The Chemical Modification of DNA for Analysis by Surface Enhanced Resonance Raman Scattering (SERRS) Spectroscopy

By

Rachel Brown

A Thesis submitted to the University of Strathclyde, Department of Pure and Applied Chemistry, in fulfilment of the degree of Doctor of Philosophy.

May 2002

Acknowledgements

Firstly, I would like to thank my supervisors Prof. Ewen Smith and Dr. Duncan Graham for all their help and guidance over the course of my Ph.D. Thanks also to AstraZeneca for funding this research in particular Dr. David Whitcombe and Dr. Neil Gibson.

I would also like to thank all my colleagues (past and present) in the Raman group for their help, enthusiasm and moral support, especially Caroline and Ben. Thanks also to the students in the lab especially Ljiljana, Arlene, Gerry, Antonio and Callum for many interesting discussions and making my time there fun.

Finally, thank you to Kenny and also my parents for all their love and support.

Abstract

The detection of specific DNA sequences is of increasing interest due to the sequencing of the human genome. This can be achieved by the use of covalently labelled oligonucleotide probes detected by sensitive analytical techniques. Surface Enhanced Resonance Raman Scattering (SERRS) spectroscopy is one such technique that provides molecularly specific information about probes at very low concentrations. The enhancement results from adsorption of a chromophore within the probe to a roughened metal surface.

Specific SERRS probes were synthesised using benzotriazole as the metal complexing agent and azo dyes as the chromophore. They were coupled to the 5' end of DNA using two strategies. Firstly, coupling was achieved *via* an amino linker, achieved by reaction of the activated carboxylic acid derivative of the SERRS label with DNA containing a free amine at the 5'end. Secondly, the phosphoramidite of the SERRS label was synthesised and incorporated as the final monomer during the solid phase synthesis of the DNA sequence.

Preliminary spectroscopic data was obtained for the labelled oligonucleotides. Ultraviolet melting studies of a DNA sequence labelled with an azobenzotriazole dye show an increase in melting temperature (T_M) of 5.42 ^{o}C over the same sequence without modification, suggesting that the label confers stability to the double helix. Initial SERRS optimisation experiments allowed the optimum sample conditions to be determined for these novel oligonucleotides. SERRS spectra have been obtained for each labelled oligonucleotide with a detection limit determined at 5×10^{-8} M.

A potential application of the labelled oligonucleotides was investigated resulting in the first ever preparation of a SERRS labelled nanoparticle probe. This provides the basis for a specific sequence detection technique based on SERRS.

Abbreviations

A	Adenine
A	Absorbance
a	Absorption Coefficient
aa	Amino Acid
Bt	Benzotriazole
bz	Benzoyl
bz Cl	Benzoyl Chloride
C	Cytosine
CDI	1,1-carbonyldiimidazole
CPG	Control Pore Glass
DBU	Diazabicyclononane
DCM	Dichloromethane
DIPEA	Diisopropylethylamine
DMAP	Dimethylaminopyridine
DMF	Dimethyl Formamide
DMSO	Dimethyl Sulfoxide
DMTr	Dimethoxytrityl
DNA	Deoxyribonucleic Acid
ds	Double Strand
EI	Electron Impact
FAB	Fast Atom Bombardment
G	Guanine
HPLC	High Performance Liquid Chromatography
ib	Isobutyryl
MALDI TOF	Matrix Assisted Laser Diffraction Time of Flight
MeCN	Acetonitrile
MeOH	Methanol
mRNA	Messenger Ribonucleic Acid
MMTr	Monomethoxytrityl

NMR	Nuclear Magnetic Resonance
RR	Resonance Raman
RNA	Ribonucleic Acid
SERS	Surface Enhanced Raman Spectroscopy
SERRS	Surface Enhanced Resonance Raman Spectroscopy
ss	Single Strand
T	Thymine
TBAF	Tetrabutylammoniun Fluoride
TBDPS	Tertiary butyldiphenylsilyl group
THF	Tetrahydrofuran
TLC	Thin Layer Chromatography
T_M	Melting Temperature
TM	Target Molecule
Tr	Trityl
tRNA	Transport Ribonucleic Acid
UV	Ultraviolet
V/v	Volume/ volume
W/v	Weight/ volume

Contents

Chapter 1

Introduction

1.1 Introduction - Deoxyribonucleic Acid, DNA

1.1.1 General

Deoxyribonucleic acid, DNA, is amongst the fundamental building blocks of life. All the information required for the existence of each species is held within the sequences of its DNA. Birth, growth, life and death are all controlled through the replication and expression of genetic codes. It is therefore very important that we attempt to understand genetic sequences and their impact on biological systems. In order to achieve this, it is necessary to develop fast and accurate analytical techniques for the analysis of DNA.

Current techniques for genomic analysis involve the use of radioactive labels, which have many drawbacks such as handling, safety issues and disposal. In addition they have a finite lifespan, some with a half-life of only a few hours. Other detection methods include luminescence and fluorescence. The spectral information obtained from such techniques is limited due to the large spectral bandwidths observed which makes distinction between three or more labels very difficult. It is therefore very favourable to use vibrational spectroscopy for the analysis of DNA since non-radioactive probes can be used which provide molecularly specific information about the analyte. This allows the detection of many probes simultaneously.

One such technique is Surface Enhanced Resonance Raman Scattering, SERRS, spectroscopy. Using this form of vibrational spectroscopy, it is possible to detect concentrations as low as 1×10^{-18} M.[1] However, there are two structural requirements of the molecule under observation: a surface complexing group and a chromophore. This thesis details the chemical modification of DNA to accommodate these requirements and subsequent SERRS analysis of successful targets.

1.1.2 DNA – Composition and Structure

DNA has three main components: 2'-deoxyribose sugar units, heterocyclic aromatic amine bases and phosphate linkages.[2] These components are joined together in a repeating pattern (Figure 1) to form an oligodeoxyribonucleotide.

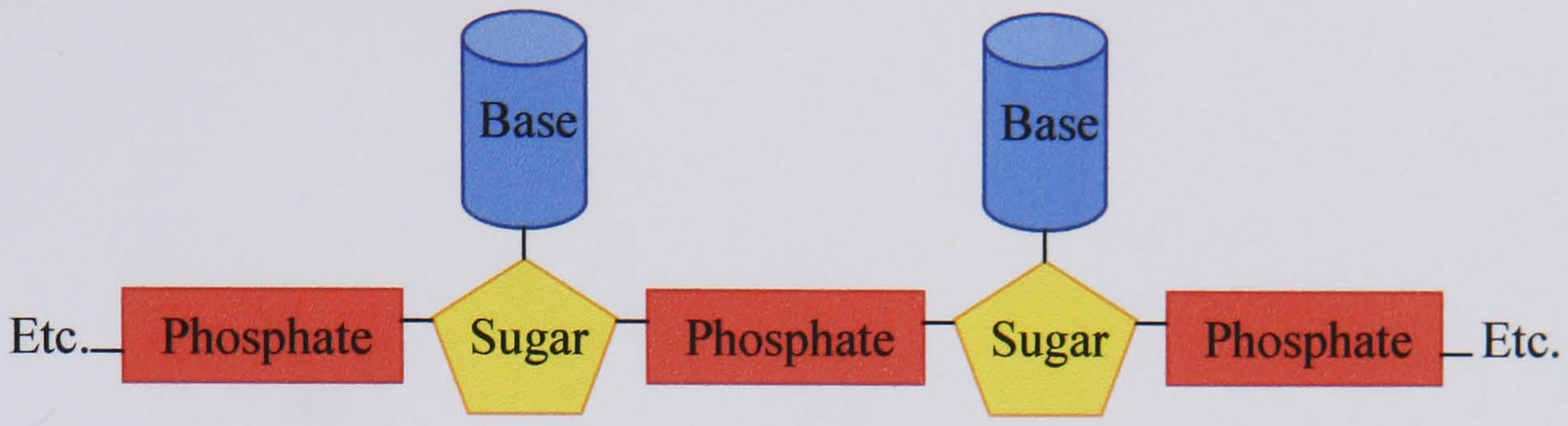


Figure 1. Primary structure of DNA

There are four aromatic bases. Guanine and adenine are purines and thymine and cytosine are pyrimidines:

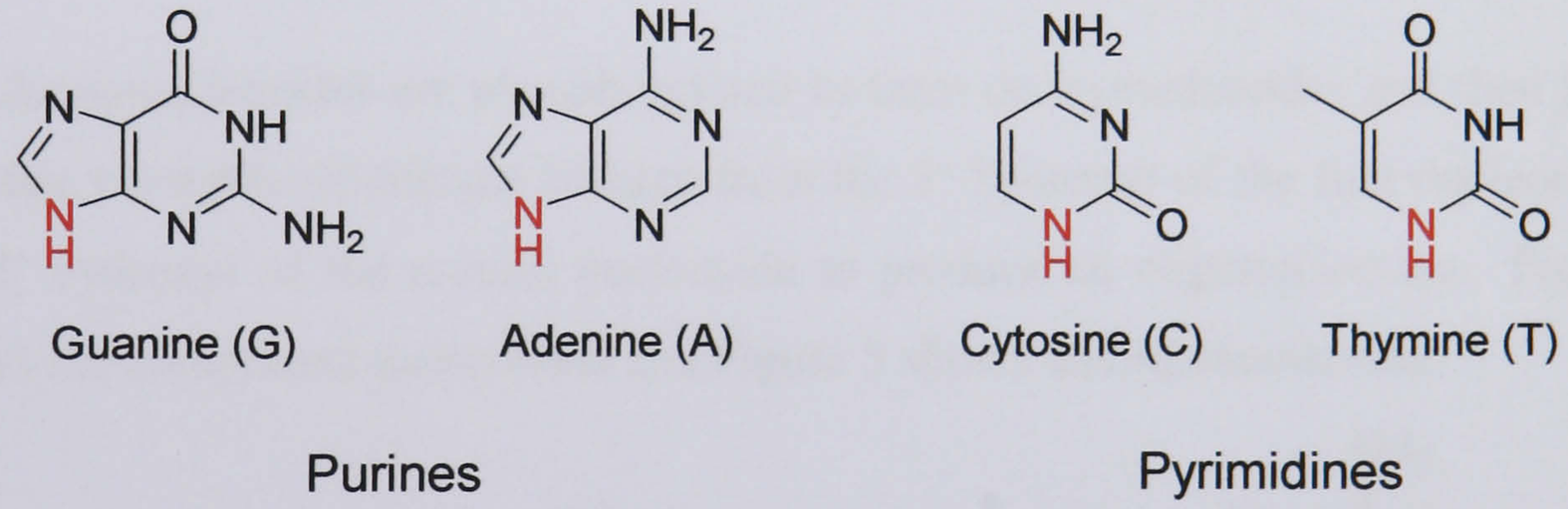
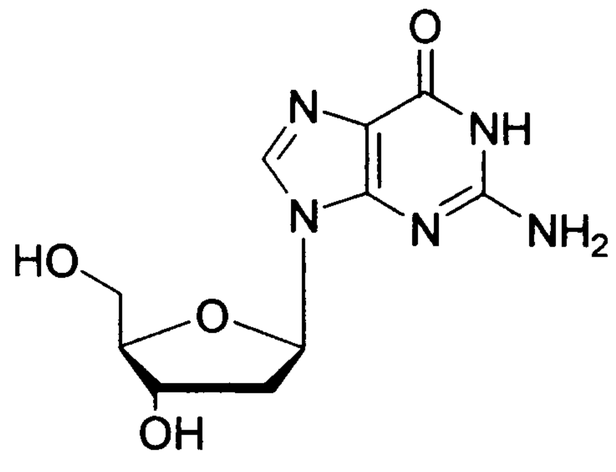
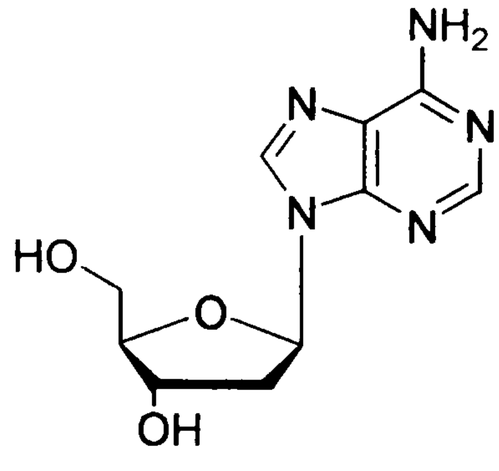


Figure 2. The DNA bases

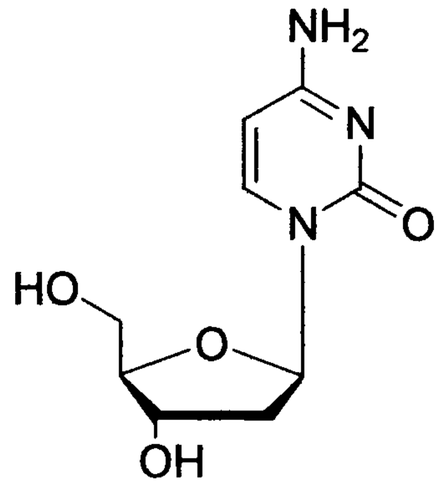
The DNA bases are linked to the sugar unit *via* an β-N-glycoside link between C1 on the sugar ring and N9 of a purine or N1 of a pyrimidine (indicated in red in Figure 2). When the base is linked to 2'-deoxyribose a deoxynucleoside is formed. Figure 3 shows the deoxynucleosides of the four bases.



