Institute, Edinburgh.

DNP labeled oligonucleotides were used in dot blot hybridization experiments for the detection of the specific insertion sequence IS900 of Mycobacterium avium subspecies paratuberculosis. PCR products were crosslinked to nylon membrane and detected by DNP probe hybridization. Detection limits were comparable with those obtained from a previously established digoxigenin detection system.

(6) Dr. H. Cubie. Regional Virology Laboratory, City Hospital, Edinburgh

(i) DNP labelled probes were synthesized for in situ hybridization experiments designed to detect Epstein Barr Virus infection in clinical tissue samples. Work carried out at the Regional Virology Laboratory compared detection limits of DNP labelled probes with those of an established digoxigenin system.

(ii) In addition to work on EBV, the Regional Virology Laboratory has carried out extensive tests on the use of DNP labelled probes to detect Human Parvovirus B19. In this case, the base sequence of the viral genome was studied and 10 sequences evenly spaced along the genome were selected. This allowed the synthesis of a "probe pool" of 10 labelled oligonucleotides, all of which were used simultaneously in the same
hybridization experiment. Labelling one pool with DNP and another pool with digoxigenin allowed a direct comparison between the two systems in a clinical setting. The result of these experiments showed an increase in sensitivity over single probes by using each pool, but most importantly the DNP pool had a similar detection sensitivity to the digoxigenin pool.

Figure (37) shows a series in situ hybridisation experiments in which the DNP pool of oligonucleotides has been used to detect Human Parvovirus B19 in a range of human tissue samples.
Figure 37: Detection of Human parvovirus B19 in clinical samples by \textit{in situ} hybridisation using DNP labelled oligonucleotide probes.
After demonstrating that simple DNP labelled phosphoramidites can be used to efficiently label synthetic oligonucleotides, we decided to investigate the possibility of using nucleoside based DNP monomers. These would allow the insertion of DNP labels along the length of an oligonucleotide during synthesis without affecting duplex formation. Indeed, well spaced single DNP labels may increase the number of antibodies which are able to bind simultaneously to a labelled probe and hence increase detection sensitivity. This alternative strategy was prompted by discussions with Cambridge Research Biosystems who expressed an interest in synthesizing modified nucleoside triphosphates for the enzymic incorporation of labels into oligonucleotides. The opportunity was taken to synthesize an amino modified 2'-deoxyuridine compound which would serve as a precursor to both a nucleoside based DNP phosphoramidite and a DNP labelled 2'-deoxyuridine 5'-triphosphate. The materials and reagents for this project were supplied by CRB and Cruachem and the work carried out jointly with Mandy Davidson of Cruachem.

7.1 Synthesis of 5-Propenyl-2'-deoxyuridine N-trifluoroacetyl-6-aminohexamide [22] (Scheme 6)

Treatment of 2'-deoxyuridine with mercuric acetate and then lithium chloride in sodium acetate buffer (pH=6.0) gave crude 5-chloromercuri-2'-deoxyuridine as a flocculent white precipitate which was then redispersed in sodium acetate buffer (pH=5.6). Subsequent addition of N-trifluoroacetyl-3-aminopropene and K₂PdCl₄ produced a thick black precipitate which was removed by passage through a column.
Scheme 6: Synthesis of 5-Propenyl-2'-deoxyuridine N-trifluoroacetyl-6-aminohexamide [22]
of celite. Treatment of the eluant with sodium borohydride, followed by silica gel chromatography gave pure 5[1-(N-trifluoroacetyl)-3-aminopropenyl] 2'-deoxyuridine [21] in 19% yield. Compound [21] was then treated with c.NH₄OH to remove the trifluoroacetyl protecting group. The crude aminopropenyl deoxyuridine derivative was then treated with succinimidyl N-trifluoroacetyl-6-aminohexanoate. Purification by silica gel chromatography gave 5-propenyl-2'-deoxyuridine N-trifluoroacetyl-6-aminohexamide [22] as a white foam in 78% yield.

7.2 Synthesis of DNP Nucleoside Monomer [25] (Scheme 7)

5-Propenyl-2'-deoxyuridine N-trifluoroacetyl-6-aminohexamide [22] was treated with conc. NH₄OH to remove the trifluoroacetyl protecting group and then treated with 2,4-dinitrofluorobenzene. Purification by silica gel chromatography gave 5-propenyl-2'-deoxyuridine N-(2,4-dinitrophenyl)-6-aminohexamide [23] in 83% yield. Compound [23] was then subject to standard tritylation and phosphitylation procedures to give the DNP 2'-deoxyuridine monomer [25] in an overall yield of 30%.

7.2.1 Synthesis of oligonucleotides containing DNP monomer [25]

DNP monomer [25] was very soluble in anhydrous acetonitrile and used as a 0.15M solution during a standard 0.2µmol oligonucleotide synthesis cycle. However, even after increasing monomer coupling times, this monomer repeatedly gave low coupling efficiencies of <90%. Unfortunately, when synthesizing small amounts (<100mg) of phosphoramidite, oxidation (which would have a negligible effect on larger quantities) can often result in poor monomer coupling efficiencies.
Scheme 7: Synthesis of DNP Nucleoside Monomer [25]
This phenomenon has been noted for even the more stable phosphoramidites when synthesized in small amounts. As only 100mg of DNP phosphoramidite was obtained from the above synthesis, this may have contributed to the poor coupling.

Unfortunately, time did not allow any further testing of monomer [25] as fresh monomer would have have been to be prepared starting again from 2'-deoxyuridine.

As well as being very expensive, it is clear that this synthetic route from 2'-deoxyuridine to the DNP monomer suffers from poor yields, particularly the first step in which 2'-deoxyuridine is converted to the aminomodified derivative [22]. Nevertheless, multiple internal labelling with DNP groups remains an interesting alternative labelling strategy, and work is currently being carried out in this group to establish a more efficient route to DNP labelled nucleoside phosphoramidites.

7.3 Synthesis of DNP Labelled 2'-Deoxyuridine 5'-Triphosphate [28]
(Scheme 8)

5-Propenyl-2'-deoxyuridine N-trifluoroacetyl-6-aminohexamide [22] was dissolved in anhydrous pyridine and treated with two equivalents of 4-toluenesulphonyl chloride to encourage a very sluggish reaction. The crude product was purified by silica gel chromatography to give 5-propenyl-5'-O-(4-toluenesulphonyl)-2'-deoxyuridine N-trifluoroacetyl-6-aminohexamide [26] as a white foam in 40% yield.

Tetra-(tetrabutylammonium)-triphosphate [29] was prepared by firstly passing sodium tripolyphosphate through a column of H+ form Dowex into a solution of tetrabutylammonium hydroxide. Sodium tripolyphosphate solution was then added via the Dowex column to bring the receiving solution to neutrality. This was lyophilized to give tetra-
Scheme 8: Synthesis of DNP labelled 2'-deoxyuridine triphosphate

Figure 37(a): Reversed phase HPLC chromatogram of crude DNP labelled 2'-deoxyuridine triphosphate (I) and diphosphate degradation product (II).
(tetrabutylammonium)-triphosphate [29] as a fluffy white solid. This was added without further purification to a solution of the tosyl compound [26] in acetonitrile and the reaction mixture incubated at room temperature for 7 days. The resultant solution was passed through a column of NH$_4^+$ form Dowex, the anisaldehyde positive fractions lyophilized and purified by cellulose chromatography to give the triphosphate [27] as a hygroscopic white solid. The trifluoroacetyl protected triphosphate [27] was then treated with ammonia to remove the trifluoroacetyl protecting group, the product dissolved after lyophilization in Na$_2$CO$_3$/NaHCO$_3$ buffer (pH=9.5), and this finally treated with DNP-NHS active ester [31] in DMSO at 45°C. Excess solvent was removed by lyophilization and the yellow product purified by reverse phase HPLC. Figure (37) shows the two product peaks obtained during preparative HPLC. These peaks (I) and (II) are thought to correspond to triphosphate and diphosphate degradation product respectively, both of which gave yellow, water soluble solids after lyophilization. Degradation may have occurred here through the elevated incubation temperature (45°C) needed to solvate both polar triphosphate and lipophilic DNP active ester in the buffer/DMSO mixture.

Samples of both the DNP-2'-deoxyuridine triphosphate [28] and the trifluoroacetyl protected 2'-deoxyuridine triphosphate have been sent to Cambridge Research Biosystems and are currently being tested as substrates for DNA polymerase.
8.0 Alternative Labelling Systems

8.1 Dansyl Labelled Oligonucleotides

Since its development by Weber (138) in 1951, the dansyl group has been used extensively to prepare fluorescent analogues of drugs, amino acids, proteins and more recently oligonucleotides (131, 139, 140). However, these compounds have invariably been used for their chromophoric properties and have not been detected immunogenically, the main drawback being the lack of commercially available anti-dansyl antibodies. We therefore decided to synthesize a multiple addition dansyl phosphoramidite similar to DNP monomer [12], and at the same time conjugate dansyl groups to proteins from which antibodies could be raised. This would allow the development of a novel detection system which might offer another alternative to digoxigenin and biotin labelling.

8.1.1 Synthesis of single addition dansyl phosphoramidite [33]

To establish the stability of the dansyl group to standard synthesis and deprotection conditions, and to investigate the general properties of dansyl labelled oligonucleotides, we decided to first synthesize a simple, single addition dansyl phosphoramidite, based on 6-aminohexanol (Scheme 9).

6-Aminohexanol in pyridine was treated with dansyl chloride and the resulting crude oil purified by silica gel chromatography. This gave N-dansyl-6-aminohexanol [32] as a green fluorescent glass in 71% yield. Standard phosphitylation procedure on compound [32] gave the single addition dansyl monomer [33] as a fluorescent green oil.
Scheme 9: Synthesis of Dansyl Monomers [33] and [36]
8.1.2 Oligonucleotide synthesis using [33]

Dansyl monomer [33] was very soluble in anhydrous acetonitrile and used as a 0.15M solution during standard DNA synthesis cycles. As this single addition monomer does not contain a dimethoxytrityl group, coupling efficiency was estimated by HPLC to be >95% (Figure 38(a)).

8.1.3 Stability of dansyl monomer [33] to DNA synthesis and deprotection conditions.

Treatment of a sample of crude ([33])p(Tp)12 with conc. NH₄OH for 12 hours at 55°C resulted in almost no oligonucleotide or label degradation (Figure 38(b)). This suggests that the dansyl group would be suitable for use in a multiple addition monomer.

8.1.4 Synthesis of multiple addition dansyl phosphoramidite [36]

Similar to DNP monomer [12], dansyl labels could now be incorporated into the previously discussed 3-aminopropylsolketal backbone to give a multiple addition dansyl monomer (Scheme 9).

3-Aminopropylsolketal was treated with dansyl chloride in methanol containing triethylamine, to give after work-up and silica gel purification, N-dansyl-1-amino-4-oxa-6,7-isopropylidene dioxyheptane [34] as a fluorescent green oil in 93% yield. The acetonide protecting group was then removed by the addition of conc. HCl in THF, with careful neutralisation required to isolate the crude diol as a free amine. Standard tritylation and phosphitylation procedures gave the multiple addition dansyl monomer [36] in 42% overall yield as a flourescent green oil.
Figure 38(a): Reversed phase HPLC chromatogram of crude ([33])p(Tp)_{11}T

Figure 38(b): Reversed phase HPLC chromatogram of crude ([33])p(Tp)_{11}T after treatment with conc. NH4OH @ 55°C for 12 hours
8.1.5 Oligonucleotide synthesis using [36]

Multiple addition dansyl monomer [36] was very soluble in anhydrous acetonitrile and was used as a 0.15M solution during standard oligonucleotide synthesis cycles. Coupling efficiencies for this monomer were routinely >97%. All syntheses were carried out "trityl-on".

8.1.6 Stability of multiple addition dansyl monomer [36] to DNA synthesis and deprotection conditions.

Oligonucleotides containing multiple dansyl labels were stable to conc. ammonia end procedures and base deprotection conditions if synthesized "trityl-on". Figure 39(a) shows a reversed phase HPLC trace, after base deprotection, of a 24-mer oligonucleotide labelled with 5 consecutive dansyl groups. Figure 39(b) shows the effect of 1,3 and 5 dansyl labels on the retention times on reversed phase HPLC of detritylated, dansyl labelled oligonucleotides.

8.1.7 Conjugation of dansyl groups to BSA and C γG

To elicit an immune response, antigens must first be attached to large carrier molecules (usually proteins) prior to immunisation. In this case, dansyl groups were attached to the proteins bovine serum albumin (BSA) and chicken-γ-globulin (C γG) by the following method:

The protein was dissolved in MES buffer (100mM, pH=6.2) followed by the addition of dansyl chloride in DMSO. The resulting mixture was incubated at 34°C overnight and the crude conjugate purified by NAP-10 column gel filtration.

The resulting dansyl-protein conjugates were sent to the Immunology Department of Unilever Research for antibody production.
Figure 39(a): Reversed phase HPLC chromatogram of a crude "trityl-on" synthesis of a (dansyl)\textsubscript{5}-24mer.

Figure 39(b): Reversed phase HPLC chromatogram of a mixed injection of purified, detritylated dansyl labelled 24-mers. From left to right: (dansyl)\textsubscript{1}-24mer, (dansyl)\textsubscript{3}-24mer and (dansyl)\textsubscript{5}-24mer. Labels were introduced via dansyl monomer [36]
The anti-dansyl antibodies are still under preparation at the time of writing.

8.1.8 Dansyl labelled oligonucleotides as fluorescent probes.

Although colorimetric detection experiments could not be carried out, we decided to investigate the fluorescence properties of multiply dansyl labelled oligonucleotides. Would multiply dansyl labelled oligonucleotides give a quantitative increase in fluorescence relative to the number of dansyl labels or would the relatively short 3-aminopropylsolketal backbones promote quenching of fluorescence by holding the dansyl labels in close proximity? In order to answer these questions, a Perkin Elmer LS50 luminescence spectrometer was used to excite the dansyl groups attached to oligonucleotides at a predetermined absorbance maximum of 335nm. Three oligonucleotides, each containing 1, 3 or 5 dansyl groups, were excited in separate solutions and the fluorescence spectra recorded. It is clear from Figure 40 that a quantitative increase in fluorescence does occur relative to the number of dansyl labels attached to the oligonucleotide. The dansyl labels must therefore be sufficiently distanced from each other to prevent quenching. In addition, it is interesting to note that as the number of dansyl groups is increased from 1 to 3 to 5, that the fluorescence maxima are blue-shifted from 522nm to 519nm to 513nm respectively. This is an effect which is commonly observed as the environment of a fluorescent species undergoes a change from a less to a more hydrophobic environment. Although no precise comparison was carried out, a qualitative estimate suggested that 5 dansyl labels yield ~20 fold less fluorescence than 1 fluorescein label.
Figure 40: Fluorescence spectrum of dansyl labelled 24mers: (dansyl)$_1$-24mer, (dansyl)$_2$-24mer and (dansyl)$_5$-24mer labelled (1), (3) and (5) respectively.
8.2 Oligonucleotide-Enzyme Conjugates

In recent years many methods have been reported which describe the covalent attachment of enzymes to oligonucleotides and their use as probes for in situ hybridisation experiments (12-15). Unfortunately, the production of oligonucleotide-enzyme conjugates is invariably expensive and usually yields only very small amounts of conjugate. In addition, some conjugation procedures can impair the activity of the enzyme or produce enzyme aggregates which can limit subsequent detection sensitivities. Nevertheless, products obtained from successful conjugation reactions can produce extremely effective non-radioactive hybridisation probes. For this reason and despite the high cost, many researchers make use of the conjugate synthesis services now offered by some biotechnology companies. Therefore, in collaboration with Oswel Research Products Ltd., and with the aim of producing a synthetic route which was both simple and relatively inexpensive, we decided to investigate the synthesis of oligonucleotide-alkaline phosphatase conjugates.

8.2.1 Synthesis of oligonucleotide-alkaline phosphatase conjugates.

As many conjugation reactions suffer from the use of unselective coupling reagents (eg DSS), we decided that the best approach would be the separate activation of the oligonucleotide and alkaline phosphatase. This type of strategy has been used for many years in the production of protein conjugates and many suitable reagents are commercially available. Thus we decided to produce a thiolated oligonucleotide and maleimidobenzoyl derivatised alkaline phosphatase, covalently crosslinking both of these via a Michael reaction (Scheme 10).
Scheme 10: Conjugation of Alkaline Phosphatase (AP) to an aminolink oligonucleotide with SPDP and MBS.
(1) Preparation of Thiolated Oligonucleotides.

As the conjugation of an oligonucleotide to an enzyme involves the crosslinking of two very large molecules, it is essential to distance these by using some type of spacer arm. This would help to minimise destabilising steric effects in subsequent hybridization experiments. For the following experiment we chose to react the commercially available active ester N-succinimidyl 3-(2-pyridyldithio)-propionate (SPDP) with a previously prepared aminolink oligonucleotide. This would introduce a disulphide moiety, and subsequently a free thiol group, onto the oligonucleotide at the end of a 13-atom linker arm.

An aminomodified oligonucleotide 19-mer probe supplied by the Oswel DNA Service (10 O.D.264) was treated with an excess of SPDP in DMSO/Na₂CO₃/NaHCO₃ buffer, pH=9.5 and incubated at room temperature for 2 hours. Preparative reverse phase H.P.L.C. (Figure 41) allowed isolation of the product as a peak (B) eluting significantly later than a mixture of minor products and unreacted starting material (A). The pure pyridyl disulphide modified oligonucleotide was then treated with a solution of dithiothreitol in concentrated aqueous ammonia at room temperature for 1 hour. It is clear from reversed phase HPLC (Figures 42 (a) and (b)) that conversion of the pyridyl disulphide oligonucleotide into a free thiol by dithiothreitol is essentially quantitative, therefore subsequent thiol oligonucleotides were purified simply by desalting on NAP-10 columns. Thiolated oligonucleotides were used for conjugation immediately to minimise oxidative dimerisation.
Figure 41: Reversed phase HPLC chromatogram of a crude SPDP derivatised 24-mer aminolink oligonucleotide
Figure 42(a) Reversed phase HPLC chromatogram of a pure 24-mer aminolink oligonucleotide derivatised with SPDP

Figure 42(b) Reversed phase HPLC chromatogram of a pure 24-mer aminolink oligonucleotide derivatised with SPDP after treatment with dithiothreitol.
(2) Activation of Alkaline Phosphatase.

Alkaline phosphatase was treated with the commercially available 3-maleimidobenzoyl-N-hydroxysuccinimide (MBS) in DMF/phosphate buffer (pH=7.0) for 1 hour at room temperature. This converted available lysine residues on the enzyme into maleimidobenzoyl derivatives (Scheme 10). The activated alkaline phosphatase was purified by passage through a NAP-10 column and concentrated using Amicon nitrocellulose centrifuge filters.