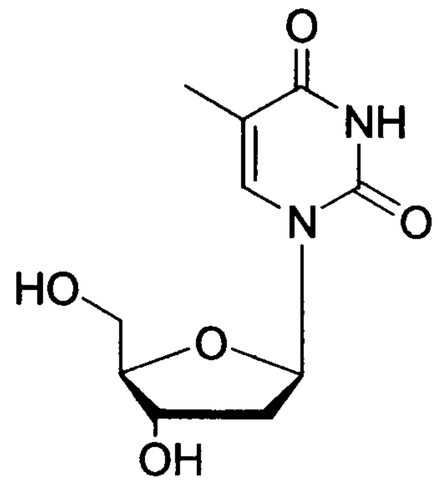
2'-deoxyguanosine
(dG)



2'-deoxyadenosine
(dA)



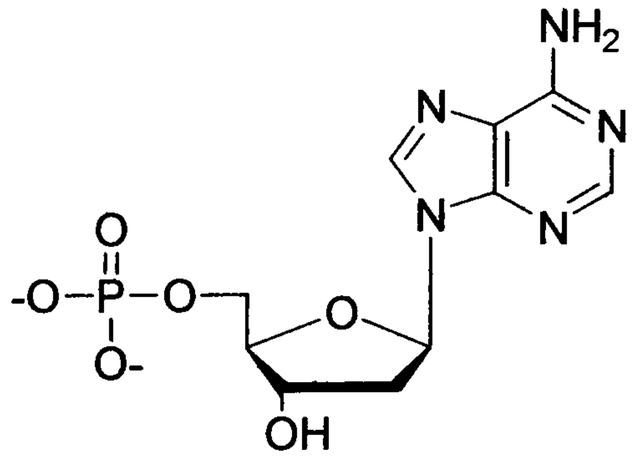
2'-deoxycytidine
(dC)



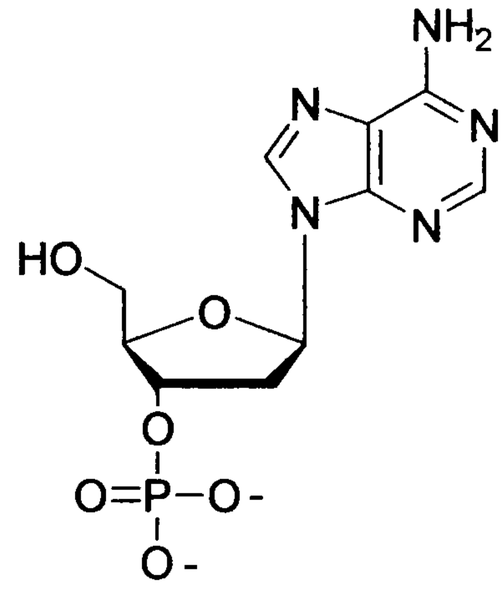
2'-deoxythymidine
(dT)

Figure 3. Deoxynucleosides

The deoxynucleosides are phosphorylated to form deoxynucleotides and then joined together *via* a phosphodiester linkage from the 3' hydroxyl of the first nucleoside to the 5' hydroxyl of the second nucleoside to produce an oligonucleotide. Figure 4 shows the component nucleotides and Figure 5 shows an oligonucleotide.



2'-deoxyadenosine 5'-monophosphate
(dAMP)



2'-deoxyadenosine 3'-monophosphate
(dAMP)

Figure 4. Deoxynucleotides.

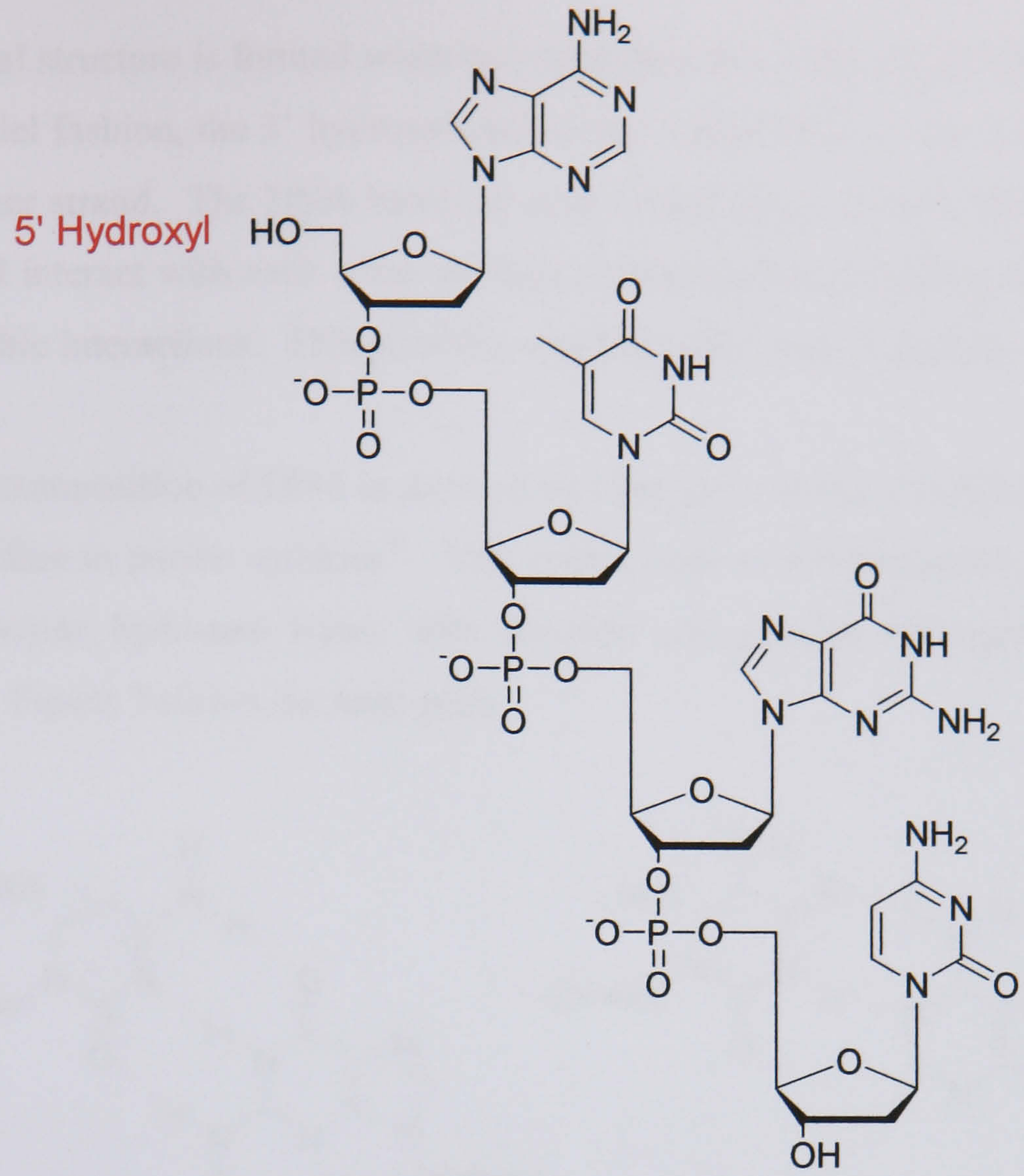


Figure 5. An oligonucleotide, with sequence of bases *5'* ATG C *3'*.

The secondary structure of DNA is a double helix, which was elucidated by Watson and Crick in 1953, shown in Figure 6.[3, 4]

Figure 6. The DNA double helix.

The helical structure is formed when two complementary oligonucleotides bind in an anti-parallel fashion, the 3' hydroxyl end of one strand binds to the 5' hydroxyl end of the other strand. The DNA bases on each strand, point towards the centre of the helix, and interact with each other through a combination of hydrogen bonding and hydrophobic interactions. This provides a hydrophobic core to the helix.

The base composition of DNA is dictated by Chargaff's Rules: DNA has equal ratios of pyrimidine to purine residues.[5] This corresponds to complementary base pairing where adenine hydrogen bonds with thymine and guanine hydrogen bonds with cytosine. Figure 7 shows the base pairs.

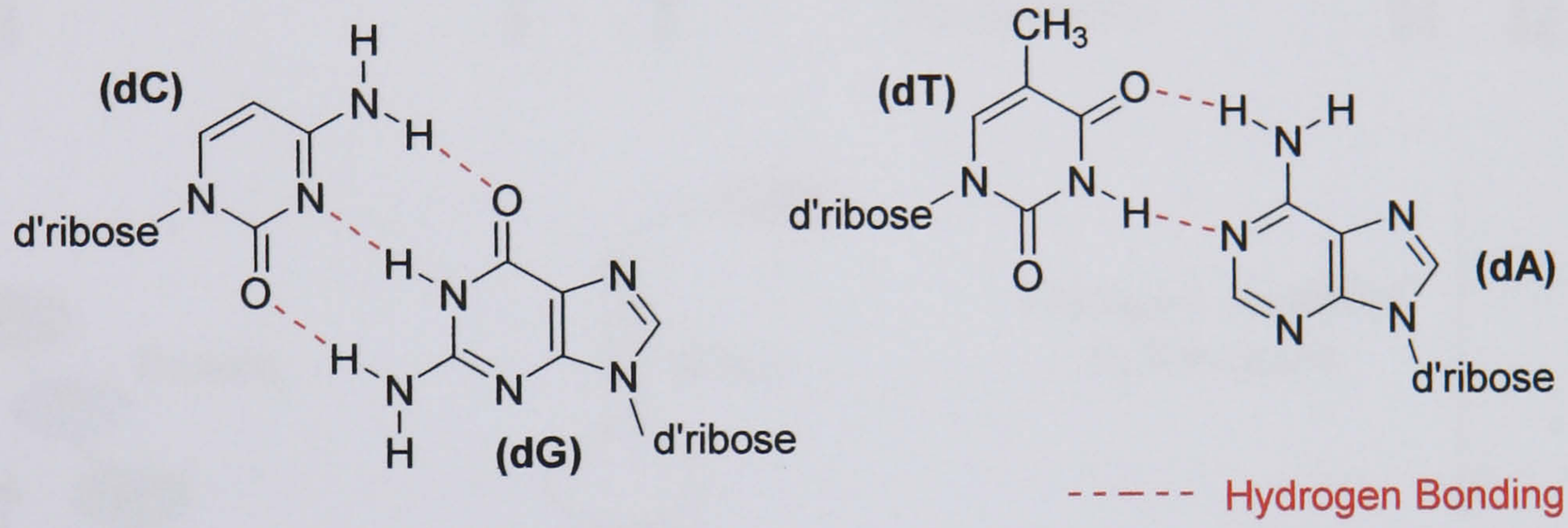


Figure 7. Base pairing.

The above hydrogen bonded molecules are flat and stack within the double helix. Each base pair has π interactions with the base pair immediately above and below, giving added stability to the helix.

1.1.3 The Central Dogma of Molecular Biology

The genetic information held within DNA is transferred into vital proteins which are required for the many biological processes which maintain a healthy functioning body. The process by which this information is transferred is called translation and is the Central Dogma of Molecular Biology.[6] Figure 8 shows a schematic of the translation process.

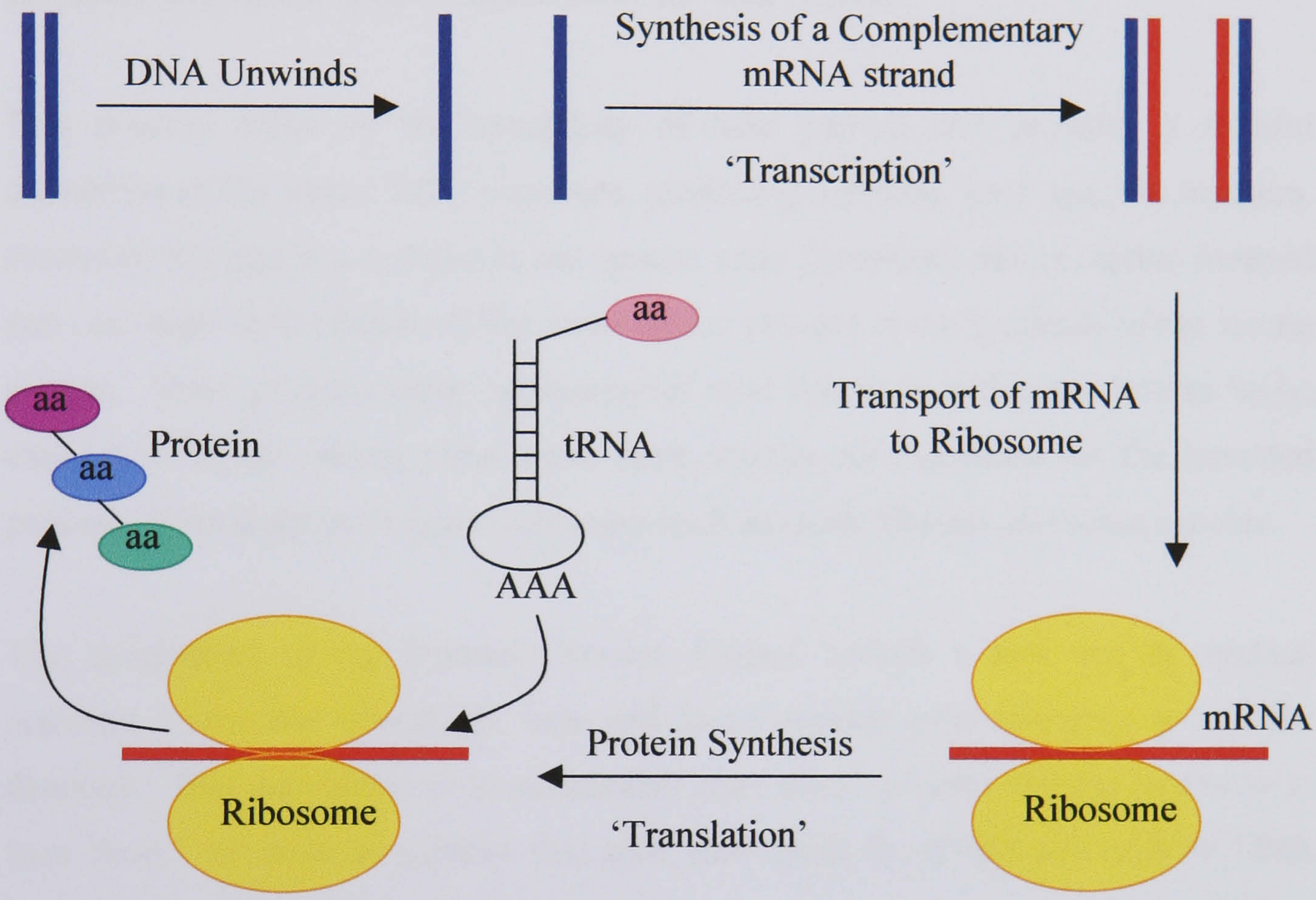


Figure 8. Translation of DNA.

The first step of this process involves the unwinding of the DNA double helix to form two single oligonucleotide strands, which then act as templates. A complementary strand of messenger RNA (mRNA) is then synthesised along the single strands from the 3' end, with a base sequence complementary to that of the DNA template (with the base thymine replaced with uridine in RNA), this process is

called transcription. The mRNA strand is then transported to the ribosome where it hybridises to transfer RNA (tRNA). tRNA consists of a triplet of bases attached covalently to a particular amino acid (aa). The sequence of the three bases, or anti-codon, varies depending on the aa however some amino acids have more than one anti-codon representing them and are therefore degenerate.