(3) Conjugation Reaction and Purification

The conjugation reaction simply involved the mixing of the thiolated oligonucleotide and the activated alkaline phosphatase and incubating overnight at room temperature. The reaction was carried out at pH=5.6 to minimise dimerisation of the thiolated oligonucleotide via disulphide formation. Purification of the resultant mixture was carried out by ion exchange FPLC (Figure 43, trace (3)). The conjugate (B) elutes significantly later than alkaline phosphatase (A) and can easily be separated from the thiolated oligonucleotide (C) and dimerised oligonucleotide (D). Although the conjugate elutes on ion exchange FPLC as two broad, overlapping peaks, these gave identical detection sensitivities during subsequent hybridisation experiments. Indeed, in hybridisation experiments similar to those carried out for DNP detection, these conjugates could be used to detect as little as 0.3 fmoles of target DNA (results not shown).

8.2.2 Conclusion

We have shown that effective oligonucleotide-alkaline phosphatase conjugates can be synthesized and purified simply and effectively by the
Figure 43: Combined ion exchange FPLC chromatograms of crude alkaline phosphatase-oligonucleotide conjugate reactions at pH's=9.0 (1), 7.0 (2) and 5.6 (3).
use of relatively inexpensive, commercially available reagents. However, the main drawback to this strategy is that the process is quite time consuming, due mainly to the number of steps involved in preparing the thiolated oligonucleotide. More effective ways of introducing thiol moieties into oligonucleotides are currently being investigated by this group.

The process outlined above has already been used to supply oligonucleotide-alkaline phosphate conjugates for many applications. These include the detection of hepatitis C virus (HCV) genomic RNA in clinical samples (Dr. Jeremy Garson, University College London Medical School) and the detection by in-situ hybridisation of nitric oxide synthetase in rat brains (Dr. Peirs Emson, MRC)

8.3 Suggestions For Future Work
(1) A full evaluation of the DNP labelling and detection system by the continued synthesis of DNP oligonucleotide probes for clinical applications.
(2) The synthesis of a more suitable nucleoside based DNP phosphoramidite via the simple 5-aminopropargyl modification of 2'-deoxyuridine, a reaction recently optimised by Duncan Graham of this group.
(3) Evaluation of the dansyl group as an immunogenic label using antibodies produced by Unilever Research.
(4) Improvement of the alkaline phosphatase-anti-DNP antibody conjugate synthesis described in Chapter 6 by the use of alternative coupling reagents such as S-acetylmercaptosuccinic anhydride (SAMSA).
9.0 Experimental

9.1 Solvents and Reagents

All solvents were of laboratory grade, except those used for the extraction and chromatography of phosphoramidites, which were of HPLC grade. Pyrogen-free, reverse osmosis purified water was used during oligonucleotide and enzyme conjugate synthesis and purification.

Anhydrous solvents

Dichloromethane and pyridine were distilled over CaH2; tetrahydrofuran and diethyl ether were distilled over sodium/benzophenone; N,N-dimethylformamide was fractionally distilled under reduced pressure over 4A molecular sieve; hexane was dried over Na wire; triethylamine and N,N-diisopropylethylamine were dried over CaH2; anhydrous acetonitrile was purchased from Applied Biosystems Inc.

Chemical reagents

2-Cyanoethyl-N,N-diisopropylphosphoramidochloridite was synthesized and purified following the procedure of Booth (141). Aminopropylsolketal was synthesized following the procedure of Misiura et al (45). 4,4’-Dimethoxytrityl chloride was obtained from Fluka and recrystallised from hexane containing 1% acetyl chloride. 4-Toluenesulphonyl chloride was obtained from Aldrich and recrystallised from 40-60 petroleum ether/chloroform. Trifluoroacetyl-3-aminopropene was obtained as a gift from Cambridge Research Biosystems. All other reagents were obtained from Aldrich or Fluka.
Biological reagents

Murine ascitic fluid containing anti-DNP antibody was supplied by Dr. Rick Randall of the Biochemistry Department, St. Andrews University. 3-Maleimidobenzoyl-N-hydroxysuccinimide, alkaline phosphatase (enzyme immunoassay grade) and anti-digoxigenin antibody-AP conjugate were obtained from Boehringer Mannheim. Anti-DNP and anti-fluorescein antibody-HRP/AP conjugates were obtained from Dakopats Ltd. PD-10 gel filtration columns were obtained from Pharmacia. All other reagents for dot blots and enzyme conjugations were obtained from Sigma.

9.2 Instrumentation

9.2.1 NMR

1H-NMR spectra were recorded on a Brucker WP-80 spectrometer (80MHz) and a Brucker WP-200 spectrometer (200.130MHz); 13C-NMR spectra were recorded on a Brucker WP-200 spectrometer (50.32MHz); 31P-NMR spectra were recorded on a Brucker WP-200 spectrometer (81MHz) or on a Jeol FX90Q spectrometer (90MHz).

9.2.2 Mass spectra

Positive ion Fast Atom Bombardment (FAB) mass spectra were recorded on a Kratos MS50 TC spectrometer using a thioglycerol or 3-nitrobenzyl alcohol (3-NOBA) matrix

9.2.3 IR spectra

IR spectra were recorded on either a Perkin-Elmer 781 or a Biorad FTS-7 fourier transform IR spectrophotometer using KBr plates and a polystyrene reference (1603 cm⁻¹ and 1029cm⁻¹).
9.3 Oligonucleotide synthesis.

Oligonucleotide synthesis was performed using cyanoethyl phosphoramidite chemistry on an Applied Biosystems 380B DNA synthesizer. All DNA synthesis reagents and nucleoside cyanoethyl-phosphoramidite monomers were supplied by Cruachem or ABI. DNP, dansyl and aminolink phosphoramidites were used as 0.15M solutions in either anhydrous acetonitrile or dichloromethane. "Trityl-on" synthesis was used throughout for phosphoramidites containing a 1,2-diol backbone. All oligonucleotides were deprotected in conc. aqueous ammonia for 8 hours at 60°C.

9.3.1 Measurement of phosphoramidite coupling efficiencies.

Coupling efficiencies were measured by comparison of the absorbance at 498nm of the dimethoxytrityl cations produced in the detritylation steps of successive synthesis cycles. The detritylation product fractions were diluted to 25ml with 0.1 M Toluene-4-sulphonic acid in acetonitrile before measurement.

9.3.2 Oligonucleotide analysis and purification.

HPLC analysis and purification of oligonucleotides was carried out on a Gilson model 306 using a Brownlee Aquapore Octyl reverse phase column (10 mm x 250 mm) with a flow rate of 3 ml/minute and the following gradient:
<table>
<thead>
<tr>
<th>Time (mins.)</th>
<th>%Buffer B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>15</td>
</tr>
<tr>
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<tr>
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<td>100</td>
</tr>
<tr>
<td>28</td>
<td>0</td>
</tr>
<tr>
<td>30</td>
<td>0</td>
</tr>
</tbody>
</table>

Buffer A: 0.1M NH₄OAc. Buffer B: 0.1M NH₄OAc with 50% acetonitrile. The desired "trityl-on" products eluted significantly later than failure sequences.

9.3.3 Detritylation of "trityl-on" oligonucleotides.

After HPLC purification "trityl-on" oligonucleotides were evaporated to dryness and dissolved in 3% aqueous acetic acid (10ml). After 30mins at 20°C the solutions were evaporated to dryness and desalted on NAP-10 columns (Sephadex G25) (Pharmacia) following manufacturers instructions.

9.4 Enzyme conjugate analysis and purification

F.P.L.C. antibody and conjugate analysis and purification was carried out on a Pharmacia GP-250 Plus system using both Mono-Q HR 5/5 anion exchange and Protein A Superose HR 10/2 columns.
Antibody purification: IgG molecules were injected onto the Protein A column in loading buffer (1.5M glycine, 3M NaCl, pH=8.9) and the antibody eluted with a flow rate of 2ml/minute and the following gradient:

<table>
<thead>
<tr>
<th>Time (mins.)</th>
<th>%Buffer B</th>
</tr>
</thead>
<tbody>
<tr>
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<td>0</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>24</td>
<td>100</td>
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<tr>
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<tr>
<td>30</td>
<td>0</td>
</tr>
<tr>
<td>35</td>
<td>0</td>
</tr>
</tbody>
</table>

Buffer A: 0.1M citric acid buffer (pH=8). Buffer B: 0.1M citric acid buffer (pH=3).

Oligonucleotide-Alkaline Phosphatase conjugate purification:
Conjugate samples were loaded onto the Mono-Q column in 2ml of Tris-HCl buffer (50mM, pH=7.4) and eluted with a flow rate of 4ml/minute and the following gradient:

<table>
<thead>
<tr>
<th>Time (mins.)</th>
<th>%Buffer B</th>
</tr>
</thead>
<tbody>
<tr>
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<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>2.5</td>
<td>30</td>
</tr>
<tr>
<td>21</td>
<td>80</td>
</tr>
<tr>
<td>21.5</td>
<td>0</td>
</tr>
<tr>
<td>25</td>
<td>0</td>
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</tbody>
</table>

Buffer A: Tris-HCl buffer (50mM, pH=7.4) Buffer B: Tris HCl buffer (50mM, pH=7.4) and 1M NaCl
**9.5 Chromatography**

Wet flash silica gel column chromatography was carried out using silica gel 60 (Fluka). Thin layer chromatography (t.l.c.) was carried out on aluminium sheets, silica 60 F254, 0.2mm layer (Merck) using the following solvent systems:

(A) Dichloromethane-methanol (99:1, v/v)
(B) Dichloromethane-methanol (95:5, v/v)
(C) Dichloromethane-methanol (9:1, v/v)
(D) Dichloromethane-methanol (85:15, v/v)
(E) Dichloromethane-methanol (80:20, v/v)
(F) Hexane-ethyl acetate (1:1, v/v)
(G) Ethyl acetate-methanol-NH4OH (5:1:1, v/v/v)
(H) Dichloromethane-ethyl acetate (1:1, v/v)
(I) Toluene-ethyl acetate (4:1, v/v)

Triethylamine (1%) was added to each of these solvent systems when compounds bearing a dimethoxytrityl group were examined by t.l.c.

Products were visualised on t.l.c. using the following techniques:
(i) UV absorption at 260nm for compounds with a chromophore.
(ii) Spraying with solutions of 10% phosphomolybdic acid in ethanol or 1% aqueous potassium permanganate, to give dark blue or white colouration respectively for oxidizable compounds.
(iii) Spraying with a solution of *para*-anisaldehyde: acetic acid: H2SO4: ethanol (5:1:1:100, v/v/v/v). Compounds with a protected or unprotected 1,2-diol function give a dark blue colour on heating.
(v) Spraying with a 1% solution of ninhydrin in ethanol. Amines give a strong purple colour on heating. Amides, tertiary amines and phthalimides give a brown colour on strong heating.
(vi) Compounds containing a dimethoxytrityl group give an orange colour on heating then exposure of the t.l.c. plate to HCl fumes.

9.6 Chemical Syntheses

3-(2,4-Dinitrophenylamino)-1,2-propanediol [1]

2,4-Dinitrofluorobenzene (10.2g, 57mmol) was added to a stirred solution of 3-amino-1,2-propanediol (4.89g, 54mmol) and triethylamine (23.4ml,0.15mol) in methanol (50ml) at room temperature. After 24 hours the reaction mixture was concentrated in vacuo, and the residue purified by wet flash column chromatography, eluting with a gradient of 0-10% methanol in dichloromethane. The appropriate fractions were collected and concentrated in vacuo to give the title compound as a yellow solid (11.36g, 82%). Rf 0.67 (solvent D). 1H n.m.r. data (CDCl3): δH 3.58-3.78 (m, 4H, CH, CH2 and OH), 3.94-4.15 (m, 2H, CH2), 4.54 (s, 1H, OH), 7.32 (d, 1H, J=9.4 Hz, DNP H-6), 8.32 (dd, 1H, J=2.8 Hz, J=9.4 Hz, DNP H-5), 9.01 (m, 2H, DNP H-3, DNP-NH). 13C n.m.r. data [(CD3)2CO]: δC 45.31 (CH2-NH), 63.19 (CH2-OH), 68.98 (CH-OH), 114.37 (Ar-CH), 122.67 (Ar-CH), 128.96 (Ar-CH), 129.41 (Ar-C), 134.66 (Ar-C), 148.09 (Ar-C). I.R. data (nujol mull) υmax: 3450 (m, OH), 3350 (m, secondary NH), 3100 (w, aromatic), 2930 (w, aliphatic), 2870 (w, aliphatic), 1620 (s, aromatic), 1590 (s, aryl nitro), 1540 (m), 1490 (m), 1420 (m), 1335 (s, aryl nitro), 1305 (s), 1235 (m), 1150 (m), 1130 (m), 1055 (m), 920 (m), 830 (w), 740 (m) cm⁻¹. F.a.b. mass spectrum: m/z 258.07259 calc. for C9H12N3O6 (M+H)+, m/z 258.07260.
**1-(4,4’-Dimethoxytrityloxy)-3-(2,4-dinitrophenylamino) propan-2-ol [2]**

Compound [1] (2.00g, 7.8mmol) was dried by coevaporation *in vacuo* with anhydrous pyridine (3x10ml) and dissolved in anhydrous pyridine (15ml). To this was added 4,4’-dimethoxytrityl chloride (2.72g, 8.0mmol) and the solution stirred at room temperature. After 12 hours the reaction was quenched with methanol (10ml) and excess pyridine removed *in vacuo*. The residue was dissolved in dichloromethane (50ml) and washed with saturated aqueous NaHCO₃ (50ml), and water (3x50ml). The organic layer was concentrated *in vacuo* and the residue was purified by wet flash silica gel chromatography, eluting with a gradient of 0-3% methanol in dichloromethane containing 2% triethylamine. The appropriate fractions were collected, concentrated *in vacuo*, and dissolved in the minimum volume of dichloromethane. Precipitation from hexane at -78 °C gave the title compound as a yellow powder (4.13g, 90%). Rf 0.52 (solvent H). ¹H n.m.r. data (CDCl₃): δH 3.31 (d, 2H, J=4.5 Hz, CH₂), 3.42-3.52 (m, 2H, CH₂) 3.77 (s, 7H, 2xOMe, OH), 4.09 (m, 1H, CH), 6.79-6.90 (m, 4H, 4xArH), 7.18-7.40 (m, 10H, 10xArH), 8.18 (dd, 1H, J=2.8 Hz, J=9.4 Hz, DNP H-5), 8.75 (bt, 1H, J=3.2 Hz, NH), 9.08 (d, 1H, J=2.8 Hz, DNP H-3). ¹³C n.m.r data (CDCl₃): δC 45.76 (CH₂-NH), 55.08 (OCH₃), 64.67 (CH₂-O), 68.75 (CH-OH), 86.53 (Ar-CH), 113.09 (Ar-CH), 114.05 (Ar-CH), 124.09 (Ar-CH), 126.91 (Ar-CH), 127.83 (Ar-CH), 128.99 (Ar-CH), 129.83 (Ar-CH), 130.03 (Ar-CH), 130.29 (Ar-CH), 135.30 (Ar-C), 144.26 (Ar-C), 148.31 (Ar-C), 158.50 (Ar-C). I.R data (nujol mull) νmax: 3440 (m, OH), 3350 (s, secondary NH), 3100 (w, aromatic), 2930 (w, aliphatic), 2850 (w), 1620 (s,aromatic), 1590 (s, aryl nitro), 1500 (s,aliphatic), 1440 (m), 1425 (m), 1335 (s, aryl nitro), 1300 (m), 1250 (s), 1175 (s), 1160 (m), 1135
2-Cyanoethyl [1-(4,4'-dimethoxytrityloxy)-3-(2,4-dinitrophenylamino) prop-2-yl] N,N-diisopropylaminophosphoramidite [3]

Compound [2] (0.50g, 0.9mmol) was coevaporated with anhydrous THF (3x10ml) and dissolved in anhydrous THF (15ml). To this solution was added N,N-diisopropylethylamine (0.6ml, 0.6mmol) and 2-cyanoethyl N,N-diisopropylphosphoramidochloridite (0.42ml, 1.8mmol). The reaction mixture was stirred at room temperature for 3 hours then quenched with ethyl acetate (30ml) and washed with saturated aqueous KCl (3x50ml). The organic layer was dried (Na2SO4), and the solvent was removed by evaporation in vacuo. The residue was purified by wet flash silica gel chromatography, eluting with a gradient of 0-18% ethyl acetate in dichloromethane containing 2% triethylamine. The appropriate fractions were collected and evaporated in vacuo and the residual oil was dissolved in the minimum volume of dichloromethane and precipitated from dry hexane at -78°C. Filtration under argon, and drying in vacuo gave the title compound as a yellow oil (0.32g, 47%). Rf 0.58 (solvent F). 31P n.m.r. data (CDCl3): δp 149.26 (s), 149.46 (s). F.a.b. mass spectrum: m/z 729.33738 calc. for C39H47N5O9 (M+H)+., m/z 729.33735.

N-tert-Butyoxy carbonyl-6-aminohexanoic acid [4]

6-Aminohexanoic acid (5.532g, 42mmol) was dissolved in water (20ml) and the pH of the solution was adjusted to ~10 by the addition of aqueous NaOH (10%, w/v). To this was added di-t-butyldicarbonate (9.20g, 42mmol) in dioxane (20ml) and the reaction mixture was stirred at room
temperature. After 6 hours the product was acidified with aqueous citric acid (30%w/v), and the resulting precipitate was extracted with ethyl acetate (3x50ml). The product was returned to the aqueous layer by washing with saturated aqueous NaHCO₃ (30ml), and this in turn was subjected to the acidification and ethyl acetate extraction procedure described above. The resulting organic layer was washed with water (50ml), dried (Na₂SO₄) and then evaporated in vacuo. This gave the title compound as a waxy solid (8.72g, 90%). Rf 0.72 (solvent E). ¹H n.m.r. data (CDCl₃): δH 1.20-1.88 (m, 15H, tBu and 3xCH₂), 2.58 (t, 2H, J=7.0 Hz, CH₂), 3.08 (bs, 2H, CH₂), 4.65 (bs, 1H, NH), 10.50 (bs, 1H, CO₂H): ¹³C n.m.r. data (CDCl₃): δC 24.18 (CH₂), 26.02 (CH₂), 28.22 (C(CH₃)₃), 28.49 (CH₂), 33.81 (CH₂-C=O), 40.17 (CH₂-NH), 79.06 (C(CH₃)₃), 155.95 (CO₂H), 178.85 (HN-C=O)). I.R data (nujol mull) νₘₐₓ: 3370 (m, secondary amide), 1710 (s, CO₂H), 1680 (s, urethane), 1510 (secondary amide), 1275 (m), 1250 (m), 1170 (m), 1040 (w), 995 (w), 940 (w), 870 (w), 720 (m) cm⁻¹. F.a.b. mass spectrum: m/z 232.15489, calc. for C₁₁H₂₂NO₄ (M+H)+., m/z 232.15487.