In the final process of translation, the mRNA and tRNA's hybridise at the ribosome according to base sequence. The attached amino acids are then bonded together by enzymatic action of the ribosome to form the final protein.

This process relies on the specificity of base pairing and provides a faithful translation of the initial DNA sequence, producing a protein for a specific function. However, if there is a mistake in the genetic code (mutation) this is carried forward and can result in the failure of the transcription process or the synthesis of the wrong protein. Since proteins carry out numerous vital functions within the human body, expression of the wrong protein can have serious consequences for the intended process. This happens in genetic diseases such as cystic fibrosis and some cancers.

The completion of the Human Genome Project heralds a new era in medical research, where the next major task will be to connect gene sequence to specific function. This will allow us to understand more about genetic diseases and how to cure them. In order to achieve this goal, new methods of fast and reliable DNA sequence detection are now necessary.

1.2 Chemical Synthesis of DNA

1.2.1 General

Initially, the drive to produce synthetic DNA was in an effort to relate base-pair sequence to function, since in earlier years the only means of obtaining DNA was from natural biological sources. Obtaining DNA from natural sources has limitations such as the cost of extracting a useful volume of material and also there is no control over the base sequence of natural DNA. Synthetic oligonucleotides provide scientists with complete control over the base sequence, with a higher throughput than from natural sources. The need for more efficient and cost-effective methods for DNA synthesis arose due to a demand for synthetic oligonucleotides as probes and primers for use in molecular biology assays.

1.2.2 Chemical Synthesis of DNA

The synthesis of DNA requires a specific strategy. The individual nucleosides are added sequentially to the growing oligonucleotide in order of the desired DNA sequence. The coupling takes place between the 5' hydroxyl of one nucleoside and the 3' hydroxyl of a second nucleoside to gradually build up the polymer of specific DNA sequence.[7]

The nucleosides can be linked together using various techniques, however the most common and most efficient is the phosphite triester, or phosphoramidite approach. Caruthers developed this highly efficient method in the early 1980's.[8] The cyclic process utilises solid phase synthesis and is carried out on a solid support, usually a glass bead with controlled pore size (CPG). The first nucleoside, with the base and sugar unit fully protected, is attached to the solid support *via* the 3' hydroxyl. This is achieved by reaction of a linker molecule with the 3' hydroxyl of the nucleoside, followed by further reaction with the free amine of the solid support. Figure 9 shows an example of this process.

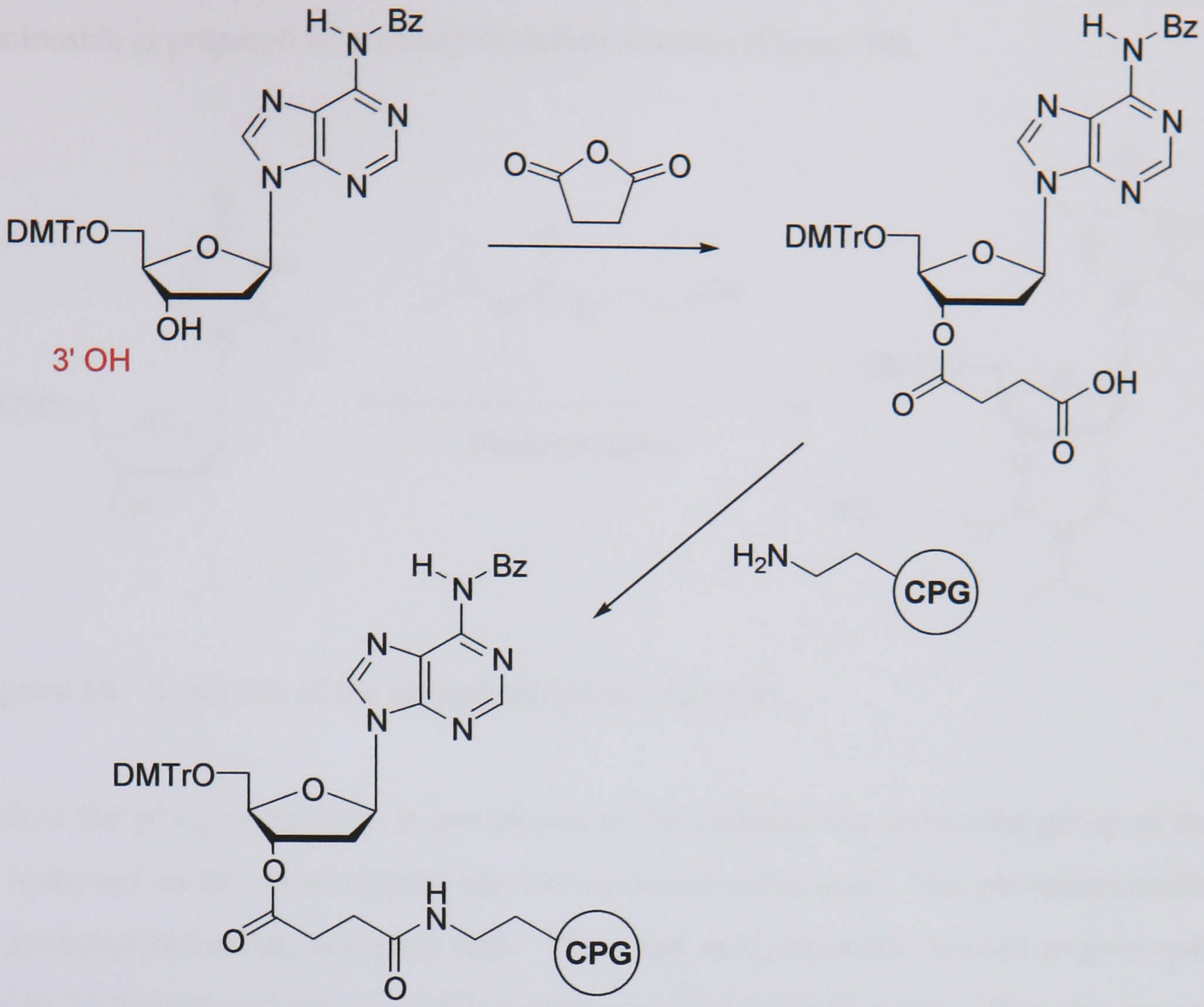


Figure 9. Attachment of the first nucleoside to the solid support

The CPG beads with the first nucleoside attached, are packed into a column, the scale can normally vary from 40nM - 10μM. All the reagents used for the synthesis are flushed through the column in excess as this ensures high yielding reactions. Since the product is bound to the solid support, removal of excess reagents can be done easily by a wash step. All the excess reagents and unreacted monomer are washed through the column, leaving the product and failure sequences on the solid support ready for the next step.

Formation of the phosphite bond between two nucleosides is known as the coupling step. This reaction takes place between the 5' hydroxyl of the nucleoside bound to the solid support, and the 3' phosphoramidite of the incoming monomer. The 2-

cyanoethyl-3'-O-N,N-diisopropylaminophosphite derivative of the corresponding nucleoside is prepared by a phosphitylation reaction (Figure 10).

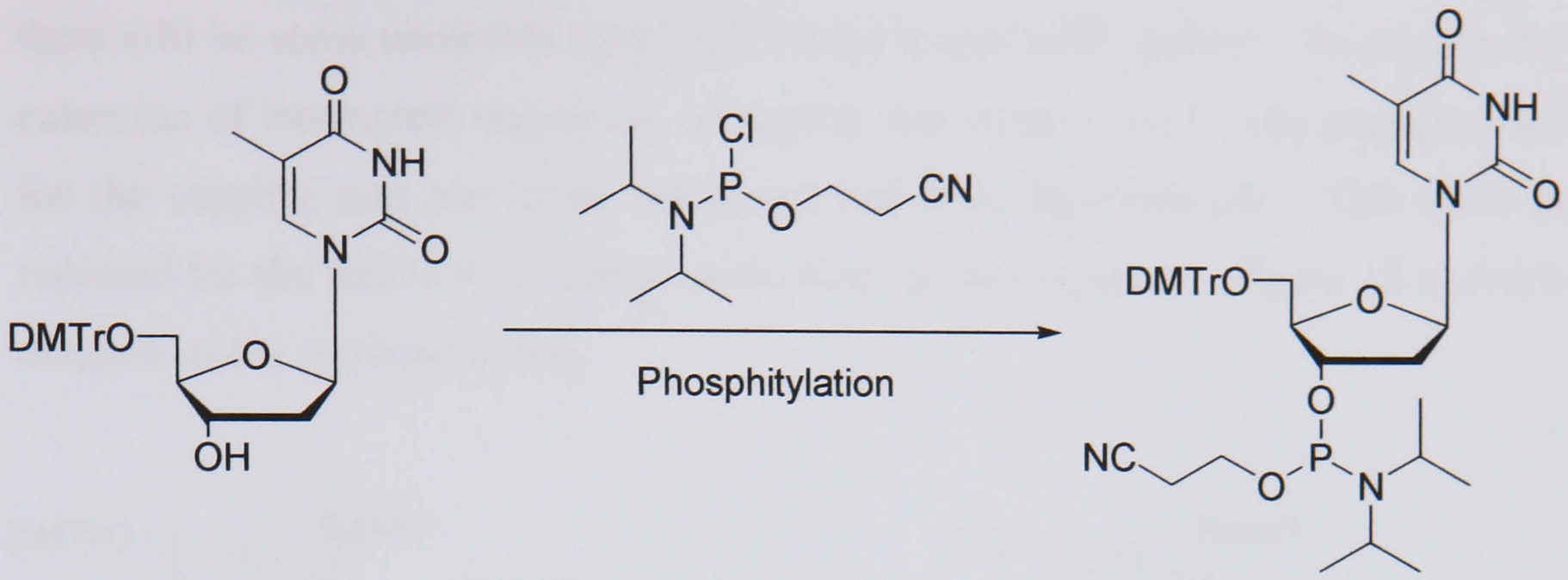


Figure 10. Synthesis of the phosphoramidite monomer.

Before the phosphoramidite is introduced to the column, the protecting group of the 5' hydroxyl on the solid support must be removed using acid. The phosphoramidite is activated before the coupling step. The weak acid, tetrazole, is used to protonate the N, N-diisopropyl group, which becomes a good leaving group. This encourages the nucleophilic attack of the 5' hydroxyl of the nucleoside bound to the solid support, to form the dinucleotide phosphite (Figure 11).

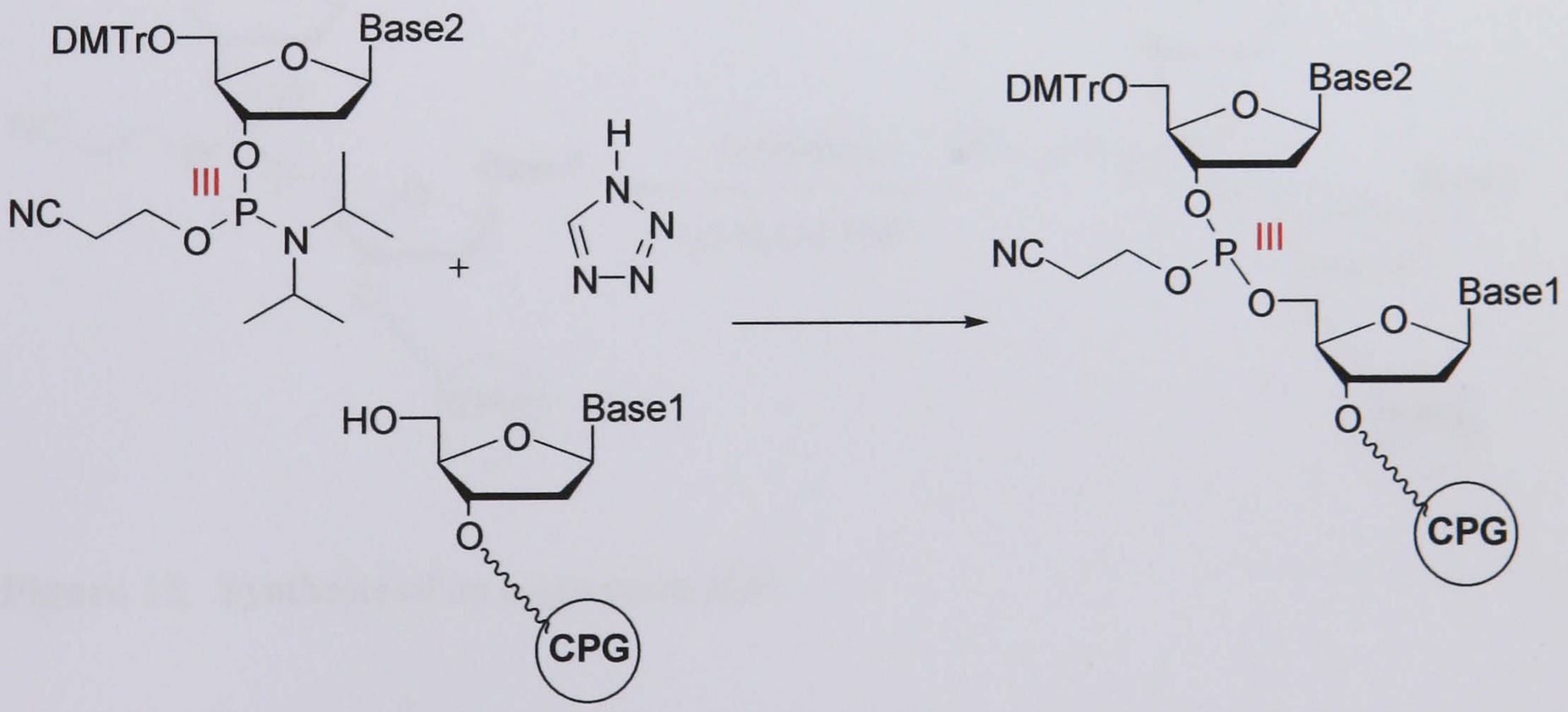


Figure 11. The coupling reaction

The resulting phosphite is then oxidised to the phosphotriester using iodine. The oligonucleotide bound to the solid support is now extended by one nucleoside. Overall the complete addition of one monomer is highly efficient (>98%), however there will be some unreacted hydroxyls bound to the solid support. To prevent the extension of incomplete sequences, a capping step is introduced. The reagents used for the capping step are acetic anhydride and N-methylimidazole. This cycle is repeated for the addition of further nucleotides to the sequence. Figure 12 shows a diagram of the synthetic cycle.

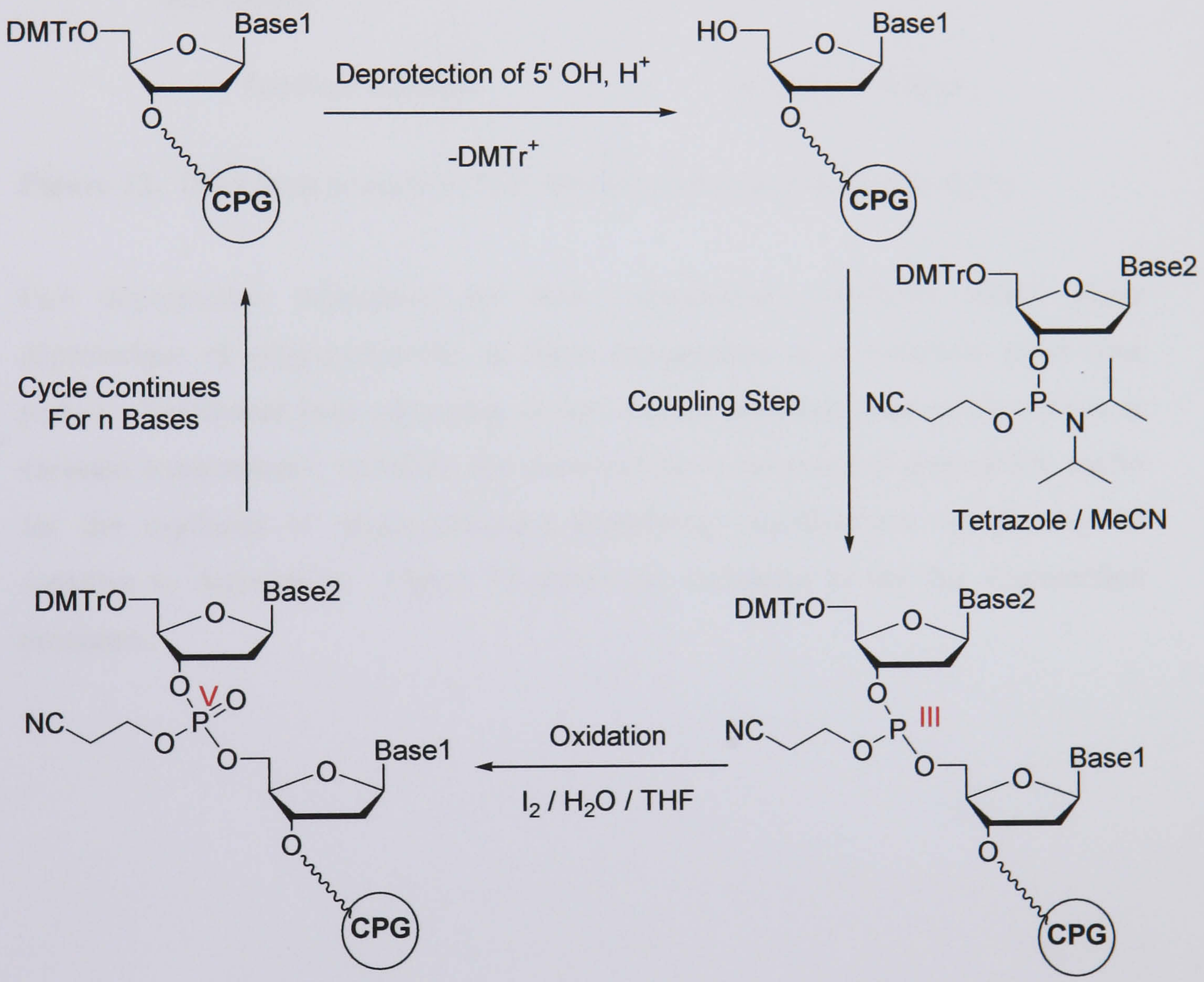


Figure 12. Synthesis of an oligonucleotide.

When the desired sequence has been synthesised the oligonucleotide is removed from the solid support. This is carried out by addition of concentrated ammonia

(NH_4OH) to the column. To carry out this synthetic cycle, an orthogonal protecting group strategy is employed. The reactive moieties of the DNA bases are protected throughout the synthesis using base labile protecting groups. Figure 13 shows the commonly used protecting groups.

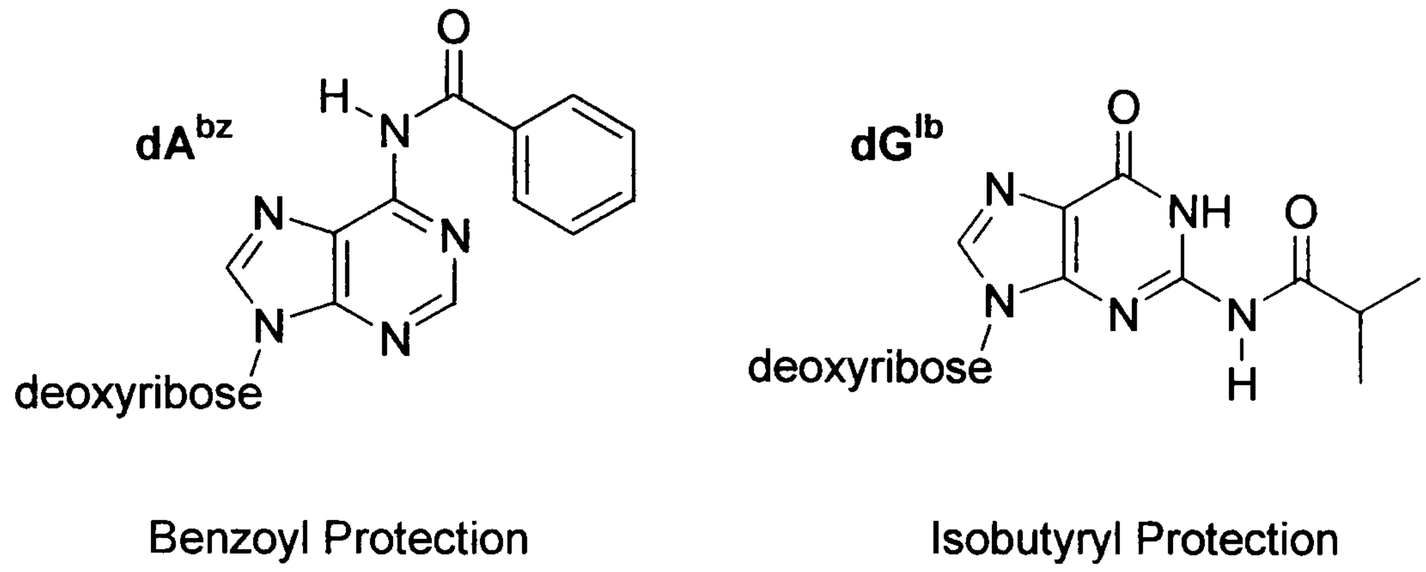
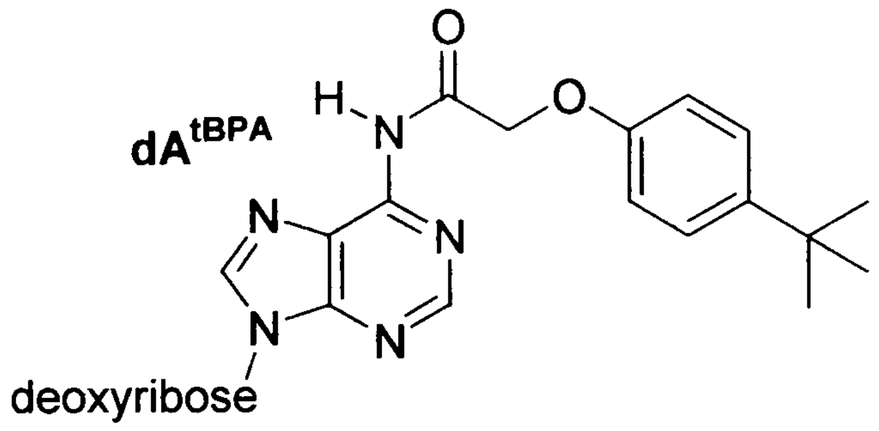


Figure 13. DNA base protection, N.B. thymine does not require protection.

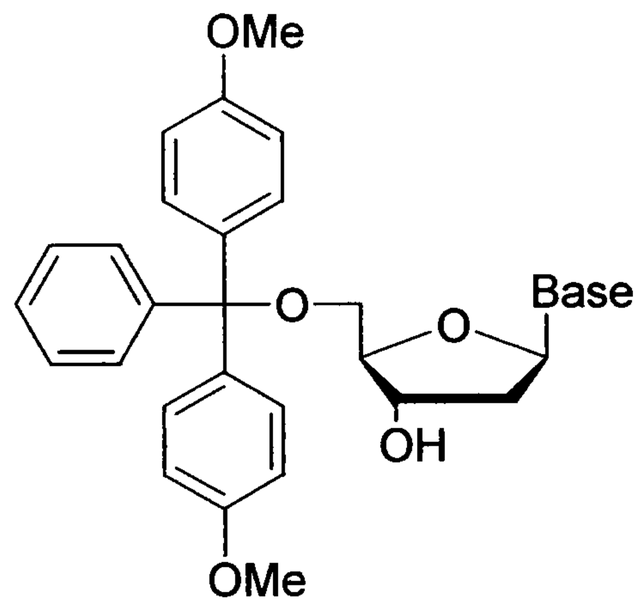
Fast deprotection monomers are now commercially available which allow deprotection of oligonucleotides at room temperature in a relatively short time period. Traditional base protecting groups require extensive deprotection times at elevated temperatures, therefore fast deprotection monomers are particularly useful for the synthesis of oligonucleotides containing modifications which may be sensitive to degradation. Figure 14 shows the structures of the fast deprotection monomer.



Tertiary-butylphenoacetamide Protection

Figure 14. Fast deprotection monomer.

The 5' hydroxyls also require protection during the synthesis but must be deprotected easily to allow further coupling of nucleosides. To do this without removal of the base protecting groups, the acid labile dimethoxy trityl group is used (figure 14).



Dimethoxytrityl (DMTr)Protection

Figure 15. 5' Hydroxyl protection.

Both de-protection and cleavage from the solid support are carried out by the addition of concentrated ammonia (NH_4OH) to the column. Cleavage of esters and the cyanoethyl group occurs quickly, however, removal of the amides requires some time. The products obtained from this step will be the desired oligonucleotide plus all the failure sequences from the synthetic cycle, which must be removed.

1.2.3 Purification of Synthetic DNA

Purification of the oligonucleotide is normally carried out by High Performance Liquid Chromatography (HPLC). There are two main types:

Ion Exchange

This technique utilises the charged nature of DNA to effect separation. Oligonucleotides of different lengths possess differing unit charges due to the negatively charged phosphate backbone, which have varying interactions with a cationic HPLC column. The longer the oligonucleotide, the greater the affinity for the column and therefore a higher salt concentration is required to neutralise the charge and effect elution. The product elution is commonly monitored by UV spectrometry, since the absorbance maxima of the DNA bases are 260 nm and 280 nm. This is an effective method of DNA purification both analytically and preparatively.

Reverse Phase

The separation of products in reverse phase HPLC arises from variations in hydrophobicity. In order to ensure maximum separation between the oligonucleotide product and the shorter failure sequences, the final dimethoxy trityl protecting group of the product may be retained during synthesis. This large hydrophobic group ensures maximum separation and is removed once the pure oligonucleotide is isolated.

These processes provide a single-strand (ss) synthetic oligonucleotide.

1.2.4 Modified DNA

It is often necessary to modify DNA to suit a particular end use. This can be done easily and there are several structural aspects of DNA which lend themselves to modification; the phosphate backbone, sugar units, bases, 3' and 5' hydroxyls. Of these, modification of the 5' hydroxyl is the most readily accessible and is often carried out for the addition of labels.

Modification at the 5' hydroxyl can be carried out in two ways both using the same initial concept. The label is attached to a linker molecule which is in turn attached to the 5' hydroxyl of the DNA sequence (Figure 16). The linker serves two purposes: to attach the label but also to act as a spacer between the DNA sequence and the label in order to maintain the correct function of both and prevent steric interference.

Figure 16. Schematic of modified DNA.

The first method to introduce a label involves the synthesis of an oligonucleotide with the linker at the 5' end. This is done by synthesis of the phosphoramidite of the linker which is added during standard automated DNA synthesis. The label can then be manually coupled to the linker. This method is outlined in more detail in Chapter 2 of this thesis.

The second method involves synthesis of the phosphoramidite of the label. The linker, containing a primary hydroxyl, is incorporated into the label to allow phosphitylation. The phosphitylated label can then be coupled directly to the DNA sequence as the final monomer during standard DNA synthesis and is outlined in more detail in Chapter 3 of this thesis.

1.2.5 Measurement of DNA Duplex Stability of Modified DNA

Modified oligonucleotides are often used as probes in a wide range of experiments for example, in the elucidation of enzymatic / DNA interactions or for the detection of specific DNA sequences.[9,10] The utility of such probes is determined by the stability of the double strand (ds) product subsequently formed. The stability of the DNA duplex is generally determined using UV – Visible spectroscopy, utilising the differing UV absorption between ss and ds DNA. At low temperatures complementary ss oligonucleotides hybridise through hydrogen-bonded base pairs to form predominantly ds DNA, a process called annealing. As the temperature of the sample increases, the increase in energy and therefore molecular vibrations disrupts the hydrogen bonding and dissociates the double helix into ss oligonucleotides, a process known as DNA melting.

The UV absorbance is monitored over the cooling / heating cycle and provides a profile of the annealing and melting processes. The point at which the concentration of ds DNA equals the concentration of ss DNA is called the melting temperature (T_m) and is used as a measure of the stability of the DNA duplex. Figure 17 shows a typical UV melting curve.