Pentachlorophenyl N-tert-butyloxycarbonyl-6-aminohexanoate [5]
To a solution of N-(tert-butyloxycarbonyl)-6-aminohexanoic acid [4] (10.00g, 43mmol) and pentachlorophenol (11.82g, 44mmol) in dry dichloromethane (30ml), was added dicyclohexylcarbodiimide (8.95g, 43mmol). The reaction mixture was protected from moisture and stirred at room temperature for 24 hours whereupon the resultant precipitate of dicyclohexylurea was removed by filtration, and the supernatant concentrated in vacuo. The residue was dissolved in diethyl ether and cooling gave the title compound as a white solid (19.72g, 95%). Rf 0.85
(solvent B). $^1$H n.m.r. data (CDCl$_3$): $\delta$H 1.38-1.68 (m, 13H, tBu and 2xCH$_2$), 1.81 (quintet, 2H, J=7.4 Hz, CH$_2$), 2.67 (t, 2H, J=7.4Hz, CH$_2$), 3.13 (quartet, 2H, J=6.2Hz, CH$_2$), 4.57 (bs, 1H, NH). $^{13}$C n.m.r. data (CDCl$_3$): $\delta$C 24.16 (CH$_2$), 26.03 (CH$_2$), 28.27 (C(CH$_3$)$_3$), 29.58 (CH$_2$), 33.33 (CH$_2$=O), 40.15 (CH$_2$-NH), 79.03 (C(CH$_3$)$_3$), 127.50 (Ar-CH), 131.29 (Ar-CH), 131.83 (Ar-CH), 143.96 (Ar-C), 155.83 (CH$_2$-CO-O) and 169.15 (HN-C=O)). I.R. data (nujol mull) $\nu_{max}$: 3370 (m, N-substituted amide), 1780 (m, aryl ester), 1680 (s, urethane), 1510 (s, N-substituted amide), 1275 (m), 1250 (m), 1170 (m), 1105 (m), 1090 (m), 1040 (w), 995 (w), 940 (w), 870 (w), 720 (m) cm$^{-1}$. F.a.b. mass spectrum: m/z 479.98836, calc. for C$_{17}$H$_{21}$N$_2$O$_4$ (M+H)$^+$. m/z 479.98836.

2,3-Dihydroxyprop-1-yl N-tert-butoxycarbonyl-6-aminohexamid [6]

3-Aminopropane-1,2-diol (0.96g, 11mmol, 1eq) was dissolved in dry DMF (15ml) and the pH was adjusted to ~9 by the addition of triethylamine. Pentachlorophenyl N-(t-butyloxycarbonate)-6-aminohexanoate [5] (5.01g, 11mmol, 1eq) was added and the solution was stirred at room temperature for 24 hours. The solvent was then evaporated in vacuo and the residue was purified by wet flash chromatography, eluting with a gradient of 0-10% methanol in dichloromethane. The appropriate fractions were combined and evaporated in vacuo to give the title compound as a pale yellow oil (2.90g,91%). Rf 0.61 (solvent C). $^1$H n.m.r. data (CDCl$_3$): $\delta$H 1.20-1.69 (m, 15H, tBu and 3xCH$_2$), 2.18 (t, 2H, J=7.4 Hz, CH$_2$), 3.02 (quartet, 2H, J=6.2 Hz, CH$_2$), 3.21-3.71 (m, 7H, CH, 2xCH$_2$ and 2xOH), 5.02 (bs, 1H, NH), 7.15 (bs, 1H, NH). $^{13}$C n.m.r. data (CDCl$_3$): $\delta$C 25.09 (CH$_2$), 131.29 (Ar-CH), 131.83 (Ar-CH), 143.96 (Ar-C), 155.83 (CH$_2$-CO-O) and 169.15 (HN-C=O)). I.R. data (nujol mull) $\nu_{max}$: 3370 (m, N-substituted amide), 1780 (m, aryl ester), 1680 (s, urethane), 1510 (s, N-substituted amide), 1275 (m), 1250 (m), 1170 (m), 1105 (m), 1090 (m), 1040 (w), 995 (w), 940 (w), 870 (w), 720 (m) cm$^{-1}$. F.a.b. mass spectrum: m/z 479.98836, calc. for C$_{17}$H$_{21}$N$_2$O$_4$ (M+H)$^+$. m/z 479.98836.
26.08 (CH₂), 28.24 (C(CH₃)₃), 29.43 (CH₂), 35.91 (CH₂-CO-N), 40.16 (NH-CH₂-CH-OH), 41.93 (CH₂-NH-C=O), 63.56 (CH₂-H₂-OH), 70.81 (NH-CH₂-CH-OH), 78.99 (C(CH₃)₃), 156.15 (HN-C=O), 174.89 (HN-C=O). I.R. data (thin film) v_max: 3300 (s, OH), 2970 (s, aliphatic), 2930 (s, aliphatic), 2860 (s, aliphatic), 1680 (s, urethane), 1640 (s, N-substituted amide), 1530 (s, N-substituted amide), 1450 (m), 1390 (m), 1360 (m), 1280 (m), 1250 (m), 1170 (m), 1110 (w), 1045 (w), 860 (w) cm⁻¹. F.a.b. mass spectrum: m/z 305.20764, calc. for C₁₄H₂₉N₂O₅ (M+H)+, m/z 305.20763.

2,3-Dihydroxyprop-1-yl 6-(2,4-dinitrophenylamino) hexamide [7]

Compound [6] (2.80g, 9.2mmol) and trifluoroacetic acid (4ml) were stirred in dichloromethane (16ml) at room temperature for 1 hour. The reaction mixture was then concentrated in vacuo and the residue was dissolved in methanol (20ml). The pH of the solution was adjusted to ~10 by the addition of triethylamine. This was accompanied by a colour change from an initial deep blue in the acidic solution, to bright yellow in excess base. 2,4-Dinitrofluorobenzene (2.02g, 11mmol) was added and the solution was stirred at room temperature for 24 hours. The mixture was then concentrated in vacuo, and the residue was purified by wet flash silica gel chromatography, eluting with a gradient of 0-2% methanol in dichloromethane. The appropriate fractions were collected and evaporated in vacuo to give a yellow oil which was dissolved in ethyl acetate (50ml) and washed with water (30ml) to remove traces of triethylamine trifluoroacetate salt. The aqueous layer was then washed with ethyl acetate (4x50ml) in order to re-extract the water soluble diol into the organic phase. The combined organic layers were dried (Na₂SO₄) and evaporated in vacuo to give the title compound as a yellow solid (2.21g,
69%). Rf 0.52 (solvent D). 1H n.m.r. data (d6-DMSO): δH 1.25-1.71 (m, 6H, 3xCH2), 2.10 (t, 2H, J=7.4 Hz, CH2), 2.94-3.65 (m, 7H, CH, 3xCH2), 3.90 (bs, 2H, 2xOH), 7.18 (d, 1H, J=9.4 Hz, DNP H-6) 7.73 (bt, 1H, J=5.4 Hz, NH), 8.20 (dd, 1H, J=2.8 Hz, J=9.4 Hz, DNP H-5), 8.77 (bt, 1H, J=6.5 Hz, DNP-NH), 8.81 (d, 1H, J=2.8 Hz, DNP H-3). 13C n.m.r. data (DMSO-d6): δC 25.18 (CH2), 26.09 (CH2), 28.06 (CH2), 35.33 (CH2-CO-NH), 42.25 (CH2-NH-C=O), 42.99 (CH2-NH), 63.83 (HO-CH2-CH), 70.75 (HO-CH2-CH), 115.47 (DNP-CH), 123.84 (DNP-CH), 129.67 (DNP-C), 130.13 (DNP-CH), 134.74 (DNP-C), 148.31 (DNP-C) and 172.85 (HN-C=O). I.R. data (nujol mull) vmax: 3300 (s, OH), 1640 (s, N-substituted amide), 1575 (s, aryl-nitro), 1530 (w, aryl-nitro), 1340 (aryl-NH), 1315 (s), 1265 (m), 1235 (m), 1200 (m), 1180 (m), 1140 (m), 920 (w), 830 (w), 740 (w), 720 (m) cm⁻¹. F.a.b mass spectrum: m/z 371.15668, calc. for C15H23N4O7 (M+H)+, m/z 371.15666.

2-Hydroxy-3-(4,4'-dimethoxytrityloxy)-prop-1-yl 6-(2,4-dinitrophenylamino) hexamide [8]

Anhydrous pyridine (3x10ml) was evaporated in vacuo from compound [7] (1.61g, 4.1mmol) which was then dissolved in anhydrous pyridine (10ml). To this was added 4,4'-dimethoxytrityl chloride (1.50g, 4.4mmol) and the solution was stirred at room temperature. After 6 hours the reaction was quenched with methanol (10ml) and excess pyridine was removed in vacuo. The residue was dissolved in dichloromethane (50ml) then washed with saturated aqueous NaHCO3 (30ml) and water (3x30ml). The organic solvent was evaporated in vacuo and the residue was purified by wet flash silica gel chromatography, eluting with 0-5% methanol in dichloromethane containing 2% triethylamine. The
appropriate fractions were combined and evaporated in vacuo to give the title compound as a yellow foam (2.61g, 85%). Rf 0.79 (solvent C). 1H n.m.r. data (CDCl3): δH 1.30-1.85 (m, 6H, 3xCH2), 2.13 (t, CH2, J=7.2 Hz, CH2), 3.00-3.52 (m, 8H, CH, 3xCH2, OH), 3.72 (s, 6H, 2xOMe), 6.00 (bs, 1H, NH), 6.70-6.89 (m, 4H, 4 aromatic CH), 7.15-7.41 (m, 10H, 10 aromatic CH), 8.20 (dd, 1H, J=2.8 Hz, J=9.4 Hz, DNP-H-5), 8.49 (bs, 1H, DNP-NH), 9.05 (d, 1H, J=2.8 Hz DNP-H-3). 13C n.m.r. data (CDCl3): δC 24.83 (CH2), 26.23 (CH2), 28.20 (CH2), 35.83 (CH2-CO-NH), 42.86 (CH2-NH-C=O), 43.13 (NH-CH2-CH), 45.81 (DNP-NH-CH2), 55.05 (Ar-OC6H3), 64.67 (HO-CH2-CH), 70.00 (HO-CH-CH2), 86.00 ((Ar)3C), 112.97 (Ar-CH), 113.81 (DNP-CH), 124.16 (Ar-CH), 126.70 (Ar-CH), 127.70 (Ar-CH), 127.86 (Ar-CH), 129.83 (Ar-CH), 130.18 (DNP-CH), 135.56 (DNP-C), 144.45 (Ar-C), 148.17 (DNP-C), 158.32 (Ar-C), 173.53 (HN-C=O). F.a.b. mass spectrum: m/z 371.15668, calc. for C15H23N4O7 (M+H)+, m/z 371.15666.

2-(2-Cyanoethyl-N,N-diisopropylphosphoramido)-3-(4,4'-dimethoxytrityloxy)-prop-1-yl 6-(2,4-dinitrophenylamino) hexamide [9]

Anhydrous THF (3x10ml) was evaporated from compound [8] (0.50g, 0.75mmol) which was then dissolved in anhydrous THF (5ml). To this solution was added N,N-diisopropylethylamine (0.5ml, 3mmol) and 2-cyanoethyl N,N-diisopropylphosphoramidochloridite (0.34ml, 1.5mmol), and the mixture was stirred at room temperature for 1 hour. The reaction was quenched with ethyl acetate (30ml), washed with saturated aqueous NaHCO3 (3x50ml), the organic layer dried (Na2SO4), then evaporated in vacuo. The residue was purified by wet flash column chromatography, eluting with a gradient of 0-20% ethyl acetate in dichloromethane.
containing 2% triethylamine. The appropriate fractions were collected, and the solvent was evaporated in vacuo. The product was then dissolved in the minimum volume of dichloromethane and precipitated at -78°C from Na-dried hexane. Filtration under argon, and drying in vacuo gave the title compound as a yellow powder (0.51g, 79%). Rf 0.75, 0.82 (diastereomers), (solvent F). 31P n.m.r. data (CDCl3): δ P 148.89 (s), 149.56 (s). F.a.b. mass spectrum: m/z 873.39522, calc. for C45H58N6O10P (M+H)+, m/z 873.39517.

1-(2,4-Dinitrophenylamino)-4-oxa-6,7-isopropylidenedioxyheptane [10]

2,4-Dinitrofluorobenzene (1.88g, 10.1mmol) was added to a solution of 3-aminopropyl solketal (2.30g, 12.1mmol) and triethylamine (3.0ml, 30mmol) in methanol (10ml) and the solution stirred at room temperature. After 24 hours the reaction mixture was evaporated in vacuo and the product was purified by wet flash silica gel chromatography, eluting with a gradient of 0-3% methanol in dichloromethane. Collection of the appropriate fractions and evaporation in vacuo gave the title compound as a yellow oil (3.42g, 94%). Rf 0.56 (solvent A). 1H n.m.r. data (CDCl3): δ H 1.27 (s, 3H, CH3), 1.33 (s, 3H, CH3), 1.99 (quintet, J=5.1Hz, 2H, CH2), 3.42-3.56 (m, 4H, 2xCH2), 3.60-3.73 (m, 3H, CH, CH2), 4.02 (dd, J=8.3Hz, J=6.4Hz, 1H, CH), 4.27 (quintet, J=6.2Hz, 1H, CH), 6.91 ( d, J=9.6Hz, 1H, DNP H-6), 8.14 (dd, J=2.6Hz, J=9.6Hz, 1H, DNP H-5), 8.77 (bt, H, DNP-NH), 8.96 (d, J=2.6Hz, 1H, DNP H-3). 13C n.m.r. data (CDCl3): δ C 25.11 (CH3) 26.60 (CH3), 28.40 (CH2), 41.70 (CH2-NH), 66.41 (CH2-O), 69.40 (CH2-O), 72.19 (CH2-O), 74.32 (CH-O), 109.21 (C(CH3)2), 113.77 (Ar-C), 123.96 (Ar-CH), 130.01 (Ar-CH), 135.45 (Ar-CH) and 148.18 (Ar-C). I.R. data (nujol mull) υmax : 3360 (m, NH),

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3100 (w, aliphatic), 2980 (m, aliphatic), 2920 (m, aliphatic), 2860 (m, aliphatic), 2350 (w), 1620 (s, nitro), 1585 (s, nitro), 1520 (m, NH), 1420 (m, aliphatic), 1370 (w), 1335 (s, aryl NH), 1305 (s), 1255 (m), 1210 (w), 1150 (m, saturated ether), 1055 (m), 920 (w), 830 (w), 740 (w) cm\(^{-1}\).
F.a.b. mass spectrum: \(m/z\) 356.14576 calc. for C\(_{15}\)H\(_{22}\)N\(_{3}\)O\(_{7}\) (M+H\(^{+}\)), \(m/z\) 356.14579.

1-(4,4'-Dimethoxytrityloxy)-4-oxa-7-(2,4-dinitrophenylamino) heptan-2-ol [11]

Concentrated hydrochloric acid (2ml) was added to a solution of \([10]\) (1.01g, 2.8mmol) in THF (5ml), and the solution was stirred at room temperature for 2 hours. The reaction mixture was then concentrated \(in\) \(vacuo\) and the residue was redissolved in THF (10ml), and concentrated once more. The resultant oil was dried by coevaporation \(in\) \(vacuo\) with anhydrous pyridine (3x5ml) then dissolved in anhydrous pyridine (10ml). 4,4'-Dimethoxytrityl chloride (1.31g, 3.9mmol) was added portionwise to the stirred solution over 30 minutes. After 12 hours the reaction was quenched with methanol (10ml), and excess pyridine removed \(in\) \(vacuo\). The residue was dissolved in dichloromethane (30ml), then washed with saturated aqueous NaHCO\(_3\) (1x50ml), saturated aqueous KCl (1x50ml) and water (1x50ml). The organic layer was dried over Na\(_2\)SO\(_4\), concentrated \(in\) \(vacuo\), and the residue purified by wet flash silica gel chromatography, eluting with a gradient of 0-50% ethyl acetate in hexane containing 2% triethylamine. The appropriate fractions were collected and evaporated \(in\) \(vacuo\) to give the title compound as a yellow foam (1.52g, 79\%). \(R_f\) 0.23 (solvent F). \(^1\)H n.m.r. data (CDCl\(_3\)): \(\delta\) 2.01 (quintet, \(J=5.2Hz\), 2H, CH\(_2\)), 2.63 (bs, 1H, OH), 3.20 (d, \(J=5.4Hz\), 2H, CH\(_2\)), 3.43-3.85 (m, 12H, 3xCH\(_2\), 2xOCH\(_3\)), 3.98-4.12 (m, 1H, CH), 6.76-6.88
(m, 4H, 4 aromatic CH), 7.13-7.43 (m, 10H, 10 aromatic CH), 8.21 (dd, J=2.8Hz, J=9.4Hz, 1H, DNP H-5), 8.83 (bt, 1H, DNP-NH), 9.08 (d, J=2.8Hz, 1H, DNP H-5). 13C n.m.r. data (CDCl3): δC 28.49 (CH2),
41.86 (CH2-NH), 55.05 (Ar-OCH3), 64.21 (CH2-O), 69.47 (CH2-O),
69.78 (CH-O), 72.92 (CH2-O), 85.97 (Ar-CH), 112.95 (Ar-CH), 113.68
(Ar-CH), 124.13 (Ar-CH), 124.13 (Ar-CH), 126.67 (Ar-CH), 127.66
(ArCH), 127.96 (Ar-CH), 128.86 (Ar-CH), 128.98 (Ar-CH), 130.13 (Ar-
CH), 135.65 (Ar-C), 135.75 (Ar-C), 144.64 (Ar-C), 148.14 (Ar-C),
158.34 (Ar-C). I.R. data (nujol mull) νmax: 3400 (m, OH), 3360 (m,
secondary NH), 3100 (w, aromatic), 2930 (m, aliphatic), 1615 (s,
aromatic), 1590 (s, aryl nitro), 1510 (s, aromatic), 1460 (m), 1450 (m),
1335 (s, aryl nitro), 1300 (s), 1250 (s), 1175 (m), 1130 (m), 1075 (m),
1030 (s), 925 (m), 830 (s), 790 (w), 750 (m), 700 (m) cm⁻¹. F.a.b. mass
spectrum: m/z 617.23731 calc. for C33H35N309 (M+H)+, m/z
617.23730.