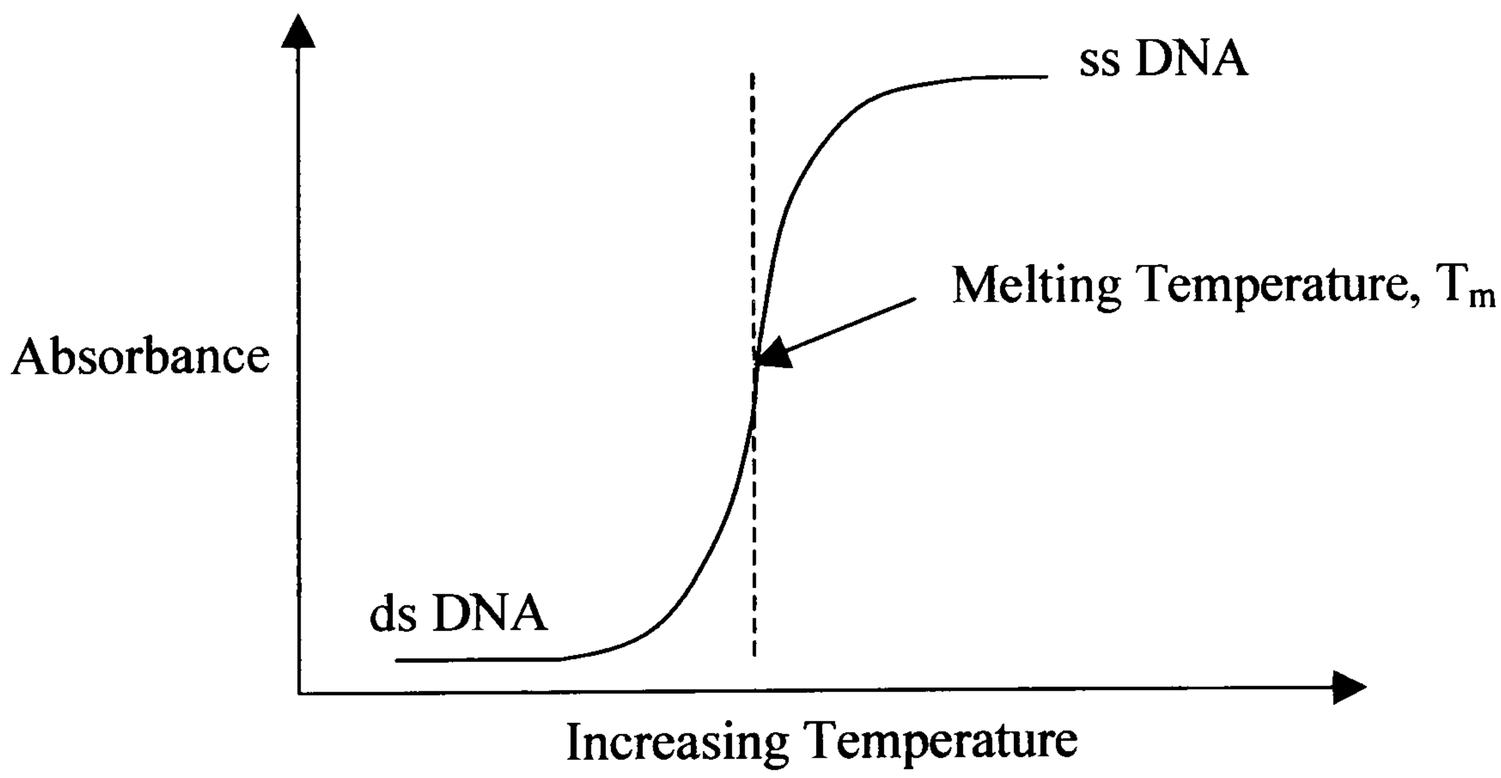


Figure 17. A typical UV melting curve.

Modified oligonucleotides can have three effects in comparison to the unmodified counterpart, an increase in stability or T_m or a decrease or no effect at all. This has implications for the ultimate use of the modified probe, therefore, the T_m must be assessed. For example if the probe is to be used as a primer and the modification introduces a disruptive effect to the duplex, a lowering of the T_m would result. A lowering of the T_m produces a less stable or distorted duplex could result in failure of enzyme recognition processes, hence the primer would not be effective.

1.3 Surface Enhanced Resonance Raman Scattering, SERRS, Spectroscopy

1.3.1 General

Surface Enhanced Resonance Raman Scattering, SERRS, spectroscopy is a highly sensitive and selective analytical technique, which has been employed in the analysis of a wide range of molecules.[11-13] Many factors make this technique ideal for the analysis of biological samples. Water is a poor Raman scatterer, which means that aqueous samples can be studied. This is ideal since nearly all biological processes occur in water. Due to the extreme sensitivity of the technique, analysis of small amounts of material is possible, which is often the case with the analysis of DNA. A further advantage of using SERRS is that mixtures of substrates can be analysed, since enhancement of the analyte alone occurs. Due to these factors there has been a substantial amount of work carried out on the SERRS of biological molecules in the past few years.[14-17]

1.3.2 Raman Scattering

In vibrational spectroscopy, there are various methods to observe transitions between vibrational states in molecules. When monochromatic light interacts with matter, among the processes which can occur are absorption or scattering. Scattering occurs when the incident photon of excitation interacts with the electron cloud of the analyte molecule. In the main these interactions are elastic, when the frequency and wavelength of the scattered photon equals that of the incident photon. This is called Rayleigh scattering.

However, one in every million collisions is inelastic and results in the transfer of energy to or from the sample to the incident photon. This is called the Raman effect.[18] Where there is a transfer of energy from the photon to the sample there is an observed decrease in the frequency of the scattered photon. This is Stokes Raman scattering. On a molecular level this involves an electron in the lowest vibrational

level of the electronic ground state becoming excited to a 'virtual level' equal to that of the incident photon, before relaxing to an excited vibrational level of the electronic ground state. During this process a photon with decreased frequency to the incident photon is emitted.

Similarly, when energy is transferred from the molecule to the photon, anti-Stokes Raman scattering occurs. An electron in a vibrational level of the electronic ground state becomes excited to a 'virtual level' before relaxing to a lower vibrational level. This causes emission of a photon with increased frequency to that of the incident photon. The three scattering processes are outlined in the diagram below (Figure 18).

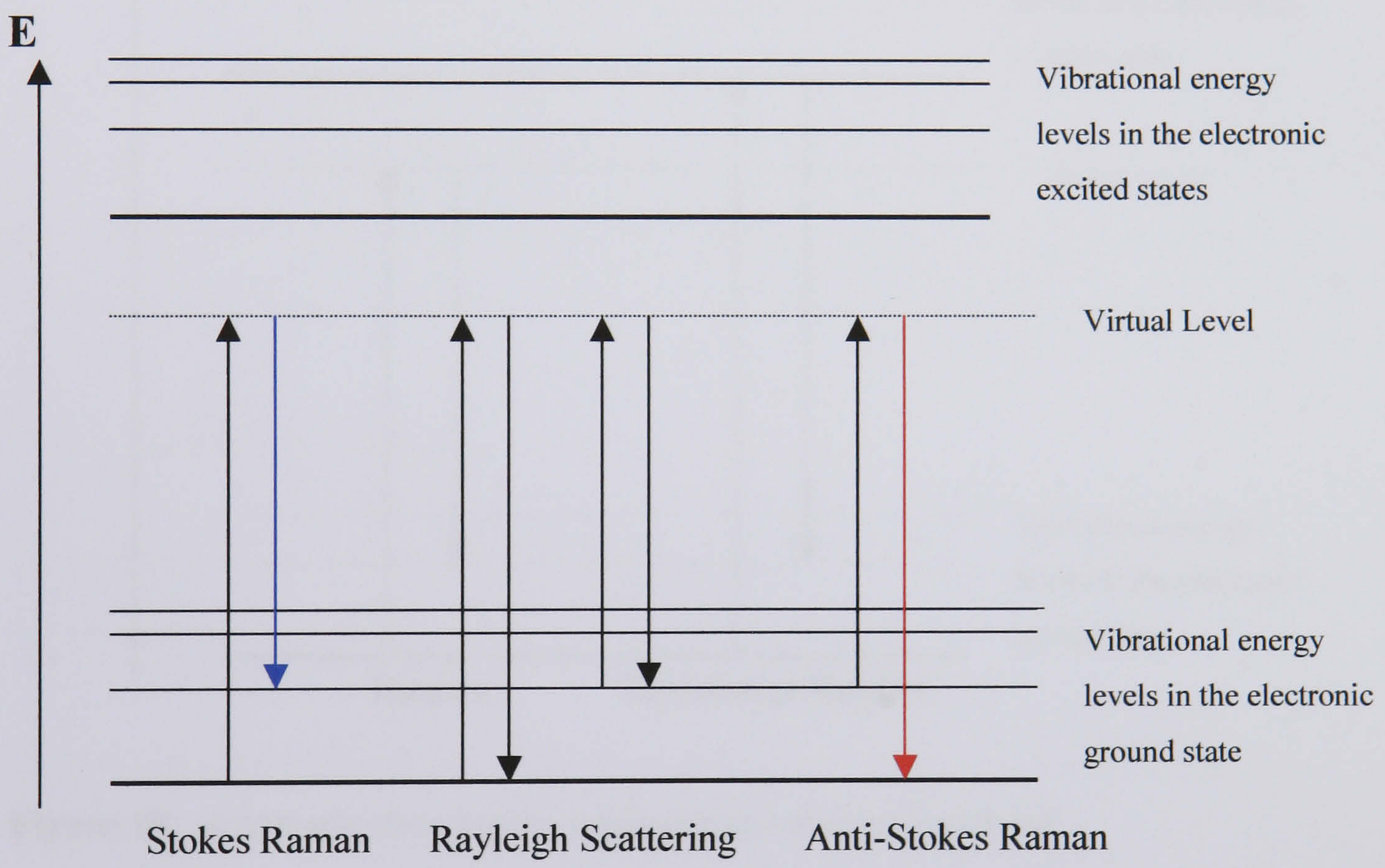


Figure 18. Schematic of energy transitions involved in scattering processes

Since the population of the vibrational ground state is higher (from the Boltzmann distribution: $N_n \propto e^{-En/kT}$), Stokes signals are much stronger than anti-Stokes and therefore are used in Raman spectroscopy.

1.3.3 Resonance Raman

Resonance Raman scattering (RR) can be achieved by the careful tuning of the frequency of the exciting radiation, so that the wavelength coincides with an electronic transition within the analyte molecule. This results in a greatly enhanced signal from the sample, up to five orders of magnitude greater than conventional Raman.[19] The frequency of electronic transitions within the molecule can be identified from UV/visible spectroscopy. Figure 19 illustrates the process of resonance Raman scattering.

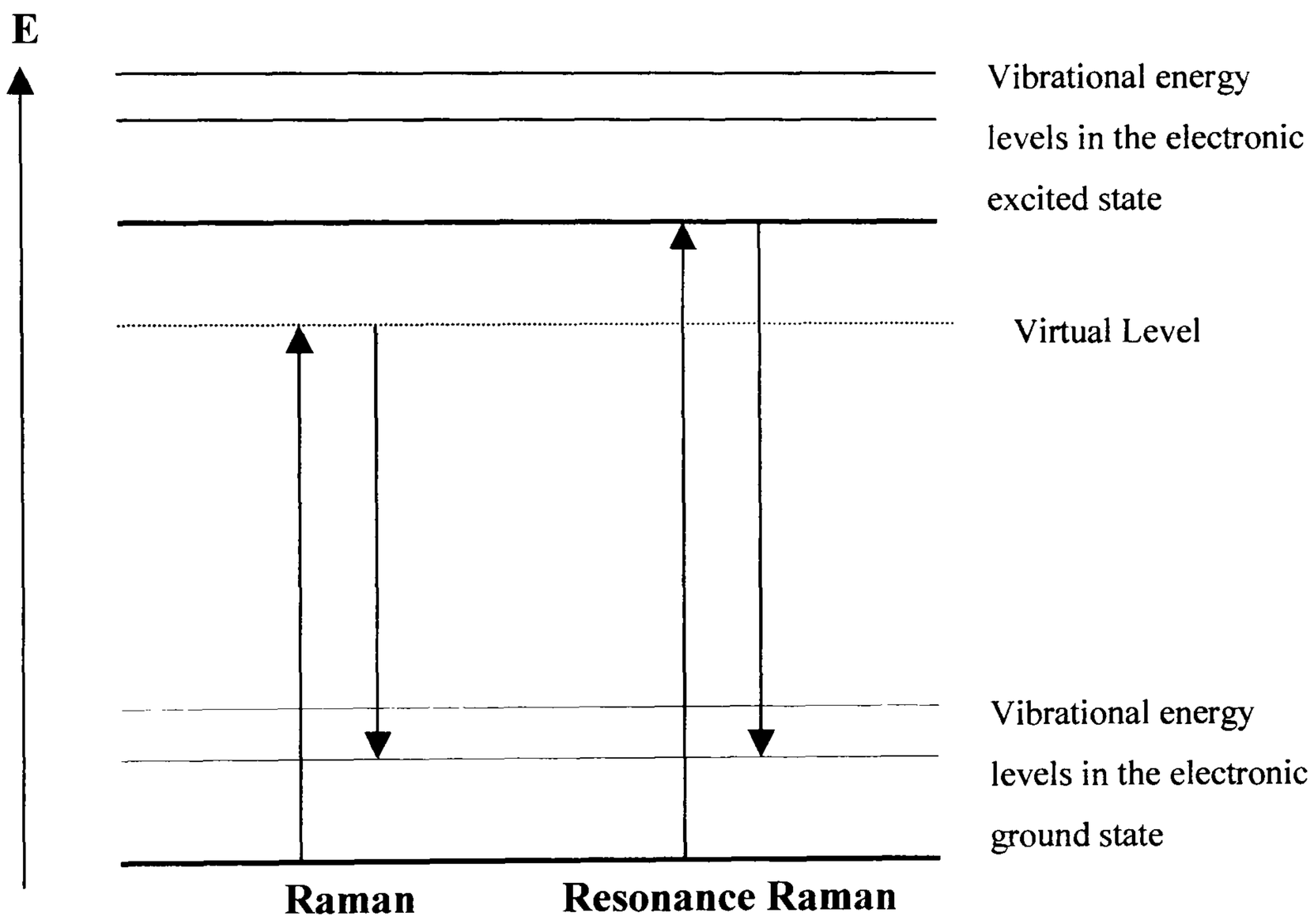


Figure 19. Schematic showing the enhancement observed with RR

1.3.4 Surface Enhanced Raman Scattering

Surface Enhanced Raman Spectroscopy (SERS) was first observed in 1974 by Fleishmann *et al.*, when they measured an enhancement of Raman signals when they adsorbed pyridine onto a roughened silver electrode.[20] It was not until 1977 that Albrecht and Creighton attributed this effect to surface enhancement.[21] A 10^6 enhancement originated from the plasmons on the metal surface. When the surface was roughened, the facets enabled enhanced scattering.

There is no single theory or proof explaining the mechanisms by which surface enhancement occurs. However, there are two main contributing components to the overall effect:

Chemical Enhancement

An interaction between the adsorbed molecule and the metal *via* a chemical bond, allows charge transfer between the energy levels of the adsorbate and the fermi level of the metal upon illumination and accounts for part of the observed enhancement.