2-Cyanoethyl [1-(4,4'-dimethoxytrityloxy)-4-oxa-7-(2,4-
dinitrophenylamino) hept-2-yl] N,N-diisopropylaminophosphoramidite [12]

Compound [11] (0.510g, 0.8mmol) was coevaporated with anhydrous
THF (3x5ml) and dissolved in anhydrous THF (5ml). To this solution was
added N,N-diisopropylethylamine (0.5ml, 3mmol) and 2-cyanoethyl-N,N-diisopropylphosphoramidochloridite (0.31ml, 1.4mmol) and the reaction
mixture was stirred at room temperature for 3 hours. The reaction was
quenched with ethyl acetate (50ml), washed with saturated aqueous
NaHCO3 (2x50ml), the organic layer dried (Na2SO4), and then
evaporated in vacuo. Purification was carried out by wet flash silica gel
chromatography using a short column of silica gel pre-equilibrated by
sonication in hexane containing 1% triethylamine. The crude product was applied in the minimum volume of hexane/ethyl acetate (1:1 v/v) and the column was eluted with ethyl acetate. The appropriate fractions were collected and evaporated in vacuo to give the title compound as a yellow foam (0.564g, 83%). Rf 0.37 and 0.44 (diastereomers), (solvent F). 31P n.m.r. data (CDCl3): δP 149.83 (s) and 150.00 (s). F.a.b. mass spectrum: m/z 818.35305 calc. for C_{42}H_{53}N_{5}O_{10}P (M+H)+, m/z 818.35298.

**N-(Trifluoroacetyl)-6-aminohexanoic acid [13]**

To a stirred suspension of 6-aminohexanoic acid (1.00g, 7.6mmol) in dry methanol (10ml) was added dry triethylamine (1.6ml, 11.5mmol) and ethyl trifluoroacetate (1.4ml, 11.5mmol). Stirring was continued at room temperature for 30 minutes and Dowex-50 resin (H+ form) (6.0g) was then added. The solution was stirred for 5 minutes, the resin was then removed by filtration, and the methanol filtrate was concentrated in vacuo to give a fluffy white solid. (1.65g, 95%). Rf 0.55 (solvent E). 1H n.m.r. data (CD3OD): δH 1.41-1.53 (m, 2H, CH2), 1.61-1.80 (m, 4H, 2xCH2), 2.40 (t, J=7.2Hz, 2H, CH2), 3.37 (t, J=7.1Hz, 2H, CH2). 13C n.m.r. data (CD3OD): δC 23.94 (CH2), 25.58 (CH2), 27.84 (CH2), 33.38 (CH2-CO2H), 38.88 (CH2-NH), 115.88 (quartet, 1JC,F=288.6Hz, 1C, CF3), 156.10 (quartet, 2JC,F=35.6Hz, 1C, CF3-CO), 174.29 (CO2H). I.R. data (nujol mull) νmax:3300 (s, N-substituted amide), 1700 (s, CO2H), 1560 (m, N-substituted amide), 1285 (m), 1250 (m), 1200 (m), 1150 (s), 1020 (m), 920 (m), 880 (m), 720 (s) cm⁻¹. F.a.b mass spectrum: m/z 228.08476 calc. for C_{8}H_{13}F_{3}N_{1}O_{3} (M+H)+, m/z 228.08476.

**2-Nitrophenyl N-(trifluoroacetyl)-6-aminohexanoate [14]**
To a solution of [13] (1.00g, 4.4mmol) in dry dichloromethane (20ml) was added triethylamine (1ml, 7.5mmol) and 2-nitrophenol (0.61g, 4.4mmol). When the reactants had dissolved, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (1.25g, 6.6mmol, 1.5eq) was added and the solution was stirred overnight at room temperature. The reaction mixture was quenched with dichloromethane (100ml) and the resultant solution was washed with 10% (w/v) citric acid (1x50ml), saturated aqueous KCl (1x50ml) and water (1x50ml). The organic layer was then dried (Na2SO4) and evaporation in vacuo gave the title compound as a waxy solid (1.41g, 76%). Rf 0.54 (solvent F). 1H n.m.r. data (CDCl3): δH 1.42-1.52 (m, 2H, CH2), 1.61 (quintet, J=7.2Hz, 2H, CH2), 1.76 (quintet, J=7.6Hz, 2H, CH2), 2.62 (t, J=7.3Hz, 2H, CH2), 3.34 (dt, J=6.7Hz, J=6.5Hz, 2H, CH2), 6.95 (bs, 1H, NH), 7.19 (dd, J=8.1Hz, J=1.3Hz, 1H, aromatic-H), 7.36 (m, 1H, aromatic-H), 7.62 (m, 1H, aromatic-H), 8.04 (dd, J=8.2Hz, J=1.6Hz, 1H, aromatic-H). 13C n.m.r. data (CDCl3): δC 23.57 (CH2), 25.64 (CH2), 28.12 (CH2), 33.38 (CH2), 39.41 (CH2), 115.67 (quartet, 1JC,F=288Hz, 1C, CF3), 124.96 (Ar-CH), 125.49 (Ar-CH), 126.48 (Ar-C), 134.68 (Ar-CH), 141.49 (Ar-C), 143.75 (Ar-C), 157.10 (quartet, 2JC,F=36.9Hz, 1C, CO-NH). I.R. data (nujol mull) υmax: 3300 (s, N-substituted amide), 1760 (s, aromatic ester), 1700 (s, CO2H), 1620 (m, aromatic), 1610 (m, aromatic), 1595 (m, aromatic), 1525 (s, aryl nitro), 1335 (s, aryl nitro), 1250 (m), 1190 (m), 1170 (m), 1120 (m), 1050 (m), 1030 (w), 1015 (w), 960 (w), 930 (w), 900 (w), 820 (w), 785 (w), 750 (w) cm⁻¹. F.a.b. mass spectrum: m/z 349.10113 calc. for C14H16F3N2O5 (M+H)+, m/z 349.10113.

1,2-Isopropylidenedioxy-4-oxa-hept-7-yl N-(trifluoroacetyl)-6-aminohexamide [15]
To a mixture of compound [14] (1.11 g, 3.2 mmol) and triethylamine (1.7 ml, 6.4 mmol) in dry dichloromethane (10 ml) was added 3-aminopropyl solketal (0.75 g, 4.0 mmol). The solution was stirred at room temperature for 12 hours and the reaction mixture was then concentrated in vacuo. The residue was dissolved in dichloromethane (100 ml) and washed with saturated aqueous KCl (2 x 50 ml) and water (2 x 50 ml). The organic layer was dried over Na2SO4, concentrated in vacuo and the residue was dissolved in the minimum volume of dichloromethane and purified by wet flash silica gel chromatography. Eluting with a gradient of 0-5% methanol in dichloromethane and pooling the appropriate fractions gave, after evaporation of the solvent in vacuo, the title compound as a clear oil (1.25 g, 99%). Rf 0.51 (solvent C).

1H n.m.r. data (CDCl3): δH 1.24-1.38 (m, 8 H, CH2 and 2 x CH3), 1.48-1.77 (m, 6 H, 3 x CH2), 2.12 (t, J= 8.0 Hz, 2 H, CH2), 3.24-3.34 (m, 4 H, 2 x CH2), 3.43 (d, J= 5.1 Hz, 2 H, CH2), 3.49-3.56 (m, 2 H, CH2), 3.67 (dd, J= 6.1 Hz, J= 8.2 Hz, 1 H, CH), 4.00 (dd, J= 6.5 Hz, J= 8.2 Hz, 1 H, CH), 4.23 (quintet, J= 5.3 Hz, 1 H, CH), 6.48 (bt, 1 H, NH), 7.25 (bt, 1 H, NH).

13C n.m.r. data (CDCl3): δC 24.45 (CH2), 25.05 (CH3), 25.76 (CH2), 26.52 (CH3), 28.04 (CH2), 28.68 (CH2), 35.81 (CH2), 37.69 (CH2), 39.13 (CH2), 66.04 (CH2-O), 70.48 (CH2-O), 71.88 (CH2-O), 74.51 (CH-O), 109.36 (C(CH3)2), 115.67 (quartet, 1JCF= 288 Hz, 1 C, CF3), 157.10 (quartet, 2JCF= 36.9 Hz, 1 C, CO-NH), 172.75 (CO-NH).

I.R. data (nujol mull) νmax: 3300 (m, N-substituted amide), 1720 (s, N-substituted amide), 1655 (s, N-substituted amide), 1550 (N-substituted amide), 1530 (s, N-substituted amide), 1210 (m), 1185 (m), 1130 (m, ether), 1075 (m, ether), 970 (w), 840 (w) cm⁻¹.

F.a.b. mass spectrum: m/z 399.21068 calc. for C17H30F3N2O5 (M+H)+, m/z 399.21066.
1,2-Isopropylidenedioxy-4-oxa-hept-7-yl 6-(2,4-dinitrophenylamino) hexamide [16]

A mixture of [15] (0.501g, 1.3mmol) and concentrated aqueous ammonia (15ml) was placed in a sealed pressure tube and heated to 70°C (water bath) with occasional swirling. After 30 minutes the previously immiscible organic oil had completely dissolved indicating complete trifluoroacetyl deprotection. The reaction mixture was then concentrated in vacuo followed by coevaporation with methanol (3x10ml) to remove residual ammonia, and the resultant oil was taken up in methanol (5ml). Triethylamine (1ml), followed by 2,4-dinitrofluorobenzene (0.280g, 1.5mmol) were added and the solution was stirred at room temperature for 12 hours. The reaction mixture was concentrated in vacuo and the residual oil was dissolved in dichloromethane (100ml), washed with saturated aqueous NaHCO₃ (1x50ml), saturated aqueous KCl (1x50ml) and water (1x50ml), dried (Na₂SO₄) and concentrated in vacuo to give the crude product which was purified by wet flash silica gel chromatography, eluting with a gradient of 0-5% methanol in dichloromethane. The appropriate fractions were collected and concentrated in vacuo to give the title compound as a yellow oil. (0.546g, 93%). Rf 0.25 (solvent B). ¹H n.m.r. data (CDCl₃): δH 1.28 (s, 3H, CH₃), 1.36 (s, 3H, CH₃), 1.38-1.48 (m, 2H, CH₂), 1.50-1.82 (m, 6H, 3xCH₂), 2.15 (t, J=7.1Hz, 2H, CH₂), 3.20-3.55 (m, 8H, 4xCH₂), 3.65 (dd, J=6.0Hz, J=8.1Hz, 1H, CH), 3.98 (dd, J=6.6Hz, J=8.2Hz, 1H, CH), 4.21 (quintet, J=5.4Hz, 1H, CH), 6.33 (bt, 1H, NH), 6.87 (d, J=9.6Hz, 1H, DNP H-6), 8.18 (dd, J=2.7Hz, J=9.5Hz, 1H, DNP H-5), 8.49 (bt, 1H, DNP-NH), 9.01 (d, J=2.7Hz, 1H, DNP H-3). ¹³C n.m.r data (CDCl₃): δC 24.85 (CH₂), 25.05 (CH₃), 2.27 (CH₂), 26.52 (CH₃), 28.22 (CH₂), 28.74 (CH₂), 35.93 (CH₂), 37.85 (CH₂), 43.11 (CH₂), 66.04 (CH₂-O),
70.55 (CH2-O), 71.86 (CH2-O), 74.47 (CH-O), 109.25 (C(CH3)2), 113.76 (DNP-CH), 124.04 (DNP-CH), 130.08 (DNP-CH), 135.63 (DNP-C), 148.12 (DNP-C), 172.24 (NH-C=O)). F.a.b. mass spectrum: m/z 469.22983 calc. for C21H33N4O8 (M+H)+. m/z 469.22983.

1-(4,4'-Dimethoxytrityloxy)-2-hydroxy-4-oxa-hept-7-yl 6-(2,4-dinitrophenylamino)hexamide [17]

Concentrated hydrochloric acid (2ml) was added to a solution of [16] (0.502g, 1.1mmol) in THF (5ml), and the solution was stirred at room temperature for 30 minutes. The reaction mixture was then concentrated in vacuo and the residue was redissolved in THF (10ml) and concentrated once more. The resultant oil was dried by coevaporation in vacuo with anhydrous pyridine (3x5ml) and dissolved in anhydrous pyridine (5ml). 4,4'-Dimethoxytrityl chloride (0.470g, 1.4mmol) was added portionwise to the stirred solution over 30 minutes and after 12 hours the reaction was quenched with methanol (10ml) and excess pyridine was removed in vacuo. The residue was dissolved in dichloromethane (50ml), then washed with saturated aqueous NaHCO3 solution (1x50ml), saturated aqueous KCl solution (1x50ml) and water (1x50ml). The organic layer was dried over Na2SO4, concentrated in vacuo and the residue was purified by wet flash silica gel chromatography, eluting with a gradient of 0-2% methanol in dichloromethane containing 2% triethylamine. The appropriate fractions were collected and evaporated in vacuo to give the title compound as a yellow foam (0.725g, 85%). 1H n.m.r. data (CDCl3): δH 1.39-1.50 (m, 2H, CH2), 1.59-1.77 (m, 6H, 3xCH2), 2.16 (t, J=7.1Hz, 2H, CH2), 2.88 (bs, 1H, OH), 3.16 (d, J=5.5Hz, 2H, CH2), 3.27-3.38 (m, 4H, 2xCH2), 3.41-3.56 (m, 4H, 2xCH2), 3.74 (s, 6H, 2xOCH3), 3.91 (m, 1H, CH), 6.29 (bt, 1H, NH), 6.75-6.85 (m, 5H, aromatic), 7.12-7.42 (m,
9H, aromatic) 8.17 (dd, J=2.7Hz, J=9.5Hz, 1H, DNP H-5), 8.50 (bt, 1H, DNP-NH), 9.10 (d, J=2.7Hz, 1H, DNP H-3). \(^{13}\)C n.m.r data (CDCl\(_3\)):
\[\delta C\]
- 24.81 (CH\(_2\)),
- 26.24 (CH\(_2\)),
- 28.17 (CH\(_2\)),
- 28.81 (CH\(_2\)),
- 35.96 (CH\(_2\)),
- 37.45 (CH\(_2\)),
- 43.08 (CH\(_2\)),
- 54.96 (Ar-OCH\(_3\)),
- 64.19 (CH\(_2\)-O),
- 69.74 (CH\(_2\)-O),
- 69.81 (CH\(_2\)-O),
- 72.27 (CH-O),
- 85.87 (C(Ar)\(_3\)),
- 112.85 (Ar-CH),
- 113.77 (DNP-CH),
- 124.01 (DNP-CH),
- 126.58 (Ar-CH),
- 127.57 (Ar-CH),
- 127.83 (Ar-CH),
- 129.76 (Ar-CH),
- 130.05 (DNP-CH),
- 135.63 (DNP-C),
- 144.52 (Ar-CH),
- 148.09 (DNP-C),
- 158.24 (Ar-C),
- 172.38 (CO-NH). F.a.b. mass spectrum: m/z 731.32918 calc. for C\(_{39}\)H\(_{47}\)N\(_4\)O\(_{10}\) (M+H\(^+\)), m/z 731.32918.

1-(4,4'-Dimethoxytrityloxy)-2-(2-cyanoethyl-N,N-diisopropylphosphoramido)-4-oxa-hept-7-yl 6-(2,4-dinitrophenylamino) hexamide [18]

Long chain DNP monomer precursor [17] (0.534g, 0.7mmol) was dried by coevaporation with anhydrous THF (3x5ml) and dissolved in anhydrous THF (5ml). To this solution was added N,N-diisopropylethylamine (0.5ml, 3mmol) and 2-cyanoethyl N,N-diisopropylchlorophosphoramidite (0.20ml, 0.9mmol) and the mixture stirred at room temperature for 2 hours. The reaction was quenched with ethyl acetate (50ml), washed with saturated aqueous KCl (2x50ml), dried (Na\(_2\)SO\(_4\)) then evaporated to an oil in vacuo. Purification was carried out by wet flash silica gel chromatography using a short column of silica gel pre-equilibrated by sonication in hexane containing 1% triethylamine. The crude product was applied in the minimum volume of 50% ethyl acetate in hexane, and eluted with ethyl acetate. The appropriate fractions were combined and evaporated in vacuo to give the title compound as a yellow foam (0.630g, 92%). R\(_f\) 0.32 and 0.38 (diastereomers), (solvent F). \(^{31}\)P
n.m.r. data (CDCl3): δP 149.66 (s) and 150.03 (s). F.a.b. mass spectrum: m/z 931.43700 calc. for C48H64N6O11P (M+H)^+, m/z 931.43704.

1-(4,4'-Dimethoxytrityloxy)-2-hydroxy-4-oxa-hept-7-yl N-trifluoroacetyl-6-aminohexamide [19]

To a solution of compound [16] (0.440g, 1.1mmol) in THF (8ml) was added 1M HCl (2ml) and the resulting mixture stirred at room temperature for 1 hour. Subsequent evaporation in vacuo gave the crude diol derivative as a pale yellow oil. This was coevaporated with anhydrous pyridine (3x10ml) and dissolved in anhydrous pyridine (3ml) before the portionwise addition of 4,4'dimethoxytrityl chloride (0.427g, 1.2mmol). The reaction mixture was then stirred at room temperature overnight. After quenching the reaction with ethyl acetate (100ml) and washing with sat. aqueous KCl (2x20ml), the crude product was purified by wet flash silica gel chromatography, eluting with a gradient of 0-5% methanol in dichloromethane. This gave the product as a colourless oil (0.540g, 75%). Rf 0.22 (solvent C) \( ^1H \) n.m.r data (CDCl3): δH 1.25-1.34 (m, 2H, CH2), 1.51-1.73 (m, 6H, 3xCH2), 2.09 (t, J=7.1Hz, 2H, CH2), 3.16 (d, J=5.6Hz, 2H, CH2), 3.25-3.34 (m, 4H, 2xCH2), 3.45-3.55 (m, 4H, 2xCH2), 3.76 (s, 1H, Ar-Cl), 3.88-4.01 (m, 1H, CH), 6.38 (bt, 1H, NH), 6.76-6.84 (m, 4H, ArH), 7.18-7.43 (m, 10H, ArH and NH). I.R. data (thin film) v\( _\text{max} \): 3320 (s, OH), 2925 (s, aliphatic), 2860 (s, aliphatic), 1710 (s, N-substituted amide), 1640 (m, aromatic), 1550 (w), 1510 (m, N-substituted amide), 1455 (w), 1440 (w), 1300 (w), 1250 (s, CF3), 1210 (m), 1175 (s), 1150 (m), 1070 (w) and 1030 (w) cm\(^{-1} \). F.a.b. mass spectrum: m/z 660.30227 calc. for C\( _{35} \)H\( _{43} \)F\( _{3} \)N\( _{2} \)O\( _{7} \) (M+H)^+, 660.30222.
3,6,9-Trioxa-11-(4,4'-dimethoxytrityloxy)-undecanol [19]
Tetraethyleneglycol (2.0g, 10.3mmol) was coevaporated with anhydrous pyridine (3x10ml) and dissolved in anhydrous pyridine (20ml). To this was added a solution of 4,4'-dimethoxytrityl chloride (3.49g, 10.3mmol) in anhydrous pyridine (10ml), dropwise and with vigorous stirring over a period of 1.5 hours. After stirring at room temperature overnight the reaction mixture was evaporated in vacuo and coevaporated with toluene (3x20ml) to remove traces of pyridine. The residue was dissolved in ethyl acetate (200ml), washed with sat. aqueous NaHCO₃ (1x50ml) and dried over Na₂SO₄. After evaporation of the organic phase in vacuo, the product was purified by wet flash column chromatography, eluting with a gradient of 0-100% ethyl acetate in hexane, to give the product as a colourless oil (2.96g, 60%). Rf 0.32 (solvent F). 1H n.m.r data (CDCl₃): δH 2.50 (bs, 1H, OH), 3.21 (t, 2H, J=5.0Hz, CH₂-OH), 3.55-3.79 (m, 14H, 7x0-CH₂), 6.76-6.84 (m, 4H, ArH) and 7.14-7.47 (m, 9H, ArH).