Electromagnetic Enhancement

Electromagnetic enhancement arises from the interaction of the scattered light from the adsorbate with the oscillating surface plasmons of the roughened metal surface, which acts as an antenna amplifying the scattered light.

The proposed mechanisms by which the enhancement occurs are complex and still the subject of debate.

A problem with the technique was that the roughened silver surface required for SERS was very difficult to characterise and gave poor reproducibility. This problem was overcome by the use of colloidal suspensions.[22-26] Various metals have been studied, such as silver, gold and aluminium, but it was found that the greatest enhancement is achieved using aggregated silver colloids.[22]

There are two common colloid preparations; these involve the reduction of silver nitrate by either sodium citrate or sodium borohydride. Both have been studied extensively. It was shown that citrate reduced colloids have a more uniform particle size distribution and are more stable.[25, 26]

There are currently two schools of thought on the best conditions for obtaining SERS signals from colloids. Nie and co-workers have observed intense signals from single colloidal particles.[27] Others have found that intense signals can be obtained when the particles are aggregated into small clusters to provide the necessary enhancement sites.[28] Aggregation can be achieved by the use of substances that reduce surface charge such as inorganic salts, acids, spermine or poly-ionic polymers such as poly(-L-lysine). However, it has been shown that use of poly-ionic polymers provides a more controlled aggregation and therefore more reproducible results.

SERS is a very useful spectroscopic tool as it provides structural and orientational information about the sample. Fluorescence is also quenched by surface adsorption, which allows a wider range of samples to be analysed.

1.3.5 Surface Enhanced Resonance Raman Scattering

Surface Enhanced Resonance Raman Scattering was first observed in 1983 by Stacy and Van Dyne, when they noticed a 10^{10} increase in signals.[29] The technique utilises both SERS and resonance Raman to give a highly sensitive and selective technique.

The requirements for SERRS activity in a molecule are a visible chromophore and a surface complexing group, for adsorption to the metal surface. A chromophore is required, since the most common sources of excitation are lasers in the visible region, therefore the electronic transition being enhanced within the analyte must be in the visible region. These requirements limit the range of molecules that can be analysed. SERRS analysis of suitable molecules provides many advantages over conventional Raman:

- Structural and electronic information is obtained
- Sensitivity is greatly increased
- Lower powered excitation radiation can be used, which reduces the possibility for sample heating and decomposition
- Fluorescence is quenched in SERRS therefore allowing the analysis of both fluorophores and non-fluorophores

When SERRS is observed, excellent sensitivity is obtained. The technique has been employed qualitatively and semi-quantitatively in several areas such as in the study of dyes and biological samples.[12, 30-34]

1.3.6 SERS and SERRS Analysis of DNA

In the early 1980s, when the strengths of the SERS and SERRS techniques began to be realised, research became focused on the area of biological analysis. To date there have been extensive studies of substances such as nucleic acids, amino acids, proteins and membranes. [30-34] Initial Raman investigations of DNA utilised the SERS effect. Individual DNA bases and nucleic acids were adsorbed onto silver surfaces and the spectra studied.[35-37] Mixtures of DNA related compounds have also been analysed.[38] Kneipp *et al* obtained SERS spectra of DNA but found that samples had to be left for long periods of time before the nucleic acid equilibrated and reached the silver surface to give a signal.[17] Other applications of SERS include the measurement of DNA adducts formed by reaction of DNA with cytotoxic compounds.[39] Vo-Dinh *et al* have developed a gene probe for the detection of specific DNA sequences and have reported sensitivities in the micromolar range. However, sample and SERRS substrate preparations are complex and time consuming.[40-42]

The use of SERRS for the analysis of DNA is an attractive proposition, since the sensitivity and stability of signals obtained using this technique provides the means of reliable analysis. SERRS has been utilised for the analysis of antitumor drugs and dyes to probe their interactions with the DNA double helix by observing spectral changes from the free intercalator species and the DNA-intercalator complex. [43-46]

The direct analysis of DNA by SERRS is not possible due to two factors:

1. DNA does not possess a chromophore in the visible region; therefore a label must be incorporated into the sequence to allow detection.
2. DNA is poly-anionic due to the phosphate backbone, and does not readily adsorb to the silver surfaces commonly used to provide SERRS enhancement.

Recent work within the group outlined the SERRS detection of modified DNA [47-49] using two fluorescent labels, HEX and rhodamine 6G.

Rhodamine 6G has amino groups in the structure of the dye, which attract to the silver surface to allow detection. For the negatively charged HEX label, the problem of surface repulsion was alleviated by the use of modified DNA bases. These contained propargyl amino groups at the end of the sequence that became positively charged in solution and attracted to the colloidal surface. This allowed detection of the modified oligonucleotide down to concentrations of 8×10^{-13} M.

From this work, it followed that greater reliability and sensitivity could be obtained if DNA could be modified to produce a more substantial interaction with the colloidal surface. It has been known for some time that benzotriazole complexes covalently with metal surfaces and indeed it is used as a corrosion inhibitor for copper.[50] Previous work has shown how benzotriazole azo dyes are excellent compounds for SERRS detection.[30] The triazole ring of benzotriazole is thought to form an irreversible bond with the colloidal surface providing surface enhancement and the azo chromophore provides the resonance component. With this in mind, this thesis outlines the modification of DNA to contain specifically designed SERRS active labels and their subsequent SERRS analysis.

1.4 Aims of Research

The primary aim of the research is to attach a specifically designed SERRS label to the 5' end of a synthetic sequence of DNA. The oligonucleotide must be modified to include a surface complexing group and a chromophore, to provide the resonant enhancement. Two methods of attachment are proposed.

1. Attachment of the SERRS label *via* an amino-linker.
2. Incorporation of the SERRS label *via* phosphoramidite chemistry.

Finally, on successful completion of the above aims, the labelled oligonucleotides will be evaluated using SERRS spectroscopy and a possible application of the probes investigated.

Chapter 2

Attachment of a SERRS Label to DNA *via* an Amino Linker

2.0 Attachment of a SERRS Label to DNA *via* an Amino Linker

2.1 Introduction

The first approach attempted for the introduction of a SERRS label to the 5' hydroxyl of DNA was *via* an amino linker. The bifunctional linker contained a primary hydroxyl to allow phosphitylation and subsequent coupling to DNA during standard automated synthesis. Secondly the amino functionality provided a reactive group at the end of the modified oligonucleotide to which the SERRS label could be covalently attached using a standard peptide coupling agent. The scheme outlined in Figure 20 shows this methodology.

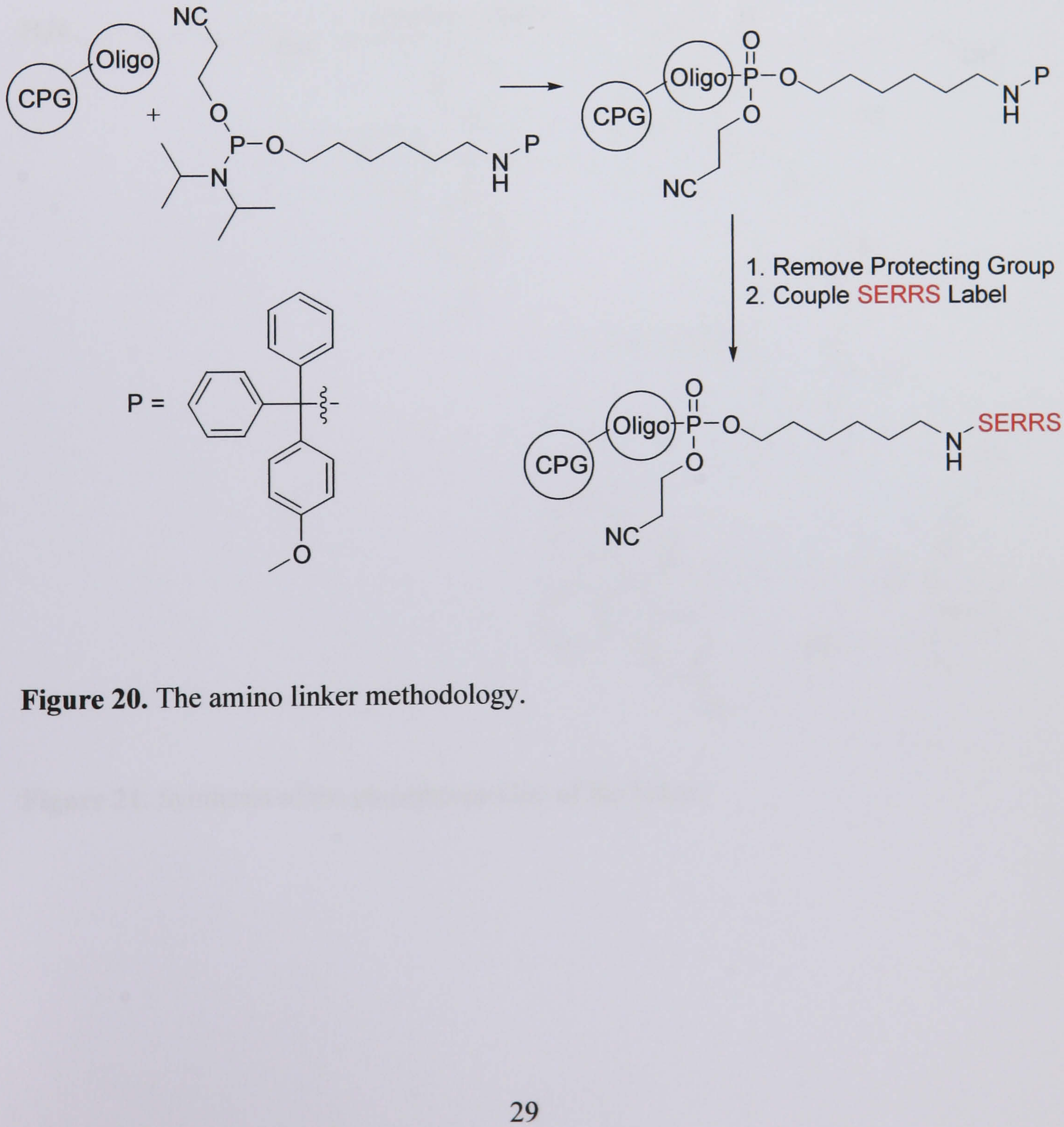


Figure 20. The amino linker methodology.

2.2 Synthesis of DNA Containing an Amino Linker

The linker used for this purpose was 6-aminohexan-ol for the properties outlined in section 2.1 of this chapter. The phosphoramidite of the linker was synthesised to allow coupling to the 5' hydroxyl as the final step during automated DNA synthesis. In order to achieve this the reactive primary amine required to be protected for the phosphitylation step and indeed throughout the synthetic cycle for the synthesis of DNA. This was carried out using a monomethoxy trityl protecting group strategy devised by Uhlmann *et al.* to give compound **[1]** in 57 % yield.[51] Phosphitylation of the protected linker gave phosphoramidite **[2]** in good yield (99 %).[52] This strategy is outlined in Figure 21.

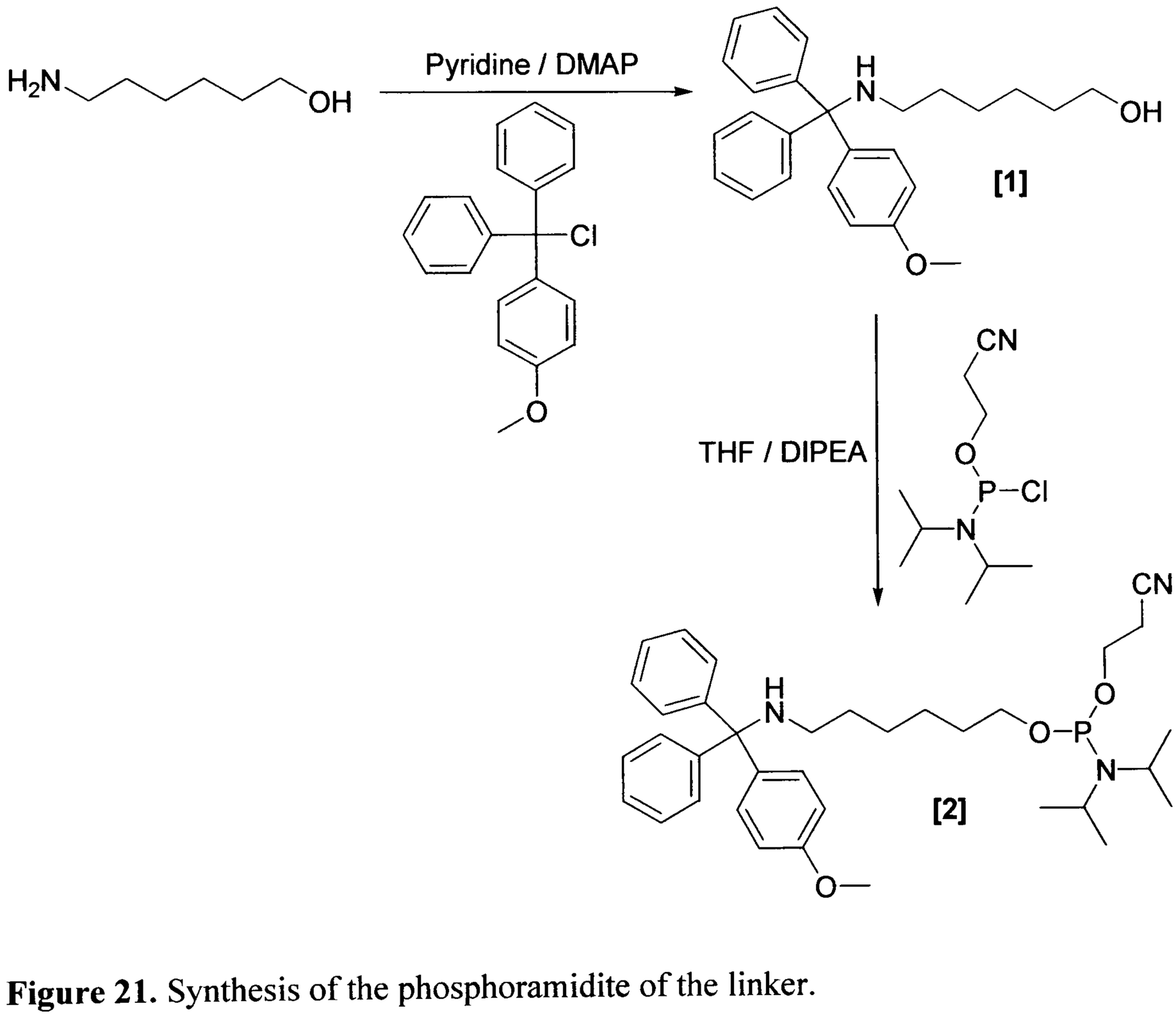


Figure 21. Synthesis of the phosphoramidite of the linker.

Phosphoramidite **[2]** was incorporated into various sequences of synthetic DNA as the final monomer during standard DNA synthesis, with an extended coupling time and double delivery of the modified monomer. The coupling efficiency of the linker was comparable to that of commercial monomers. The final monomethoxy trityl protecting group was removed using trichloroacetic acid as the final step in the automated synthesis resulting in a primary amine at the 5' end of the oligo. The primary amine was then free to couple to the carboxylic acid derivative of the SERRS active label to form a covalent amide linkage between the oligo and the label.

2.3 Synthesis of SERRS Active Labels

The aim of this piece of work was to synthesise a series of SERRS labels that were suitable for further derivatisation in order to couple to DNA. Previous work by Graham *et al.* on the SERRS analysis of DNA labelled with commercially available fluorescent dyes, indicated that stronger interactions between the labelled DNA and the silver colloidal surface provided improved enhancement of the SERRS spectrum. This work utilised DNA probes modified with propargyl amino groups at the 5' terminus, which in solution can form electrostatic interactions with the negatively charged colloidal particles.[47-49]

From this work it followed that greater reliability and sensitivity could be obtained if the DNA probe could be modified to produce a more substantial interaction with the colloidal surface. It has been known for some time that benzotriazole complexes strongly with metal surfaces and indeed the benzotriazole group is thought to displace the citrate layer on the surface of the colloid to form an irreversible bond between the triazole ring and the silver surface.[50] Unlike the attachment of propargyl amino groups through electrostatic attraction, a chemical bond is formed which is an irreversible process and is therefore more robust.

A starting point for the synthesis of the SERRS labels was the benzotriazole group, since one of the most important requirements of the label is excellent surface complexing properties. Secondly, the label must possess a chromophore in the visible region to provide the resonance component of the SERRS spectrum. For this, two well known classes of dye were investigated, the azo and the fluorescein. Incorporation of the benzotriazole group into both azo and fluorescein dyes was attempted.

2.3.1 Synthesis of Azobenzotriazole Dyes

The azo chromophore was chosen for several reasons. The dyes are relatively simple to prepare and the nature of the diazonium coupling reaction (figure 22) provides great flexibility for the synthesis of many different dyes with varied functional groups.

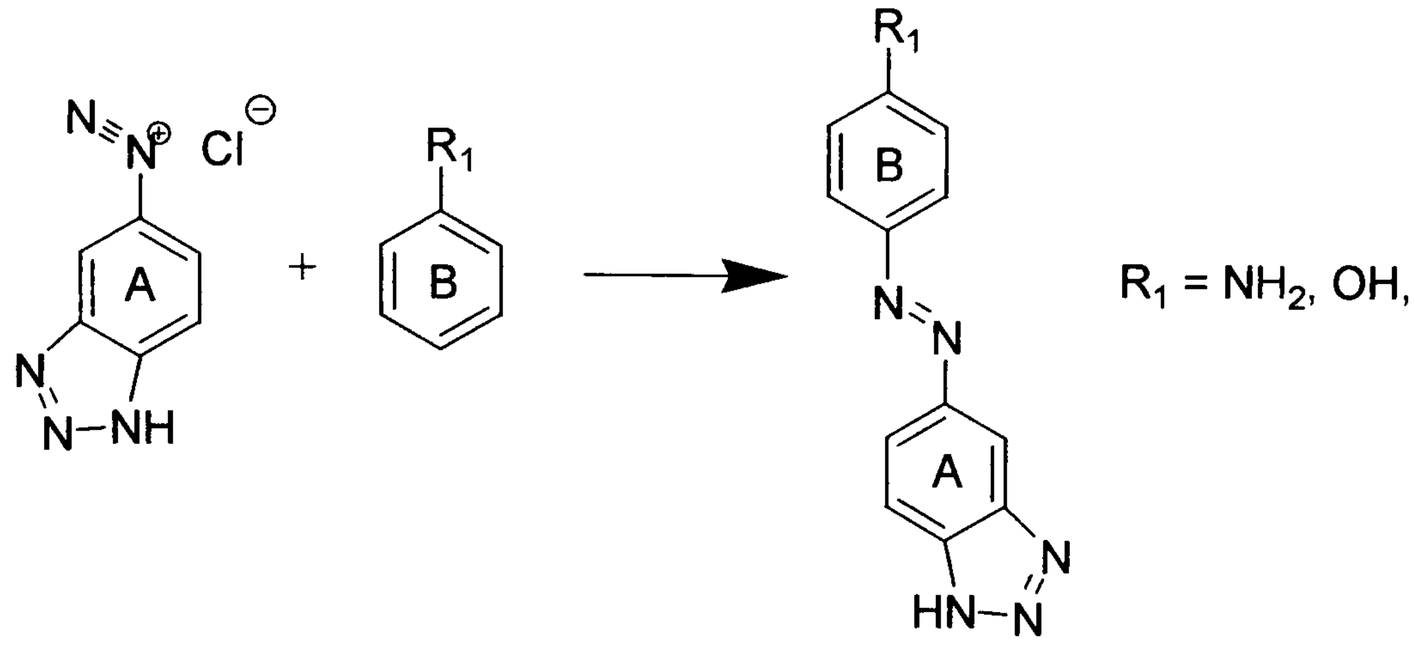


Figure 22. Schematic of the diazonium coupling reaction.

This flexibility is essential for the work detailed in this thesis, since two important factors must be accommodated, i.e. introduction of the benzotriazole group and a means of attachment of the dye to DNA. Introduction of the benzotriazole group is possible by two methods. By forming the diazonium salt (compound A in figure 22) of the benzotriazole group then coupling to a suitable coupling agent (compound B in figure 22). Alternatively a diazonium salt could be coupled to a benzotriazole compound. Greater flexibility and ease of synthesis can be achieved by formation of the benzotriazole diazonium salt then coupling to a wide range of different coupling components.

The benzotriazole diazonium salt was synthesised by a standard diazotisation reaction. In each case, 5-aminobenzotriazole was dissolved in 50% aqueous hydrochloric acid and diazotised by the addition of sodium nitrite solution at 0°C (Figure 23).

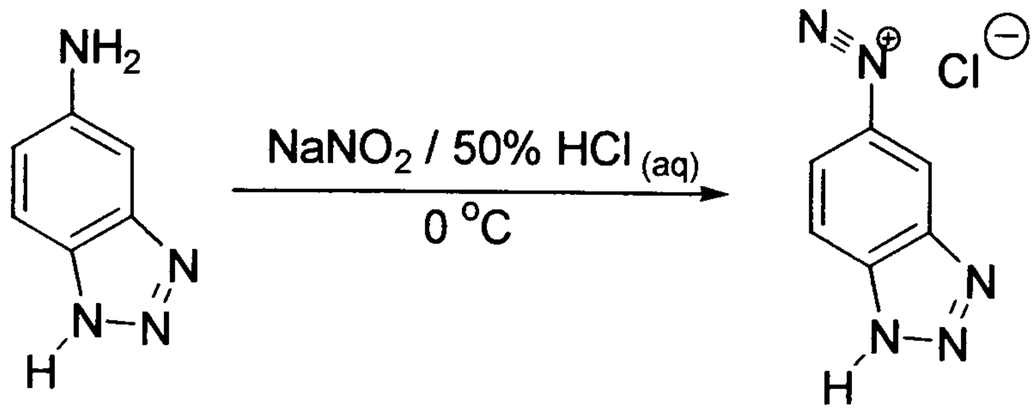


Figure 23. Standard diazotisation of 5-aminobenzotriazole.

The azobenzotriazole dyes were then produced by electrophillic attack of the diazonium ion on the chosen coupling components, which were dissolved in buffer solutions. The general coupling reaction is shown in figure 24.

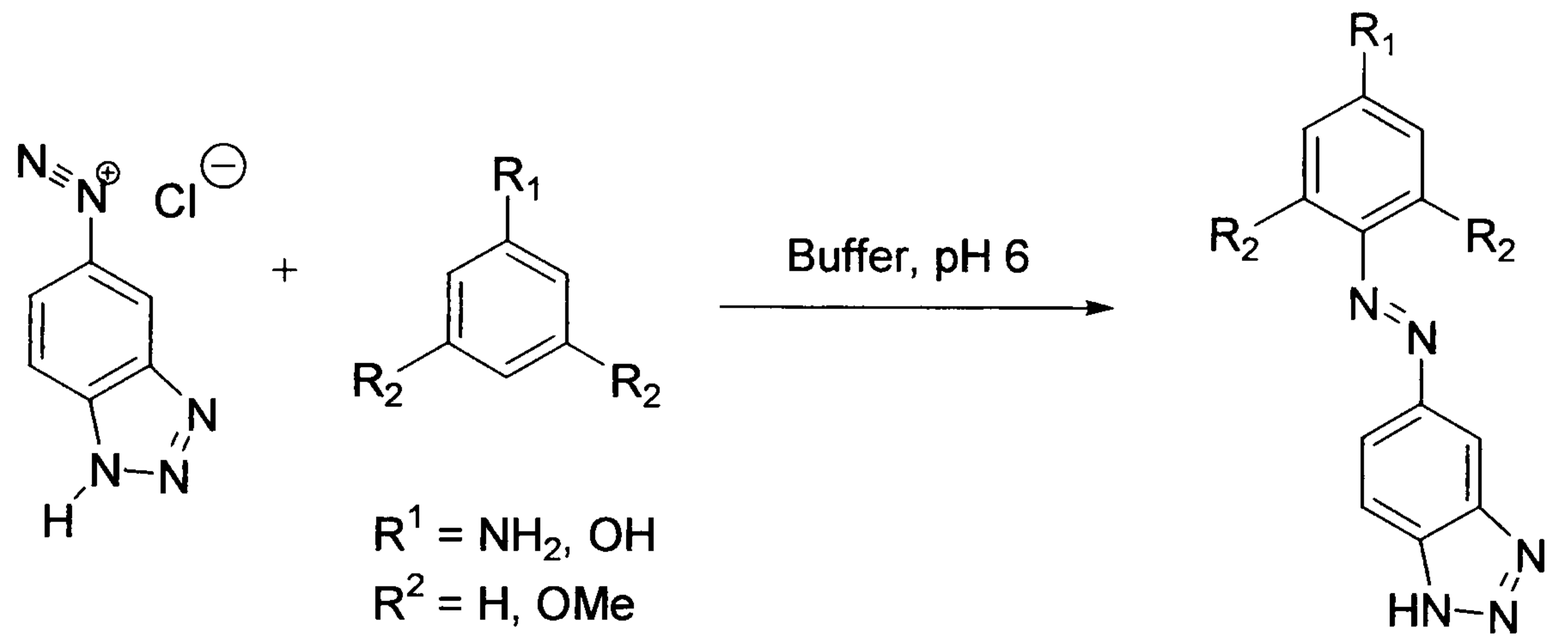


Figure 24. Coupling reaction to form the azobenzotriazole dye.

There are two main requirements of the coupling component. The compound must contain a suitable functional group to allow derivatisation to the carboxylic acid and subsequent coupling to the amino modified oligonucleotide. Secondly the compound must be aromatic and electron rich or contain electron–donating substituents (R_1 in the above diagram) para to a site suitable for derivatisation, since the benzotriazole diazonium is a weak electrophile.

The first dye synthesised was N-[2-(4'-5''-azobenzotriazolyl)naphthalen-1-yl)-amino-ethyl]succinamic acid **[4]**, shown in Figure 25. The coupling component, N-(1-naphthyl)-ethylenediamine, was chosen since it has an electron rich naphthyl ring in addition to a secondary amine that donates electron density into the ring *via* the lone pair of electrons. Due to this activation, electrophillic attack of the benzotriazole diazonium in sodium acetate buffer produced dye **[3]** in 73% yield.

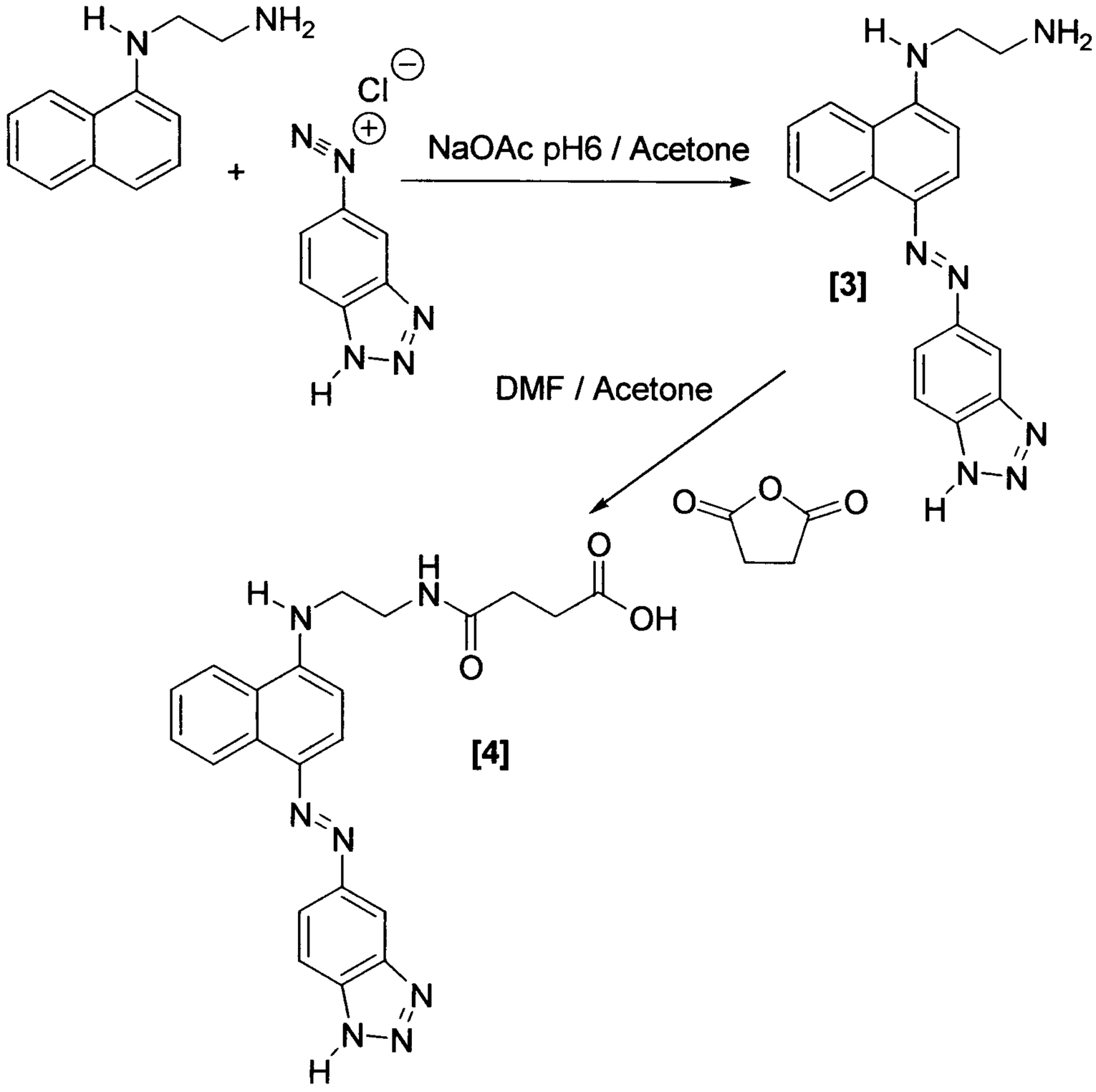


Figure 25. Synthesis of N-[2-(4'-5''-azobenzotriazolyl)naphthalen-1-yl)-amino ethyl] succinamic acid.