1-(2-Cyanoethyl-(3,6,9-trioxa-11-(4,4'-dimethoxytrityloxy)-undecanyl)-N,N-diisopropylphosphoramidite [20]
Compound [19] (0.510g, 1.0mmol) was coevaporated with anhydrous THF (3x5ml) and dissolved in anhydrous THF (5ml). To this solution was added N,N-diisopropylethylamine (0.9ml, 5.0mmol) and 2-cyanoethyl N,N-diisopropylphosphoramidochloridite (0.32, 1.4mmol) and the reaction mixture stirred at room temperature for 2 hours. The reaction was then quenched with dichloromethane (100ml), washed with sat. aqueous KCl (1x50ml), dried (Na₂SO₄) then evaporated to an oil in vacuo.
Purification was carried out by wet flash silica gel chromatography, eluting with 0-50% acetonitrile in dichloromethane containing 1% triethylamine. Evaporation in vacuo gave the product as a colourless oil
To a solution of 2'-deoxyuridine (5.00g, 22mmol) in 0.1M sodium acetate buffer (1000ml, pH=6.0) was added mercuric acetate (16.0g, 50mmol), and the mixture heated at 50°C for 4 hours. After cooling on ice for 1 hour, lithium chloride (3.95g, 93mmol) was added to the flask with vigorous stirring and a colourless, gelatinous precipitate filled the reaction vessel. Once the reaction mixture had returned to room temperature the precipitate was collected by centrifugation using a Sorvall macrocentrifuge fitted with 2x300ml tubes. The resultant pellets were broken up and washed with ice cold 0.1M NaCl (2x500ml), ethanol (2x500ml) and diethyl ether (2x500ml), centrifugation being carried out after each washing. The crude 5'-chloromercurideoxyuridine was then redispersed in 0.1M sodium acetate (1500ml, pH=5.6) with gentle heating and vigorous stirring. To the finely dispersed suspension was added N-trifluoroacetyl-3-aminopropene (21.2g, 138mmol) and a solution of K_2PdCl_4 (7.2g, 22mmol) in H_2O (100ml). The resultant thick black suspension was stirred at room temperature overnight. After filtration of the reaction mixture through a pad of celite (packed dimensions: diameter=10cm, depth=5cm), NaBH_4 (3x100mg) was added, and the resultant pale green turbid solution concentrated in vacuo to approximately 100ml. Ethyl acetate (6x200ml) was used to extract the product from the aqueous phase to give, after evaporation in vacuo of the organic phase, a thick green oil. Purification of the product by wet flash silica gel chromatography, eluting with a gradient of 0-20% methanol in dichloromethane, gave the product as a white foam (1.52g, 19%). R_f 0.43
(solvent D). 1H n.m.r data (d6-DMSO): δH 2.05-2.51 (m, 2H, 2'-H), 3.59 (m, 2H, 5'-H), 3.75-3.92 (m, 3H, 4'-H and N-CH2), 4.16-4.28 (m, 1H, 3'-H), 5.13 (t, 1H, 5'-OH), 6.12-6.25 (m, 2H, 1'-H and C=C-H), 6.39-6.53 (m, 1H, C=C-H), 8.06 (s, 1H, 6-H), 9.63 (bt, 1H, NH-CH2) and 11.45 (bs, 1H, 3-H). 13C n.m.r data (d6 DMSO): δC 39.84 (2'-C), 61.22 (5'-C), 70.33 (4'-C), 84.57 (3'-C), 87.67 (1'-C), 110.08 (5-C), 116.12 (quartet, J=288.3Hz, CF3), 124.23 (C=C), 124.42 (C=C), 138.28 (6-C), 149.68 (4-C) 156.60 (quartet, J=36.1Hz, CO-CF3) and 163.26 (2-C). U.V spectrum (ethanol): (λmax=239.2nm, ε=9045), (λmax=286.9nm, ε=6585). F.a.b. mass spectrum: m/z 379.09919 calc. for C14H16F3N3O6 (M+H)+, m/z 379.09912.

5-(3-Aminopropenyl)-2'-deoxyuridine N-trifluoroacetyl-6-aminohexamid [22]
5-[1-(N-trifluoroacetyl-3-aminopropenyl]) deoxyuridine (1.01, 2.7mmol) was dissolved in conc. aqueous ammonia (20ml) and the mixture placed in a pressure tube and incubated in a water bath at 60°C for 3 hours. The contents of the tube were then evaporated to an oil in vacuo, coevaporated with methanol (20ml), redissolved in water (100ml) and washed with ether (2x100ml). The aqueous layer was evaporated in vacuo, followed by coevaporation with acetone (2x100ml) to give a colourless gum. The crude aminopropenyl deoxyuridine derivative was then dissolved in anhydrous DMF (20ml) followed by the addition of triethylamine (0.5ml) and succinimidyl N-trifluoroacetyl-6-aminohexanoate (3.5ml of crude filtrate). The latter was prepared by adding N-hydroxysuccinimide (1.15g, 10mmol) and dicyclohexylcarbodiimide (2.27g, 11mmol) to a stirred solution of N-trifluoroacetyl-6-aminohexanoic acid (2.3g, 10mmol) in anhydrous DMF.
(10ml) at room temperature, stirring was continued overnight. Once the resulting precipitate of dicyclohexylurea had been removed by filtration, the supernatant containing the crude active ester could be used directly. Reaction at room temperature overnight and purification of the reaction mixture by wet flash silica gel chromatography, eluting with 0-15% methanol in dichloromethane, gave the product as a white foam (1.02g, 78%). Rf 0.47 (solvent C). $^1$H n.m.r data (d6-DMSO): $\delta_H$ 1.13-1.31 (m, 2H, CH$_2$), 1.41-1.59 (m, 4H, 2xCH$_2$), 2.06-2.16 (m, 4H, COCH$_2$ and 2'-H), 3.21 (m, 2H, NCO-CH$_2$), 3.65-3.75 (m, 3H, 4'H and CH$_2$NCOCF$_3$), 4.27 (m, 1H, H-3'), 5.08 (t, 1H, 5'-OH), 5.24 (d, J=4.0Hz, 1H, 3'-OH), 6.10-6.20 (m, 2H, 1'-H and C=C-H), 6.34-6.45 (m, 1H, C=C-H), 7.94 (t, 1H, NH-CH$_2$), 8.01 (s, 1H, 6-H), 9.35 (bt, 1H, NHCOCF$_3$) and 11.35 (bs, 1H, 3-H). $^{13}$C n.m.r. data (d6-DMSO): $\delta_C$ 24.95 (CH$_2$), 25.98 (CH$_2$), 28.10 (CH$_2$), 35.31 (CO-CH$_2$), 39.16 (N-CH$_2$), 40.02 (N-CH$_2$), 40.96 (2'-C), 61.23 (5'-C), 70.34 (4'-C), 84.49 (3'-C), 87.62 (1'-C), 110.51 (5-C), 118.95 (quartet, J=288.3Hz, CF$_3$), 122.40 (C=C-H), 126.98 (C=C-H), 137.32 (6-C), 149.64 (4-C), 156.62 (quartet, J=36.5Hz, COCF$_3$), 162.19 (2-C) and 171.91 (NHC0).

5-(Aminopropenyl)-2'-deoxyuridine N-(2,4-dinitrophenyl)-6-aminohexamide [23]

Compound [22] (0.250g, 0.51mmol) was dissolved in conc. aqueous ammonia (8ml), placed in a pressure tube and heated at 60°C for 2hrs. The solution was then evaporated in vacuo, coevaporated with methanol (3x10ml) and finally dissolved in methanol (10ml). To this was added triethylamine (0.3ml, 2.0mmol) followed by dinitrofluorobenzene (0.095g, 0.051mmol), and the reaction mixture stirred at room temperature overnight. After evaporation in vacuo, the residue was taken up in
dichloromethane (50ml), washed with sat. aqueous KCl (2x25ml), and the crude product purified by wet flash silica gel chromatography, eluting with a gradient of 0-15% methanol in dichloromethane. This gave the product as a yellow solid (0.253g, 83%). Rf 0.52 (solvent C). 1H n.m.r data (CDCl3): δH 1.32-1.38 (m, 2H, CH2), 1.51-1.66 (m, 4H, 2xCH2), 2.06-2.13 (m, 4H, COCH2 and 2'-H), 3.49 (m, 2H, NCO-CH2), 3.55-3.65 (m, 1H, 4'H), 3.72-3.79 (m, 2H, NH-CH2) 4.25 (m, 1H, H-3'), 5.12 (t, 1H, 5'-OH), 5.25 (d, J=4.0Hz, 1H, 3'-OH), 6.10-6.17 (m, 2H, 1'-H and C=C-H), 6.34-6.43 (m, 1H, C=C-H), 7.20 (d, 1H, J=9.4Hz, DNP H-6), 7.97-8.01 (m, 2H, CONHCH2 and DNP H-5), 8.23 (dd, 1H, J=9.4Hz and J=2.8Hz, DNP-H-5), 8.80-8.83 (m, 2H, DNP-NH and DNP-H-3) and 11.39 (bs, 1H, 3-H). 13C n.m.r. data (CDCl3): δC 29.95 (CH2), 30.95 (CH2), 32.91 (CH2), 40.24 (CO-CH2), 44.94 (N-CH2), 45.85 (N-CH2), 47.72 (2'-C), 66.12 (5'-C), 75.25 (4'-C), 89.34 (3'-C), 92.50 (1'-C), 120.26 (Ar-CH), 127.18 (C=C-H), 128.73 (ArCH), 127.89 (Ar-C), 131.85 (C=C-H), 135.01 (ArCH), 139.74 (Ar-C), 142.30 (6-C), 149.64 (4-C), 153.31 (Ar-C) 164.15 (2-C) and 174.54 (NHCO). F.a.b mass spectrum: m/z 562.20234 calc. for C24H30N6O10 (M+H)+, m/z 562.20234

5-(Aminopropenyl)-5'-O-(4,4'-dimethoxytrityl)-2'-deoxyuridine N-(2,4-dinitrophenyl)-6-aminohexamide [24]

Compound [23] (0.250g, 0.51mmol) was coevaporated with anhydrous pyridine (3x10ml) and dissolved in anhydrous pyridine (5ml). To this was added 4,4'-dimethoxytrityl chloride (0.119g, 0.35mmol) and the reaction mixture stirred at room temperature. After 12 hours another 0.5 equivalents of 4,4'-dimethoxytrityl chloride (0.057g, 0.17mmol) was added and the reaction mixture stirred for a further 6 hours. Excess pyridine was then removed by evaporation in vacuo followed by
coevaporation with toluene (3x20ml). The resulting residue was purified by wet flash silica gel chromatography, eluting with a gradient of 0-10% methanol in dichloromethane, to give the product as a yellow foam (0.100g, 34%) R_f 0.56 (solvent C). F.a.b. mass spectrum: m/z 864.33303 calc. for C_{45}H_{48}N_{6}O_{12} (M+H)^+, m/z 864.33302

5-(Aminopropenyl)-3'-O-(2-cyanoethyl-N,N-diisopropylphosphoramido)-5'-O-(4,4'-dimethoxytrityl)-2'-deoxyuridine N-(2,4-dinitrophenyl)-6-aminohexamide [24]

Compound [24] (0.100g, 0.12mmol) was coevaporated with anhydrous THF (3x5ml) and dissolved in anhydrous THF (2.0ml). To this solution was added N,N-diisopropylethylamine (0.1ml, 0.5mmol) and 2-cyanoethyl N,N-diisopropylphosphoramidochloridite (0.5ml, 0.25mmol). The reaction mixture was stirred at room temperature for 1hr, then quenched with ethyl acetate (50ml). This was washed with sat. aqueous NaHCO_3 (2x20ml), the organic phase dried (Na_2SO_4), and the solvent removed by evaporation in vacuo. The residue was purified by wet flash silica gel chromatography, using silica prequilibrated with 1% triethylamine in dichloromethane, and eluting with 0-50% acetonitrile. This gave the product as a yellow foam (0.100g, 81%). R_f 0.49 (solvent J). 31P n.m.r. data (CDCl_3): δ_p 146.33 (s). F.a.b. mass spectrum: m/z 1064.44095 calc. for C_{54}H_{65}N_{8}O_{13}P (M+H)^+, m/z 1064.44087.

5-(Aminopropenyl)-5'-O-(4-toluenesulphonyl)-deoxyuridine N-trifluoroacetyl-6-aminohexamide [26]

To a stirred solution of compound [25] (0.202g, 0.4mmol) in anhydrous pyridine (5ml) at 0°C was added 4-toluenesulphonyl chloride (0.085g, 0.45mmol) and dry triethylamine (0.15ml, 0.8mmol). Stirring was
continued at 0°C for 1 hour, then the solution allowed to warm gradually to room temperature and finally left to stir overnight. After this time, a further addition of 4-toluencesulphonyl chloride (0.080g, 0.42mmol) was required to encourage reaction. Stirring was continued for a further 6 hours, after which time the reaction mixture was evaporated in vacuo and coevaporated with toluene (3x10ml) to remove traces of pyridine. The residue was purified by wet flash silica gel chromatography, eluting with a gradient of 10% methanol in dichloromethane, to give the product as a white foam (0.105g, 40%).

5-(Aminopropenyl)-5'-O-(triphosphate)-deoxyuridine N-trifluoroacetyl-6-aminohexamide [27]

To a stirred solution of compound [26] (0.095g, 0.19mmol) in dry acetonitrile (300ml) was added tetra (tetrabutylammonium) triphosphate [30] (0.351g, 0.30mmol) and stirring continued at room temperature for 7 days. The mixture was then diluted H2O (5ml) and applied to a column of Dowex 50X-8 (NH4+ form) and eluted with water. The anisaldehyde positive fractions were lyophilised and the resulting solids extracted with a solution of acetonitrile: ammonium bicarbonate (100mM): conc. ammonia (7:3:2). The soluble portions were purified by wet flash cellulose chromatography, eluting with the same solution. Once again, the anisaldehyde positive fractions were pooled, acetonitrile removed by evaporation in vacuo, and the remaining solution lyophilised to give the product as a hygroscopic white solid (55mg).

5-(Aminopropenyl)-5'-O-(triphosphate)-deoxyuridine N-(N-(2,4-dinitrophenyl)-6-carboxyhexylamine)-6-aminohexamide [28]
TFA-CAP-AA-dU TP (3mg) was dissolved in conc. ammonia (2ml) and the solution incubated at 25°C overnight. Ammonia was then removed by evaporation in vacuo and the remaining solution lyophilised to give a white solid. This was dissolved in Na$_2$CO$_3$/NaHCO$_3$ buffer (0.5M, 0.5ml) and added to a stirred solution of DNP-NHS active ester [31] (10mg) in DMSO (0.5ml) and the reaction mixture incubated at 45°C overnight. H$_2$O and DMSO were then removed by lyophilisation, the residue dissolved in H$_2$O (1ml), washed with ethyl acetate (2x1ml) and finally purified by reverse phase H.P.L.C.

**Tetra (tetrabutylammonium) triphosphate [29]**
Aqueous sodium tripolyphosphate (0.2M, 50ml) chilled to 4°C was passed through a column of Dowex 50X-8 ion exchange resin (H$^+$ form) into a chilled aqueous solution of tetrabutylammonium hydroxide (0.75M, 30ml) with rapid stirring. Water was added to the ion exchange column until the eluant was no longer acidic. Sufficient sodium tripolyphosphate solution was then added via the column to bring the pH of the receiving solution to neutrality. The resultant solution was lyophilised to give a highly hygroscopic, fluffy white solid. The product was used without purification and stored at -20°C.

**N-(2,4-dinitrophenyl)-6-aminohexanoic acid [30]**
To a suspension of 6-aminohexanoic acid (1.01g, 7.7mmol) in methanol (20ml) containing triethylamine (3ml, 21mmol) was added 2,4-dinitrofluorobenzene (1.43g, 7.7mmol), and the reaction mixture stirred at room temperature overnight. After removal of methanol and excess triethylamine in vacuo, the resultant yellow solid was redissolved in methanol (50ml), and the solution neutralised by the careful addition of
c.HCl. The addition of dichloromethane (300ml), followed by washes with aqueous citric acid (10% w/v) (3x100ml) and water (100ml), and finally concentration of the organic phase in vacuo yielded a bright yellow solid. The crude product was recrystallised from ethanol to give yellow needles. (2.11g, 95%). Rf 0.44 (Solvent B). $^1$H n.m.r. data (d6-DMSO): $\delta_H$ 1.31-1.40 (m, 2H, CH$_2$), 1.50-1.66 (m, 4H, 2xCH$_2$), 2.22 (t, J=6.9Hz, 2H, COCH$_2$), 3.42-3.57 (m, 2H, N-CH$_2$), 7.18 (d, 1H, J=9.4 Hz, DNP H-6), 8.20 (dd, 1H, J=2.8 Hz, J=9.4 Hz, DNP H-5), 8.79 (d, 1H, J=2.8Hz, DNP H-3), 8.83 (t, 1H, J=5.9Hz, DNP-NH), 12.00 (bs, 1H, CO$_2$H) $^{13}$C n.m.r. data (d6-DMSO): $\delta_C$ 24.25 (CH$_2$), 25.91 (CH$_2$), 27.91 (CH$_2$), 33.64 (CO-CH$_2$), 42.77 (N-CH$_2$), 115.33 (ArCH), 123.72 (ArCH), 129.60 ArC), 130.00 (ArCH), 134.67 (ArC), 148.20 (ArC) and 174.55 (CO$_2$H). F.a.b. mass spectrum: m/z 297.09615 calc. for C$_{12}$H$_{15}$N$_3$O$_6$ (M+H)$^+$, m/z 297.09609.

**Succinimidyl 6-(2,4-dinitrophenyl)-hexanoate [31]**

N-(2,4-dinitrophenyl)-6-aminohexanoic acid [30] (0.501g, 1.7mmol) was dissolved in anhydrous DMF (5ml) followed by the addition of N-hydroxy succinimide (0.212g, 1.8mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (0.48g, 2.6mmol) and the resultant solution stirred at room temperature overnight. The reaction mixture was then evaporated to dryness in vacuo, the residue taken up in ethyl acetate (100ml), and this washed with sat. aqueous KCl (50ml) and water (50ml). Drying (Na$_2$SO$_4$) and concentration of the organic phase in vacuo produced a crude yellow solid. This was purified by wet flash silica gel chromatography, eluting with a gradient of 0-10% methanol in dichloromethane, to give the product as a fine yellow powder (0.491g, 74%). Rf 0.61 (solvent A). $^1$H n.m.r. data (CDCl$_3$) $\delta_H$ 1.41-1.55 (m, 2H,
CH2), 1.58-1.76 (m, 4H, 2xCH2), 2.69 (t, J=7.0Hz, 2H, CO-CH2), 2.81 (s, 4H, 2xCOCH2), 3.37-3.48 (m, 2H, N-CH2), 7.19 (d, 1H, J=9.4 Hz, DNP H-6), 8.20 (dd, 1H, J=2.8 Hz, J=9.4 Hz, DNP H-5), 8.80 (d, 1H, J=2.8Hz, DNP H-3), 8.85 (t, 1H, J=5.9Hz, DNP-NH). $^{13}$C n.m.r. data (d6-DMSO): δC 23.96 (CH2), 25.35 (CH2), 25.54 (2xCH2), 27.59 (CH2), 30.15 (CH2), 42.63 (CH2), 115.31 (ArCH), 123.71 (ArCH), 129.64 (ArC), 129.98 (ArCH), 134.68 ArC), 148.19 (ArC), 169.05 (CO) and 170.36 (2xCO). F.a.b. mass spectrum: m/z 394.11250 calc. for C$_{16}$H$_{18}$N$_{4}$O$_{8}$ (M+H)$^+$, m/z 394.11246.

**N-Dansyl-6-aminohexanol [32]**

To a solution of 6-aminohexanol (0.65g, 5.5mmol) in dry pyridine (25ml) was added triethylamine (1.5ml, 11mmol) and dansyl chloride (1.50g, 5.5mmol). The reaction mixture was stirred at room temperature for 24hrs, then evaporated to dryness and coevaporated with toluene (3x10ml) to remove excess pyridine. The resulting oil was taken up in dichloromethane (50ml) and washed with water (3x20ml). The organic layer was evaporated to dryness, and the crude product purified by wet flash silica gel chromatography, eluting with a gradient of 0-5% methanol in dichloromethane. This gave the product as a green fluorescent glass (1.38g, 71%). $R_f$ 0.45 (solvent A). $^{1}$H n.m.r. data (CDCl$_3$) : δH 1.0-1.5 (m, 8H, 2CH$_3$, CH$_2$), 2.1 (bs, 1H, OH), 2.8-3.0 (m, 8H, 4CH$_2$), 2.4 (t, 2H, CH$_2$), 5.2-5.3 (t, 1H, NH), 7.0-8.5 (m, 6H, ArH). $^{13}$C n.m.r. data (CDCl$_3$): δC 24.80 (CH$_2$), 25.82 (CH$_2$), 29.16 (CH$_2$), 32.09 (CH$_2$), 42.87 (CH$_2$), 45.22 (N(CH$_3$)$_2$), 62.29 (O-CH$_2$), 114.98 (ArCH), 118.67 (ArCH), 123.02 (ArCH), 128.13 (ArCH), 129.31 (ArCH), 129.44 (ArC), 130.10 (ArC), 134.71 (ArCH) and 151.72 (ArC). I.R. data (thin film) $\nu_{max}$: 3353 (s, OH), 2932 (s, aliphatic), 2859 (m, aliphatic), 1569 (m),
1463 (m), 1392 (w), 1316 (m), 1142 (m), 1056 (m), 817 (w), 728 (w) and 624 (m) cm\(^{-1}\). F.a.b. mass spectrum: m/z 350.16647 calc. for C\(_{18}\)H\(_{26}\)N\(_2\)O\(_3\)S (M+H)\(^+\), m/z 350.16641.

2-Cyanoethyl (N-dansyl-6-aminohexyl) N,N-diiisopropylamino phosphoramidite [33]

Compound [32] (0.522g; 1.5mmol) was dissolved in anhydrous THF (5ml) and to this solution was added N,N-diisopropylethylamine (1.08ml, 6mmol) and 2-cyanoethyl N, N-diisopropylphosphoramidochloridite (0.39ml, 1.8mmol). After 1hr the reaction was quenched by the addition of ethyl acetate (50ml). The resulting solution was washed with sat. aqueous KCl (4x20ml), the organic phase dried (Na\(_2\)SO\(_4\)), filtered and evaporated \(\text{in vacuo}\). The product was applied to a silica gel column pre-equilibrated with hexane containing 1% triethylamine and eluted with a 0-40% gradient of ethyl acetate to give a green fluorescent oil (0.573g, 70%). R\(_f\) 0.53 (solvent D). \(\text{\textsuperscript{31}P n.m.r. data (CH}_3\text{CN solvent; (CD}_3\text{)}_2\text{CO lock) } \delta p 145.5\) (s). F.a.b. mass spectrum: m/z 550.27432 calc. for C\(_{42}\)H\(_{54}\)N\(_5\)O\(_9\)P (M+H)\(^+\), m/z 550.27427.

N-dansyl-1-amino-4-oxa-6,7-isopropylidene dioxyheptane [34]

Aminopropylsolketal (1.10g, 5.8mmol) was added to a solution of dansyl chloride (1.58g, 5.8mmol) and triethylamine (1.5ml, 23mmol) in methanol (5ml) and the reaction mixture stirred at room temperature overnight. The reaction was then quenched with dichloromethane (100ml), washed with sat. aqueous KCl (3x20ml) and evaporated to dryness to give the crude product as a dark green oil. This was purified by wet flash silica gel chromatography, eluting with a 0-5% gradient of methanol in dichloromethane, to give the product as a green fluorescent oil (2.28g,
93%). Rf 0.43 (solvent G). $^1$H n.m.r. data (CDCl$_3$): $\delta$H 1.33 (s, 3H, CH$_3$), 1.38 (s, 3H, CH$_3$), 1.62 (quintet, $J$=5.2 Hz, 2H, CH$_2$), 2.85 (s, 6H, N(CH$_3$)$_2$), 2.98 (quartet, $J$=6.1 Hz, 2H, CH$_2$), 3.23-3.30 (m, 2H, CH$_2$), 3.38 (t, $J$=5.8 Hz, 2H, CH$_2$), 3.58 (dd, $J$=6.4 Hz, $J$=8.2 Hz, 1H, CH), 3.96 (dd, $J$=6.4 Hz, $J$=8.2 Hz, 1H, CH), 4.05-4.15 (m, 1H, CH), 5.56 (t, $J$=5.9 Hz, 1H, SONH), 7.15 (d, $J$=7.4 Hz, 1H, ArH), 7.45-7.53 (m, 2H, 2xArH), 8.20 (d, $J$=6.0 Hz, 1H, ArH), 8.30 (d, $J$=8.6 Hz, 1H, ArH), 8.51 (d, $J$=8.6 Hz, 1H, ArH). $^{13}$C n.m.r. data (CDCl$_3$): $\delta$C 25.13 (CH$_3$), 26.49 (CH$_3$), 28.68 (CH$_2$), 41.26 (CH$_2$-NH), 45.19 (NCH$_3$), 66.12 (CH$_2$-O), 69.43 (CH$_2$-O), 71.65 (CH$_2$-O), 74.39 (CH-O), 109.33 (C(CH$_3$)$_2$), 114.93 (Ar-CH), 118.81 (ArCH), 122.99 (Ar-CH), 127.96 (Ar-CH), 129.33 (Ar-CH), 129.59 (ArC), 134.64 (ArCH), 151.55 (ArC). I.R. data (thin film) $\nu_{max}$: 3292 (m, aromatic), 2983 (m), 2936 (s, aliphatic), 2870 (s, aliphatic), 2835 (m), 1608 (m), 1574 (m), 1508 (s), 1458 (m), 1408 (w), 1322 (s), 1250 (s), 1145 (s), 1090 (s), 944 (w), 835 (m), 792 (s), 703 (w) and 625 (s) cm$^{-1}$. F.a.b. mass spectrum: m/z 422.18755 calc. for C$_{21}$H$_{30}$N$_2$O$_7$S (M+H)$^+$, m/z 422.18754.

**N-dansyl-1-(4,4'-Dimethoxytrityloxy)-4-oxa-6-amino-heptan-2-ol [35]**

To a solution of [34] (0.752g, 1.8mmol) in THF (5ml) was added conc. aqueous HCl (1ml) and the reaction stirred vigorously for 2hrs at room temperature. The THF, water and excess HCl were then removed by careful evaporation *in vacuo* and the residual oil dissolved once more in THF (10ml). Final traces of HCl were neutralised by the dropwise addition of triethylamine until the solution was basic by pH paper. The resulting solution was coevaporated with anhydrous pyridine (3x10ml) and dissolved in anhydrous pyridine (5ml). To this solution, portionwise
and with stirring, was added 4,4'-dimethoxytrityl chloride (0.627g, 2.0mmol). The reaction mixture was stirred at room temperature for 24hrs then quenched with methanol (1ml) and the solvent removed by evaporation in vacuo. The resulting dark green oil was coevaporated with toluene (3x10ml), taken-up in dichloromethane (50ml) and washed with sat NaHCO3 (20ml). The organic phase was dried (Na2SO4), filtered and evaporated in vacuo. The product was then pre-adsorbed onto Na2SO4 and applied to a wet-flash silica gel chromatography column which had been equilibrated with hexane containing 1% triethylamine. The column was then eluted with a gradient of 0-100% ethyl acetate in hexane, to give a fluorescent green foam (0.65g, 54%). Rf 0.69 (solvent G). ¹H n.m.r. data (CDCl₃): δH 1.61 (quintet, J=5.2 Hz, 2H, CH₂), 2.86 (s, 6H, N(CH₃)₂), 2.93-3.02 (m, 2H, CH₂), 3.13-3.19 (m, 2H, CH₂), 3.31-3.41 (m, 2H, CH₂), 3.52-3.65 (m, 1H, CH), 3.75 (s, 6H, 2xOCH₃), 3.96 (m, 2H, 2xCH), 5.56 (dt, J=5.9Hz, J=49.6Hz, 1H, SONH), 6.79-6.83 (m, 4H, ArH), 7.10-7.55 (m, 12H, ArH), 8.21 (d, J=6.0Hz, 1H, ArH), 8.33 (t, J=8.5Hz, 1H, ArH), 8.52 (d, J=8.5Hz, 1H, ArH). ¹³C n.m.r. data (CDCl₃): δC 28.73 (CH₂), 41.03 (CH₂-NH), 45.20 (NCH₃) 54.99 (OCH₃), 66.00 (CH₂-O), 69.21 (CH₂-O), 71.59 (CH₂-O), 74.42 (CH-O), 114.96 (Ar-CH), 118.82 (ArCH), 123.02 (Ar-CH), 126.57 (Ar-CH), 127.58 (Ar-CH), 128.07 (ArCH), 128.92 (ArCH), 129.24 (ArCH), 129.38 (ArCH), 129.55 (ArCH), 129.82 (ArCH), 129.6 (ArCH), 134.73 (ArC), 135.67 (ArC), 139.28 (ArC), 144.55 (ArC), 147.16 (ArC), 151.50 (ArC), 158.22 (ArC) and 158.32 (ArC). F.a.b. mass spectrum: m/z 684.28688 calc. for C₃₉H₄₄N₂O₇S (M+H)⁺, m/z 684.28692.

2-Cyanoethyl-[N-dansyl-1-(4,4'-Dimethoxytrityloxy)-4-oxa-6-aminohept-2-yl] N,N-diisopropylamino phosphoramidite [36]
Compound [35] (0.605g, 0.9mmol) was coevaporated with anhydrous THF (3x10ml) and dissolved in anhydrous THF (5ml). To this solution was added N,N-diisopropylethylamine (0.5ml, 3.0mmol) and 2-cyanoethyl-N,N-diisoproplyphosphoramidochloridite (0.24ml, 1.1mmol,) and the reaction mixture stirred at room temperature for 1hr. The reaction was quenched with ethyl acetate (100ml), washed with saturated aqueous NaHCO3 (2x30ml), the organic layer dried (Na2SO4), and then evaporated in vacuo. Purification was carried out by wet flash silica gel chromatography using a short column of silica gel pre-equilibrated by sonication in hexane containing 1% triethylamine, and eluting with a gradient of 0-100% ethyl acetate. The appropriate fractions were collected and evaporated in vacuo to give the title compound as a fluorescent green foam (0.599g, 78%). Rf 0.44 and 0.53 (diastereomers), (solvent D). 31P n.m.r. data (CDCl3): δP 147.59 (s) and 148.01 (s). F.a.b. mass spectrum: m/z 884.39479 calc. for C48H61N5O8PS (M+H)+, m/z 884.39477.

1-(4,4'-Dimethoxytrityloxy)-2-hydroxy-4-oxa-hept-7-yl N-trifluoroacetyl-6-aminohexamide [37]

To a solution of compound [15] (0.440g, 1.1mmol) in THF (8ml) was added 1M HCl (2ml) and the resulting mixture stirred at room temperature for 1 hour. Subsequent evaporation in vacuo gave the crude diol derivative as a pale yellow oil. This was coevaporated with anhydrous pyridine (3x10ml) and dissolved in anhydrous pyridine (3ml) before the portionwise addition of 4,4'dimethoxytrityl chloride (0.427g, 1.2mmol). The reaction mixture was then stirred at room temperature overnight. After quenching the reaction with ethyl acetate (100ml) and washing with sat. aqueous KCl (2x20ml), the crude product was purified.
by wet flash silica gel chromatography, eluting with a gradient of 0-5% methanol in dichloromethane. This gave the product as a colourless oil (0.540g, 75%). Rf 0.22 (solvent C) $^1$H n.m.r data (CDCl$_3$): $\delta_H$ 1.25-1.34 (m, 2H, CH$_2$), 1.51-1.73 (m, 6H, 3xCH$_2$), 2.09 (t, J=7.1Hz, 2H, CH$_2$), 3.16 (d, J=5.6Hz, 2H, CH$_2$), 3.25-3.34 (m, 4H, 2xCH$_2$), 3.45-3.55 (m, 4H, 2xCH$_2$), 3.76 (s, 1H, Ar-OCH$_3$), 3.88-4.01 (m, 1H, CH), 6.38 (bt, 1H, NH), 6.76-6.84 (m, 4H, ArH), 7.18-7.43 (m, 10H, ArH and NH). I.R. data (thin film) $\nu_{max}$: 3320 (s, OH), 2925 (s, aliphatic), 2860 (s, aliphatic), 1710 (s, N-substituted amide), 1640 (m, aromatic), 1550 (w), 1510 (m, N-substituted amide), 1455 (w), 1440 (w), 1300 (w), 1250 (s, CF$_3$), 1210 (m), 1175 (s), 1150 (m), 1070 (w) and 1030 (w) cm$^{-1}$. F.a.b. mass spectrum: m/z 660.30227 calc. for C$_{35}$H$_{43}$F$_3$N$_2$O$_7$ (M+H)$^+$. 660.30222.

1-(4,4'-Dimethoxytrityoxy)-2-(2-cyanoethyl-N,N-diisopropylphosphoramido)-4-oxa-hept-7-yl N-trifluoroacetyl-6-aminohexamide [38]

Compound [37] (0.480g, 0.7mmol) was coevaporated with anhydrous THF (3x5ml) and dissolved in anhydrous THF (5ml). To this solution was added diisopropylethylamine (1ml, 3.5mmol) and 2-cyanoethyl N,N-diisopropylchlorophosphoramidite (0.3ml, 1.4mmol) and the reaction mixture stirred at room temperature for 2 hours. The reaction was quenched with dichloromethane (100ml), washed with sat. aqueous NaHCO$_3$ (1x20ml), sat. aqueous KCl (1x20ml) and H$_2$O (1x20ml). Once dried (Na$_2$SO$_4$) the organic phase was evaporated in vacuo and the residue purified by wet flash silica gel chromatography, eluting with dichloromethane. The product was then precipitated into hexane at -78°C under argon and the resultant solid evaporated in vacuo to give a
colourless oil (0.420g, 70%). Rf 0.55 (solvent H). $^{31}$P n.m.r. data (CDCl$_3$): $\delta$P 148.72 (s) and 149.63 (s).

9.7 Bioconjugations

9.7.1 Purification of mouse IgG anti-DNP antibody (K3)

All samples of anti-DNP antibody used in the following conjugation reactions were purified beforehand from murine ascitic fluid by the following method:

Ice cold saturated aqueous (NH$_4$)$_2$SO$_4$ solution (410µl) was added slowly and with rapid stirring to crude mouse ascites fluid (500µl) and the resulting solution allowed to warm slowly to room temperature. The white flocular precipitate obtained as the solution reached a concentration of 45% (NH$_4$)$_2$SO$_4$ was then pelleted by centrifugation at 1000g for 2 minutes and the pale brown supernatant solution discarded. The off-white pellet of crude antibody was then resuspended in ice cold saturated aqueous (NH$_4$)$_2$SO$_4$ solution (1000µl) and the suspension centrifuged once more. After careful removal of the supernatant, the remaining pellet of semi-purified antibody was dissolved in loading buffer (1.5M glycine, 3M NaCl, pH=8.9, 2ml) and traces of (NH$_4$)$_2$SO$_4$ removed using a PD-10 column equilibrated and eluted with the same buffer. The antibody was then purified by F.P.L.C. using a Protein A superose column, eluting with a gradient of 0.1M citric acid buffer, pH=8 to pH=3. The appropriate fractions were collected and citric acid removed immediately by passage through the required number of PD-10 columns equilibrated and eluted with PBS (0.1M, pH=7.4). The purified antibody was then adjusted to the desired concentration by centrifugation with nitrocellulose centrifuge filters (30,000 M.Wt. cut off) (Millipore).
Samples of antibody not required for immediate use were then lyophilised or stored frozen at -20°C. The purity of antibody samples was verified by 12% polyacrylamide gel electrophoresis:

**Gel Setup:**

SE250 Mighty Small II Vertical Slab Unit (Hoefer Scientific Instruments). Polyacrylamide gels (80x75x1.5mm) were precast on a SE245 Mighty Small Dual Gel Caster. Power supply: Hoefer PS500XT Power Pack.

**Stock Solutions and Reagents**

A. 30% acrylamide/bisacrylamide in water. (29% w/v acrylamide and 1% w/v N,N'-methylenebisacrylamide)
B. 1.5M Tris buffer pH8.8
C. 10% (w/v) sodium dodecyl sulphate (SDS) solution in water.
D. 10% (w/v) ammonium persulphate in water
E. N,N,N',N'-tetramethylethylene diamine (TEMED)
F. Disruption buffer: 2ml 20% (w/v) SDS, 1ml β-mercaptoethanol, 1ml 1.0M Tris buffer pH7.0, 1ml 50% glycerol in water and 0.1% (w/v) bromophenol blue.

**Gel Preparation**

To prepare 25ml of a 12% polyacrylamide gel A (10.0ml), B (6.3ml) and C (0.25ml) were dissolved in water (8.2ml). D (0.25ml) and E (0.01ml) were added immediately before pouring the gel. Antibody (1-8μg) was dissolved in disruption buffer (F, 10μl) and heated in a boiling water bath for two minutes. After cooling, the solutions were loaded into the wells of the prepared gel and run at 20mA constant
current. Antibody heavy and light chains were visualised by staining the
gel with Coomassie Blue (0.01M in a 20% methanol, 20% acetic acid,
60% water (v/v/v) solution).

9.7.2 Conjugation of mouse IgG anti-DNP antibody (K3) to
horseradish peroxidase

Horeseradish peroxidase (10.1mg) was dissolved in H2O (1770µl)
to give a brown solution with a concentration of 5.65mg/ml. Similarly,
NaI04 (50.2mg) was dissolved in H2O (1173µl) to give a concentration
of 42.8mg/ml. Aliquots of the peroxidase (1000µl) and periodate (202µl)
solutions were then mixed in a sealed 3ml glass vial, protected from light
by wrapping in foil and mixed end over end for 30 minutes at room
temperature. The resultant green solution was then passed down a PD-10
column equilibrated and eluted with sodium acetate buffer (1mM,
pH=4.5). Once the eluant containing the brown activated peroxidase had
been collected, the pH of the solution was adjusted to pH=9.5 by small
(20µl) additions of Na2CO3/NaHCO3 buffer (0.2M, pH=9.5). An aliquot
of the activated peroxidase solution (150µl) was then added to a solution
of mouse IgG anti-DNP antibody (1mg) in H2O (1ml) in a 3ml glass vial.
The vial was then foil wrapped and mixed end over end at room
temperature. After 2.5 hours 47µl of a solution of NaBH4 (4mg) in Milli-
Q (1ml) was added to the reaction mixture and mixing continued for a
further 2 hours. Ice cold saturated aqueous (NH4)2SO4 solution (979µl)
was then added and the resultant precipitate centrifuged at 1000g for 10
minutes. After discarding the supernatant, the faintly brown pellet was
resuspended in 40% aqueous (NH4)2SO4 solution (2x1.5ml) and
centrifuged once more. The supernatant was again discarded, the final
pellet dissolved in PBS (0.1M, pH=7.4, 2ml) and traces of (NH4)2SO4
removed using a PD-10 column equilibrated and eluted with PBS. The resultant anti-DNP antibody-horseradish peroxidase conjugate solution (0.25mg/ml) was stored at 4°C in foil wrapped containers.

9.7.3 Conjugation of mouse IgG anti-DNP antibody (K3) to alkaline phosphatase

Glutaric dialdehyde, 25% aqueous solution (100ml) was added to phosphate buffer (50mM, pH=7.2, 11.9ml) and the solution stirred for 10 minutes. Alkaline phosphatase (100ml of 10mg/ml in phosphate buffer) was then activated by the addition of the buffered glutaric dialdehyde solution (400μl). Mixing end over end was continued at room temperature for 1 hour. Mouse IgG anti-DNP antibody (100μl of 4mg/ml in PBS) was then added to the activated alkaline phosphatase solution and the reaction mixture mixed at room temperature for 2 hours. The conjugate was purified by a Sephacryl 200 gel filtration column equilibrated with PBS (50mM, pH=7.4), the appropriate fractions being pooled and stored at 4°C.

9.7.4 Conjugation of dansyl groups to BSA and CyG

To a gently warmed solution of protein (10mg) in MES buffer (0.1M, pH=6.2, 2ml) was added dansyl chloride (21mg) in DMSO (2ml) and the resulting mixture incubated at 34°C overnight. The crude conjugate solution was then purified using NAP-10 columns, eluting with ethanol/water (1:1). Ethanol was removed from the pooled fractions by rotary evaporation and the protein residue redissolved in H2O. The purified conjugate solutions were stored frozen at -20°C.
9.7.5 Conjugation of oligonucleotides to alkaline phosphatase

(i) Preparation of Thiol Modified Oligonucleotides

An aminomodified oligonucleotide 19-mer probe supplied by the Oswel DNA Service (10 O.D.\textsuperscript{264}) was evaporated \textit{in vacuo} and redissolved in Na\textsubscript{2}CO\textsubscript{3}/NaHCO\textsubscript{3} buffer (0.5M, pH=9.5, 100µl). To this was added N-succinimidyl 3-(2-pyridyldithio)-propionate (2mg) in DMSO (100µl). Slight cloudiness in the reaction mixture was removed by the addition of a further 100µl of buffer and gentle warming. The resulting solution was protected from light and allowed to stand at room temperature for 2 hours. Preparative reverse phase H.P.L.C., eluting with a gradient of 0-40% acetonitrile in 0.1M NH\textsubscript{4}OAc buffer, allowed isolation of the product as a peak eluting significantly later than a mixture of minor products and unreacted starting material. The pure product was collected, desalted on a NAP-10 column, and the resulting solution kept frozen at -20°C until required.

(ii) Activation of Alkaline Phosphatase

To a solution of alkaline phosphatase (1mg) in phosphate buffer (50mM, pH=7, 500µl) was added 3-maleimidobenzoyl-N-hydroxysuccinimide (0.3mg) in DMF (50µl) and the solution incubated at room temperature for 1 hour. Phosphate buffer (50mM, pH=5.6, 350µl) was then added, and the mixture applied to a NAP-10 column equilibrated and eluted with the same phosphate buffer. The activated alkaline phosphatase solution was concentrated down to 200µl using nitrocellulose centrifuge filters (30,000 M.Wt. cut off) and the resultant solution used immediately in the next step.
(iii) Conjugation Reaction and Purification

The pyridyl disulphide modified oligonucleotide (7 O.D.'s) from (i) was evaporated in vacuo and redissolved in concentrated aqueous ammonia (1.5ml). This was followed by the addition of dithiothreitol (10mg) and the reaction mixture incubated at room temperature for 1 hour.

Purification of the thiol modified oligonucleotide was carried out by reverse phase H.P.L.C., eluting with a gradient of 0-20% acetonitrile in NH₄OAc buffer. The product was desalted on a NAP-10 column, evaporated in vacuo, and followed by the immediate addition of the 200μl of activated alkaline phosphatase solution from (ii). The reaction mixture was incubated at room temperature for 2 hours. Purification of the oligonucleotide-alkaline phosphatase conjugate was carried out by F.P.L.C. ion exchange chromatography, using a Mono-Q 5/5 HR20 anion exchange column. Elution with a gradient of 0-1.0M NaCl in Tris-HCl buffer (50mM, pH=7.4) gave the product as mixture of 3 discernible products eluting between 0.4-0.5M NaCl. The appropriate fractions were pooled and stored in the eluting buffer at 4°C.

9.8 Immunoblot Experiments

9.8.1 Solutions used

(1) PBS/EDTA/EGTA: Phosphate buffered saline solution (0.1M, pH=7.4) (Sigma) containing 1mM EDTA and 1mM EGTA.
(2) PBS/EDTA/EGTA/TWEEN: Phosphate buffered saline solution containing 1mM EDTA, 1mM EGTA and 0.5% Tween-20.
(3) Blocking solution: 10% solution of fat-free skimmed milk in PBS/EDTA/EGTA/TWEEN
(4) PBS/N₃:Phosphate buffered saline solution containing 0.01% NaN₃
PBSIN3ITWEEN: Phosphate buffered saline solution containing 0.01% NaN₃ and 0.5% Tween-20.

9.8.2 Membrane fixing of oligonucleotides

Solutions of labelled oligonucleotides of the appropriate concentrations were made up and 0.5μl of each solution spotted onto nylon (Hybond-N) filters. After leaving the membrane to dry at room temperature for 10 minutes, the oligonucleotides were fixed to the filters by U.V. irradiation at 300nm for 5 minutes. The filters were blocked by incubation with blocking solution for 1 hour and washed with PBS/EDTA/EGTA/TWEEN (3x2min) and PBS/EDTA/EGTA (1x2min).

9.8.3 Oligonucleotide hybridisation

Labelled oligonucleotides were hybridised to complementary membrane bound oligonucleotides by blocking the filters with blocking solution, and then incubating the filter at room temperature for 1 hour with a solution containing a 10-100 fold excess of the labelled probe.

9.8.4 Label detection

(1) DNP detection:

(a) Secondary antibody system: The filters were incubated at room temperature for 1 hour with a 1:200 dilution of anti-DNP antibody (K3) ascitic fluid in PBS/EDTA/EGTA then washed with PBS/EDTA/EGTA/TWEEN (3x2min) and PBS/EDTA/EGTA (1x2min). The filters were then incubated with a 1:1000 dilution of goat-anti-mouse IgG horseradish peroxidase conjugate solution (Sigma) for 1 hour, then washed with PBS/EDTA/EGTA/TWEEN (3x2min) and PBS/EDTA/EGTA (1x2min)
(b) Primary antibody-HRP conjugate system: The filters were incubated at room temperature for 1 hour with a 1:250 dilution of commercially available, anti-DNP antibody-HRP conjugate (Dako) in PBS/EDTA/EGTA, then washed with PBS/EDTA/EGTA/TWEEN (3x2min) and PBS/EDTA/EGTA (1x2min).

(c) K3 anti-DNP antibody-HRP conjugate system: The filters were incubated at room temperature for 1 hour with a 1:100 dilution of K3 anti-DNP antibody-HRP conjugate in PBS/EDTA/EGTA, then washed with PBS/EDTA/EGTA/TWEEN (3x2min) and PBS/EDTA/EGTA (1x2min).

(d) Anti-DNP antibody-alkaline phosphatase conjugate system: The filters were incubated at room temperature for 1 hour with a 1:80 dilution of anti-DNP antibody-alkaline phosphatase conjugate (not commercially available, supplied by Dako) in PBS/N3, then washed with PBS/N3/TWEEN (3x2min) and PBS/N3 (1x2min).

(2) Biotin detection: The filters were incubated for 1 hour with a 1:1000 dilution of avidin-HRP conjugate solution in PBS/EDTA/EGTA, then washed with PBS/EDTA/EGTA/TWEEN (3x2min) and PBS/EDTA/EGTA (1x2min).

(3) Digoxigenin detection: The filters were incubated at room temperature for 1 hour with a 1:1000 dilution of anti-digoxigenin antibody-alkaline phosphatase conjugate (Boehringer Mannheim) in PBS/N3, then washed with PBS/N3/TWEEN (3x2min) and PBS/N3 (1x2min).

(4) Fluorescein detection:

(a) Anti-fluorescein antibody-alkaline phosphatase conjugate system: The filters were incubated at room temperature for 1 hour with a 1:250 dilution of anti-fluorescein antibody-alkaline phosphatase conjugate (Dako) in PBS/N3, then washed with PBS/N3/TWEEN (3x2min) and PBS/N3 (1x2min).
9.8.5 Colour reactions

(1) **Horseradish peroxidase**: 3,3'-Diaminobenzidine tetrahydrochloride (10mg) was dissolved in Tris-HCl buffer (50mM, pH=7.6, 20ml) followed by the addition of hydrogen peroxide (0.06% final concentration). The filters were incubated with DAB solution at room temperature for 10-30 minutes and the reaction stopped by washing the filter several times with water.

(2) **Alkaline phosphatase**: Sigma FAST-BCIP/NBT tablets were used as directed by the manufacturer, incubating the filters at room temperature for 30min-12hrs. The reaction was stopped by washing with water.
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Synthesis and antibody-mediated detection of oligonucleotides containing multiple 2,4-dinitrophenyl reporter groups

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ABSTRACT
A series of non-nucleoside-based 2,4-dinitrophenyl (DNP) phosphoramidites have been prepared and used in the multiple labelling of oligonucleotides during solid-phase synthesis. The length of spacer arm between the DNP label and the oligonucleotide phosphate backbone, and the number of attached DNP groups have both been varied in order to determine the optimum conditions for anti-DNP antibody binding. Detection using enzyme-linked colorimetric techniques showed sensitivity equivalent to that obtainable using biotinylated oligonucleotides.

INTRODUCTION
Detection of oligonucleotide probes can be achieved by the addition of non-radioactive reporter groups, thereby precluding the hazardous, expensive and time consuming introduction of radiolabels such as $^3$H, $^{32}$P, $^{35}$S and $^{125}$I. Despite these advantages, non-radioactive labelling of oligonucleotides has been limited by its poor sensitivity relative to standard radiolabelling techniques. A solution to this problem is provided by the attachment of multiple labels and this has facilitated the visualisation of oligonucleotide probes in quantities previously obtainable only by radiolabelling (1,2). The requirements for the multiple attachment of non-radioactive labels are: (i) the aqueous solubility of the oligonucleotide must be unaffected by the introduction of lipophilic reporter groups; (ii) the reporter groups must not interfere with oligonucleotide hybridisation; (iii) the addition of reporter groups should be controllable to allow the attachment of a defined number of labels; and (iv) the labelling procedure should be simple and the materials inexpensive.

The most commonly used non-radioactive labelling group is biotin, which has been incorporated into oligonucleotides enzymically (3,4), photochemically (5), by reaction with amino-functionalised oligonucleotides (6–10), and by incorporation of biotinylated phosphoramidites during solid-phase synthesis (11,11–14). The main drawbacks are that biotin and its derivatives are expensive, the synthesis of biotinylated phosphoramidites is made difficult by the poor solubility of biotin, and endogenous biotin occurs at high levels in certain tissues, making it unsuitable for some types of in situ hybridisation. Detection of biotinylated oligonucleotides commonly employs enzyme linked immunosorbent assay (ELISA) methodology, with colorimetric or chemiluminescent visualisation via avidin or streptavidin conjugated to reporter enzymes such as alkaline phosphatase or horseradish peroxidase. Oligonucleotides can also be labelled with these enzymes directly, although the oligonucleotide-enzyme conjugate synthesis and purification procedures are time consuming (15) and such conjugates are unsuitable for use in techniques such as P.C.R. Fluorescent labelling of amino-modified oligonucleotides using fluorescein isothiocyanate (FITC) and other fluorescent dyes is now routine (15–19). However, the detection of oligonucleotides labelled in this way requires specialised equipment.

Recently, the hapten digoxigenin has been used as a non-radioactive reporter group (2,20–22). The labelled oligonucleotides are detected by digoxigenin-specific polyclonal sheep antibodies conjugated to alkaline phosphatase. Labelling with digoxigenin involves the enzymic incorporation of digoxigenin-labelled deoxyuridine-triphosphate into DNA, or via the reaction of a digoxigenin active ester with 5'-amino modified oligonucleotides. However the chemical labelling procedure is expensive and allows only the introduction of a single label whereas the enzymic method does not allow the controlled insertion of a specific number of labels.

An inexpensive label which can be detected immunogenically is the 2,4-dinitrophenyl group (DNP group). The DNP group has been introduced into oligonucleotides via the action of deoxynucleotidyl terminal transferase or DNA polymerase on a DNP-aminohexyl derivative of ATP, and by the reaction of Sanger's reagent (2,4-dinitrofluorobenzene) with oligonucleotides containing an aminohexyl derivative of adenosine (23). DNP groups have been introduced photochemically (24) and by reaction with brominated bases (25). Both single and multiple

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DNP groups have been incorporated into oligonucleotides using DNP phosphoramidites during solid phase synthesis, although in both examples of multiple addition the DNP group was unstable in aqueous ammonia under normal base-catalysed deprotection conditions (12,26).

Oligonucleotides labelled with a multiple addition DNP phosphoramidite have been previously synthesised by this group. These oligonucleotides, which had the DNP groups directly linked to the nitrogen atoms of a 3,6-diazaoctane backbone gave much lower sensitivity on detection with a monoclonal IgG mouse anti-DNP antibody than those labelled with a single addition DNP phosphoramidite separated from the DNA backbone by a hexyl spacer (26). This suggested to us that a spacer arm is necessary for maximum sensitivity using the above antibody. The aim of this work was to develop a range of simple DNP phosphoramidites with various spacer arms for the poly-labelling of oligonucleotides during standard solid phase synthesis.

RESULTS AND DISCUSSION

The first step in the synthesis of a 2,4-dinitrophenyl multiple addition phosphoramidite is the construction of the backbone onto which the DNP label, a dimethoxytrityl group and a phosphorus group can be attached. There are two basic requirements for this backbone: (i) Two hydroxyl functions, preferably one primary and one secondary to give the necessary specificity for the sequential addition of the dimethoxytrityl and phosphoramidite moieties. (ii) An amino group separated from the backbone by a linker to allow attachment of the DNP group.

Synthesis of DNP phosphoramidites

DNP phosphoramidite [1c]. (Scheme 1) 3-Aminopropylglycerol was converted to its acetone derivative [2a] in a yield of 85% by reaction with anhydrous acetonitrile in the presence of anhydrous potassium phthalimide in DMF to give the phthalimide [2b] in 96% yield. Subsequent removal of the phthalimide group with hydrazine hydrate in methanol gave, after distillation, the amine [2c] in 96% yield. Subsequent removal of the phthalimide group with hydrazine hydrate in methanol gave, after distillation, the amine [2c] in 96% yield. Subsequent removal of the phthalimide group with hydrazine hydrate in methanol gave, after distillation, the amine [2c] in 96% yield. The amine was reacted with 2,4-dinitrofluorobenzene to give the DNP phosphoramidite [1c] in 94% yield.

DNP phosphoramidite [2g]. (Scheme 2) 1,2,6-Trihydroxyhexane was converted to its tosylate derivative [2d] in a yield of 85% by reaction with anhydrous toluene under normal base-catalysed deprotection conditions (12,26).

DNP phosphoramidite [3c]. (Scheme 3) This compound has a 1-O-(3-aminopropyl) glycerol backbone, originally developed by Miura et al (1) for use in the construction of multiple addition phosphoramidites. No alterations were made to the original experimental procedure to prepare 3-aminopropyl solketal. This was treated with 2,4-dinitrofluorobenzene to give, after wet flash column chromatography the DNP phosphoramidite [3a] in 94% yield. Subsequent removal of the
isopropylidene protecting group followed by addition of a dimethoxytrityl group gave the DNP monomer precursor [3b] in 79% yield. Reaction of [3b] with 2-cyanoethyl-N,N-disopropyl phosphoramidochloridite gave the DNP phosphoramidite [3c] in 83% yield.

**Scheme (4) Synthesis of phosphoramidite [4f]**

DNP phosphoramidite [4f]. (Scheme 4) 6-Aminohexanoic acid was reacted with ethyl trifluoroacetate to give a near quantitative yield of the trifluoroacetyl (TFA) protected amino acid [4a]. This was converted to the 2-nitrophenyl active ester [4b] which was reacted with 3-aminopropyl solketal to give the TFA-acetonide [4c] in quantitative yield. Heating this compound in conc. aqueous ammonia in a sealed pressure tube at 70°C cleanly removed the TFA protecting group and subsequent reaction of the crude free amine with 2,4-dinitrofluorobenzene and triethylamine gave the DNP-acetonide [4d]. Removal of the isopropylidene group by acidic treatment followed by the standard tritylation and phosphitylation procedures, gave the DNP phosphoramidite [4f] in an overall yield of 78%.

**Figure 1. Reversed phase H.P.L.C. chromatogram of a mixed injection of purified 5'-DMTr-multiple DNP labelled 24-mers and unlabelled reference sequence: From left to right: unmodified 24-mer, (DNP)3-24mer, (DNP)5-24mer. DNP labels were introduced via phosphoramidite [3c].**

oligonucleotides to elute much later than failure sequences. Additional lipophilicity is gained by the presence of the terminal DMTr-group and this was left attached to the oligonucleotide during H.P.L.C. purification. The effect of increasing the number of DNP groups on oligonucleotide retention times is shown in Figure (1). After H.P.L.C. purification the oligonucleotides were detritylated with acetic acid and desalted by gel-filtration on Sephadex G-25. DNP labelled oligonucleotides are bright yellow in colour.

**DNP label detection.** The detection of DNP labelled oligonucleotides was carried out simply and conveniently using the following procedure:

(i) Known amounts of DNP labelled 24-mer probes were fixed
to a nylon membrane by UV irradiation. (The DNP labels were found to be stable to prolonged irradiation).

(ii) After blocking with non-fat skimmed milk the membrane was incubated with a mouse anti-DNP monoclonal antibody, followed by incubation with a goat anti-mouse horseradish peroxidase conjugate.

(iii) Subsequent incubation with 3,3'-diaminobenzidene/H₂O₂ allowed colorimetric visualisation of the fixed oligonucleotides.

The detection of biotin labels was carried out similarly, substituting step (ii) for an incubation with an avidin-horseradish peroxidase conjugate.

It is clear from Figure 2 that three DNP labels gave better sensitivity than a single DNP group. However, increasing the number of labels from three to five did not give any further improvement (Figures 2, 5, 7). Why do three DNP groups give optimum sensitivity? Clearly, it is unlikely that more than one antibody molecule can bind simultaneously to a short string of DNP groups due to the huge size of an IgG molecule relative to the DNP monomer units. In addition, the space between the two Fab regions of an IgG molecule is very large so it is not possible for both arms of the antibody to bind to a single short oligonucleotide.

Does the steric bulk of the oligonucleotide hinder the interaction between some of the DNP groups and the antibody? To investigate this a tetraethylene glycol monomer was prepared (Figure 3) and incorporated during oligonucleotide synthesis between the 5'-end of the oligonucleotide and the DNP labelling groups. Although the tetraethylene glycol spacer arm separates the DNP groups from the oligonucleotide it does not produce any enhancement in colorimetric detection (Figure 4). As tetraethylene glycol is similar in length to two DNP monomer units and is sterically undemanding, the lack of increased signal with this spacer suggest that the steric bulk of the oligonucleotide does not hinder the antibody-hapten interaction. It is possible that the increased sensitivity achieved by three DNP groups relative to one arises from co-operativity between the DNP groups. When one DNP group occupies the antibody binding site, its neighbours all lie very close to the site. Thus there is a high local concentration of haptens around the antibody binding site. Under these circumstances it is reasonable to assume that three DNP groups may be as effective as five.

Significantly, labelling with three or more DNP groups gave detection limits comparable with those of biotin labelled oligonucleotides (Figures 5 & 6). A much lower background was obtained with no significant loss of signal using a commercially available anti-DNP polyclonal antibody-horseradish peroxidase conjugate (Dakopats, results not shown).

All four phosphoramidite monomers 1c, 2g, 3c and 4f were attached singly and multiply to synthetic oligonucleotides to investigate the relationship between sensitivity of detection and
spacer arm length. Figure 7 shows that there is no significant difference in detection sensitivity between any of the monomers. Therefore, our results show that the optimum DNP oligonucleotide-labelling system will consist of three DNP labels attached to an oligonucleotide probe via a spacer arm of any reasonable length.

**Conclusion**

These results augur well for the establishment of the DNP hapten as a useful alternative to currently available non-radioactive labels such as biotin or digoxigenin. The combined advantages of minimal background signal, ease of introduction and low cost offer a system of practical and commercial viability. The uses of DNP labelled oligonucleotides as probes in applications such as the detection of Polymerase Chain Reaction products and in situ hybridisation are currently under investigation. The results of these studies, which are so far extremely encouraging, will be published elsewhere.

**EXPERIMENTAL**

**Anhydrous solvents:** Dichloromethane was distilled over CaH₂; tetrahydrofuran and diethyl ether were distilled over sodium/benzophenone; N,N-dimethylformamide was fractionally distilled under reduced pressure over 4A molecular sieves; pyridine was distilled over CaH₂; acetone was distilled over anhydrous CaSO₄ (Sikkon Fluka Universal Dessicant); hexane was dried over Na wire; triethylamine and N,N-diisopropylidene dioxyhex-1-yl p-toluenesulphonate [2b]. To a solution of 1,2,6-trihydroxyhexane (37g; 0.27mol) in anhydrous acetone (250ml) was added anhydrous sodium sulphate (100g) and conc. aqueous HCl (5ml). The reaction was stirred overnight at 20°C. Lead (II) carbonate (basic form) (100g) and Na₂CO₃ (1g) were added and the reaction was set aside for 48 hrs. Solids were then filtered off and the solvent was evaporated in vacuo to give a pale yellow liquid. Na₂CO₃ (1g) was added to the liquid to maintain basic conditions and the product was purified by kugelrohr distillation to give a clear liquid (b.pt. 120°C at 0.038 mm Hg), (39.92%,85%). RF 0.46 (solvent A). H N.m.r. data (CDCl₃): δH 1.24 (3 H, s, CH₃), 1.29 (s, 3 H, CH₃), 1.24-1.60 (m, 6 H, 3 CH₂), 2.91 (brs, 1 H, OH), 3.36-3.52 (m, 3 H, CH, CH₂), 3.87-4.01 (m, 4 H, 2CH₂); F.a.b. mass spectrum: m/z 175.13341 calc. for C₉H₁₀O₂ (M+H)+, m/z 175.13341.

**N-phthaloyl-5,6-isopropylidene dioxyhexylamine [2c].** To a solution of (0.0116mol; 2.14g) in anhydrous pyridine (50ml), cooled to 0°C, was added toluene sulfonyl chloride (0.014mol; 2.74g). After 2h at 20°C the solution was evaporated to an oil and coevaporated with toluene three times. The residue was taken-up in dichloromethane and washed with water (20ml) and sat. aq. KCl (3×20ml). The organic phase was dried (Na₂SO₄), filtered and evaporated in vacuo. The product was purified by wet-flash silica gel chromatography, eluting with 10% ethyl acetate in toluene, to give the product as a clear pale yellow oil (2.87g, 61%). RF 0.30 (solvent B). H N.m.r. data (CDCl₃): δH 1.24 (3 H, s, CH₃), 1.29 (s, 3 H, CH₃), 1.24-1.63 (m, 6 H, 3 CH₂), 2.36 (s, 3 H, CH₃), 3.87-3.97 (m, 4 H, 2CH₂), 7.26 (d, 2 H, Ar-H₃, Ar-H₅, J=7.84 Hz), 7.69 (m, 2 H, Ar-H₂, Ar-H₆). F.a.b. mass spectrum: m/z 329.14224 calc. for C₁₆H₂₅O₂S (M+H)+, m/z 329.14226.

**Supplementary data.** Syntheses of all compounds in schemes 2 and 3 are described in detail. Full experimental details for the synthesis of the compounds in schemes 1 and 4 and Figure 7 are available on request. H N.m.r. and i.r. spectral data are also available for most compounds.
5,6-Isopropylidenedioxyhexylamine [2d]. To a solution of [2c] (2.501g; 8.25mmol) in methanol (10ml) was added hydrazine hydrate (9.08mmol; 0.44ml). The reaction was stirred for 16hrs. at 20°C, during which time a white precipitate formed. The solution was evaporated to dryness, and the residue was dissolved in diethyl ether and washed with 2M NaOH (30ml). The aqueous phase was extracted with diethyl ether (3×20ml) and the combined organic phase was dried (MgSO₄), filtered and evaporated in vacuo to give a white solid. The product was purified by kugelrohr distillation to give a clear liquid (b. pt. 100°C at 0.0076 mmHg), (0.720g; 51%).

1-(2,4-Dinitrophenylamino)-5,6-isopropylidenedioxyhexane [2e]. To a solution of compound [2d] (0.5g; 2.89mmol) in methanol (10ml) was added triethylamine (0.012mol; 1.61ml) and a solution of 2,4-dinitrofluorobenzene (2.89mmol; 0.538g) in methanol (5ml). The reaction was stirred at 20°C for 16h, then evaporated to dryness, and coevaporated with toluene (3×20ml).

The residue was purified by wet-flash silica gel chromatography, eluting with a gradient of 0 to 50% ethyl acetate in dichloromethane methanol. The reaction mixture was evaporated in vacuo, and the product was purified by wet flash column chromatography, eluting with a gradient of 0 to 3% methanol in dichloromethane methanol. The appropriate fractions were evaporated in vacuo to give the title compound as a yellow oil (3.42g, 94%).

1-(2,4-Dinitrophenylamino)-4-oxa-6,7-isopropylidenedioxy-heptane [3a]. 2,4-Dinitrofluorobenzene (1.88g, 10mmol) was added to a solution of 3-aminopropyl solketal (2.30g, 12mmol) and triethylamine (3.0ml, 22mmol) in methanol (10ml) and the solution was stirred at room temperature. After 24 hours the reaction mixture was evaporated in vacuo, and the product was purified by wet flash column chromatography, eluting with a gradient of 0 to 3% methanol in dichloromethane methanol. The appropriate fractions were evaporated in vacuo to give the title compound as a yellow oil (3.42g, 94%).

1-(2,4-Dinitrophenylamino)-4-oxa-6,7-isopropylidenedioxy-heptan-2-ol [2f]. To a solution of [2e] (1.00g; 2.95mmol) in tetrahydrofuran (10ml) was added water (4ml) and conc. aqueous HCl (1ml). The reaction to give the diol was complete after 45min. Rf (diol) 0.39 (solvent C). The solvent was removed by evaporation in vacuo and the residual oil was coevaporated with anhydrous pyridine (4×10ml), and then dissolved in anhydrous pyridine (20ml). To this solution was added 4,4'-dimethoxytrityl chloride (3.25mmol; 1.1g). The reaction mixture was stirred at 20°C for 16h then quenched with methanol (1ml).

The solvent was removed by evaporation and the residue was coevaporated with toluene (3×10ml), taken-up in dichloromethane and washed with sat. aqueous NaHCO₃ (20ml) and sat. aqueous KCl (3×20ml). The organic phase was dried (MgSO₄), filtered and evaporated in vacuo. The product was dissolved in hexane:ethyl acetate:1:1 and applied to a wet-flash silica gel chromatography column which had been equilibrated with hexane containing 20% ethyl acetate and 1% triethylamine. The column was then eluted with a gradient of 20% 50% ethyl acetate in hexane, to give a yellow foam (1.274g, 72%).

1-(4,4'-Dimethoxytrityl)-6-(2,4-dinitrophenylamino) hexan-2-ol [3b]. Concentrated hydrochloric acid (2ml) was added to a solution of [3a] (1.01g, 2.8mmol) in THF (5ml), and the solution was stirred at room temperature for 2 hours. The reaction mixture was then concentrated in vacuo and the residue was dissolved in THF (10ml), and concentrated once more. The resulting solution was washed with sat. aqueous KCl (3×10ml), sat. aqueous NaHCO₃ (1×50ml), sat. aqueous KCl (1×50ml) and water (1×50ml). The organic layer was dried over Na₂SO₄, concentrated in vacuo, and the residue was purified by wet flash column chromatography, eluting with a gradient of 0 to 50% ethyl acetate/1:1.
in hexane containing 2% triethylamine. The appropriate fractions were collected and evaporated in vacuo to give the title compound as a yellow foam (1.52g, 79%). Rf 0.23 (solvent D). 'H n.m.r. in hexane containing 2% triethylamine. The appropriate fractions were collected and evaporated in vacuo.

\[
\text{2-Chloroethyl} \quad [1-(4,4'-dimethoxytrityloxy)-4-oxa-6-(2,4-dinitrophenylamino) hept-2-yl] \quad N,N\text{-diisopropylamino phosphoramidite} \quad \text{(3c). Compound} \quad \text{[3b]} \quad \text{(0.510g, 0.8mmol, 1eq) was coevaporated with anhydrous THF (3 x 5ml) and dissolved in anhydrous THF (5ml). To this solution was added N,N-diisopropylethylamine (0.5ml, 3mmol) and 2-cyanoethyl-N,N-diisopropylphosphoramidochloridite (0.31ml, 1.4mmol) and the mixture was stirred at room temperature for 3 hours. The reaction was quenched with ethyl acetate (50ml), washed with sat. aqueous NaHCO₃ (2 x 50ml), the organic layer dried (Na₂SO₄), and then evaporated in vacuo.}
\]

The crude product was purified by wet flash column chromatography on a short column of silica gel which was pre-equilibrated by sonication in hexane/triethylamine (99:lv/v). The product was applied to the column in the minimum volume of hexane/ethyl acetate (1:1 v/v) and eluted with ethyl acetate. The appropriate fractions were evaporated in vacuo.

\[
\text{Oligonucleotide synthesis. Oligonucleotide synthesis was performed using cyanoethyl phosphoramidite chemistry on an Applied Biosystems 380B DNA synthesizer. All DNA synthesis reagents and cyanoethyl-phosphoramidite monomers were supplied by Cruachem. DNP phosphoramidites were used as 0.15M solutions in anhydrous acetonitrile. 'Trityl-on' synthesis was carried out throughout and all oligonucleotides were deprotected in conc. aqueous ammonia for 5 hours at 60°C.}
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**Measurement of phosphoramidite coupling efficiencies.** Fractions from successive detritylations were diluted to 25ml with 0.1M 4-toluene-sulphonic acid in acetonitrile and coupling efficiencies were measured by comparison of absorbances at 498nm.

**Oligonucleotide analysis and purification.** HPLC analysis and purification of 'trityl-on' oligonucleotides was carried out on a Gilson model 306 using a Brownlee Aquapore Octyl reverse phase column (10 mm x 250 mm) with a flow rate of 3ml/minute and the following gradient:

<table>
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<th>Time (mins.)</th>
<th>%Buffer B</th>
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<td>3</td>
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<td>28</td>
<td>0</td>
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Buffer A: 0.1M NH₄OAc. Buffer B: 0.1M NH₄OAc with 50% acetonitrile. The desired 'trityl-on' products eluted significantly later than failure sequences.

**Detritylation of 'trityl-on' oligonucleotides.** After HPLC purification, 'trityl-on' oligonucleotides were evaporated to dryness and dissolved in 3% aqueous acetic acid (10ml). After 30mins at 20°C the solutions were evaporated to dryness in vacuo and oligonucleotides were desalted on NAP-10 columns (Sephadex G25, Pharmacia) following the manufacturer's instructions.

**Dot blot experiments.** Solutions used: (1) PBS/EDTA/EGTA: Phosphate-buffered saline solution (Sigma) containing 1mM EDTA and 1mM EGTA. (2) PBS/EDTA/EGTA/Tween: Phosphate-buffered saline solution (Sigma) containing 1mM EDTA and 1mM EGTA and 0.5% Tween-20. (3) Blocking solution: 10% solution of fat-free skimmed milk in PBS/EDTA/EGTA. (4) Mouse anti-DNP antibody ascitic fluid: containing approx. 7mg.ml⁻¹ of monoclonal anti-DNP antibody. (5) Avidin-HRP conjugate solution: a 0.2mg.ml⁻¹ solution of avidin-horseradish peroxidase conjugate (Sigma) in PBS. (6) DAB solution: a 0.5mg.ml⁻¹ solution of 3,3'-diaminobenzidine tetrahydrochloride (Sigma) in 50mM Tris—HCl, pH 7.6. Hydrogen peroxide (0.06% final conc.) added just before use. Solutions of DNP and DNP/biotin labelled oligonucleotides of the appropriate concentrations were made up and 0.5μl of each solution was spotted onto nylon (Hybond N) filters (Amersham). The oligonucleotides were fixed to the membrane by UV irradiation for 5 min. The filters were blocked by incubation with blocking solution for 1h and washed with PBS/EDTA/EGTA/Tween (3 x 2min) and PBS/EDTA/EGTA (1 x 2min).

**DNP detection.** The filters were incubated for 1h with a 1:200 dilution of anti-DNP antibody ascitic fluid in PBS/EDTA/EGTA. then washed with PBS/EDTA/EGTA/Tween (3 x 2min) and PBS/EDTA/EGTA (1 x 2min). The filters were then incubated with a 1:1000 dilution of goat-anti-mouse IgG solution (Sigma) for 1h, then washed with PBS/EDTA/EGTA/Tween (3 x 2min) and PBS/EDTA/EGTA (1 x 2min).

**Biotin detection.** The filters were incubated for 1h with a 1:1000 dilution of avidin-HRP conjugate solution in PBS/EDTA/EGTA then washed with PBS/EDTA/EGTA/Tween (3 x 2min) and PBS/EDTA/EGTA (1 x 2min).

**Colour reaction.** The filters were incubated with DAB solution for 10—30 min. The reaction was stopped by washing the filter several times with water.

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