The primary aliphatic amine of the substrate provides the means of further derivatisation. This aliphatic side chain containing a primary amine was required since previous unpublished work by Graham *et al*[53] on the attempted derivatisation of N-[4-(5'-azobenzotriazoyl)naphthalen-1-yl]amine proved unsuccessful. This was

due to the reduced nucleophilicity of the primary amine due to the electron-withdrawing nature of the benzotriazole group. Introduction of the primary aliphatic amine provided the means for facile further derivatisation of the dye for addition to the amino oligonucleotide. In this case derivatisation was carried out by reaction with succinic anhydride to produce the carboxylic acid derivative **[4]** in 66% yield.

The second coupling agent used was 2-amino-4,6-dimethoxypyrimidine. This compound was chosen since the amine and methoxy groups add electron density. The primary amine can also be derivatised for addition to DNA, again using succinic anhydride to form the carboxylic acid. In this case the primary aromatic amine was expected to be sufficiently nucleophilic to derivatise due to the increased electron density provided by the pyrimidine ring. Figure 26 shows the synthesis of N-[5-(5`-azobenzotriazoyl)-4,6-dimethoxy-pyrimidin-2-yl]-succinamic acid **[6]**.

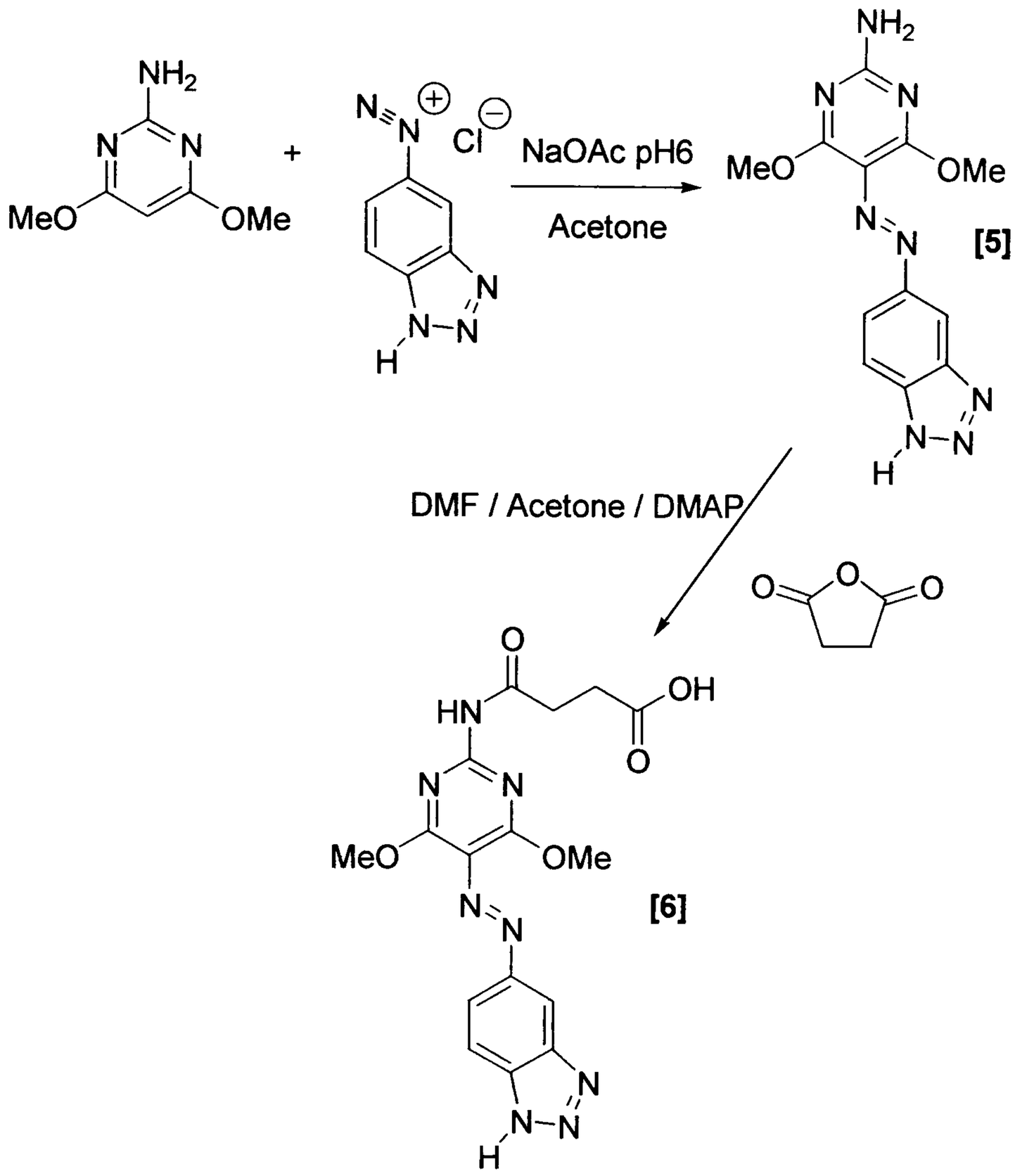


Figure 26. N-[5-(5'-Azobenzotriazoyl)-4,6-dimethoxy-pyrimidin-2-yl]-succinamic acid.

The diazotisation of 5-aminobenzotriazole and coupling to 2-amino-4,6-dimethoxypyrimidine was carried out on a small scale initially (50 mg) as a test. The expected azo dye **[5]** was obtained but also an unknown red compound was isolated. The reaction was repeated on a large scale in order to characterise both products, however, the reaction predominantly yielded the desired product **[5]** (64%). It was thought that the formation of side product could be linked to pH, since the concentrated hydrochloric acid used in the diazotisation was in higher ratio to coupling buffer in the small-scale reaction.

To test this a series of test coupling reactions were carried out under carefully controlled pH conditions, at pH 1 and pH 6. As expected the reaction carried out at pH 1 produced more of the side product, whereas the reaction at pH 6 produced predominantly the desired azo dye.

^{1}H NMR analysis of the side product showed the presence of 5 aromatic protons: a singlet at δ_H 9.06 (H1) and four doublets at 8.46 (H2), 8.07 (H3), 8.01 (H4) and 7.18 (H5). The coupling constants of the doublets were 8.95 Hz for H2 and H4 and 9.02 Hz for H3 and H5. This suggests that H2 is positioned ortho to H4 on the aromatic ring and similarly H3 is ortho to H5. Initially, it was thought that an internal rearrangement reaction was occurring to produce the following compound (figure 27).

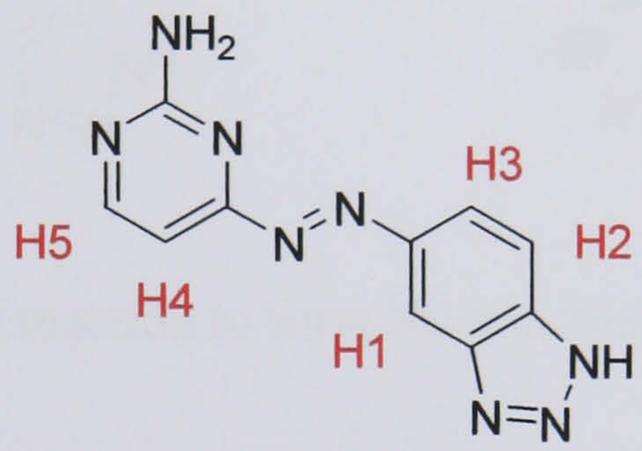


Figure 27. Proposed structure of side product.

This structure agrees with the observed ^{1}H NMR data. However a proposed mechanism was unknown. During the synthesis of subsequent azo dyes, the same side product was isolated, irrespective of coupling agent. This suggests that formation of the side product is independent of coupling agent and dependent on the benzotriazole diazonium. It is possible that the benzotriazole diazonium coupled to unreacted 5-aminobenzotriazole starting material through the primary amine to form a triazene compound (figure 28).

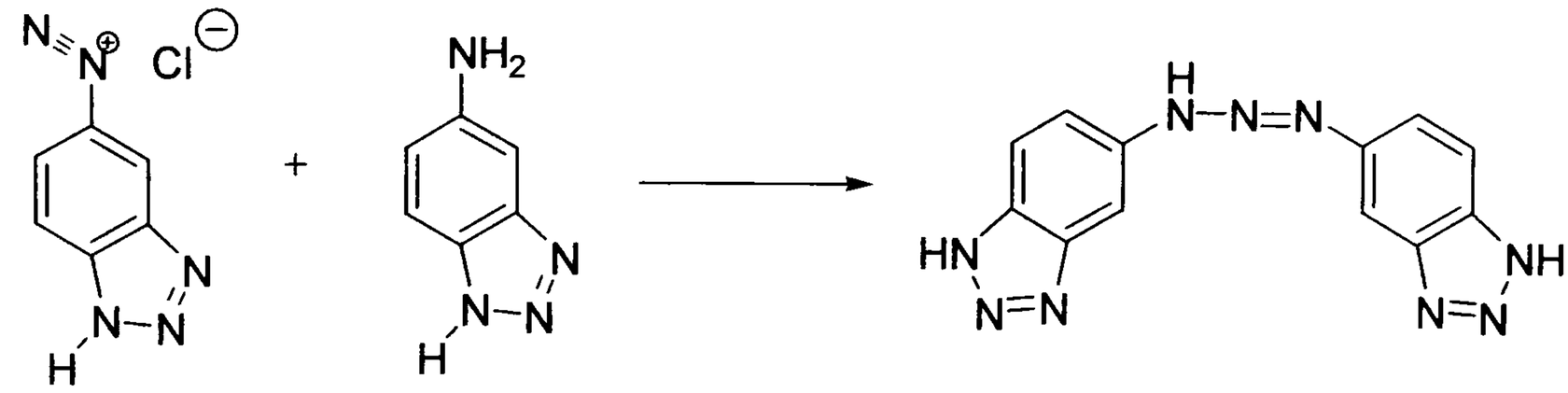


Figure 28. Formation of a triazene.

Since the reaction is carried out in conc. hydrochloric acid, this allows rearrangement of the triazene by a 1/C-hydro-3/N-arylazo-interchange to form the following azo dye (figure 29).[54]

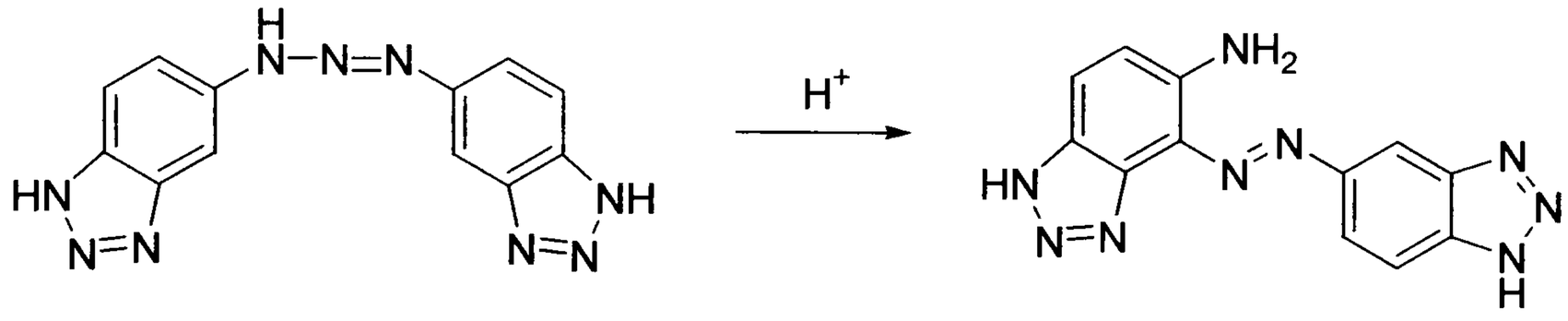


Figure 29. Rearrangement reaction to form the azo dye.

This rearranged structure agreed with the ^{1}H NMR spectra obtained. In addition mass spectral analysis confirmed the presence of the molecular ion. The formation of this side product could be due to incomplete diazotisation of the starting material which led to steps being taken to improve this process for future work.

The final step in the synthesis was functionalisation of the amine to a carboxylic acid and was carried out by reaction of compound **[5]** with succinic anhydride.

Previous analogous azobenzotriazole dyes proved unreactive, due to the electron-withdrawing nature of the azobenzotriazole reducing the nucleophilicity of the reacting amine. The reaction of compound **[5]** with succinic anhydride was carried out in DMF with catalytic amounts of DMAP to improve reaction yields. The desired product **[6]** was obtained in 82 % yield.

The final dye synthesised used 3,5-dimethoxyphenol as the coupling agent. In this case the methoxy groups activate the ring towards electrophilic attack and the phenol provides the means of derivatisation *via* ether synthesis. The following reaction route (Figure 30) outlines the synthesis of [3,5-dimethoxy-4-(5'-azobenzotriazoyl) phenoxy]-pentanoic acid **[9]**.

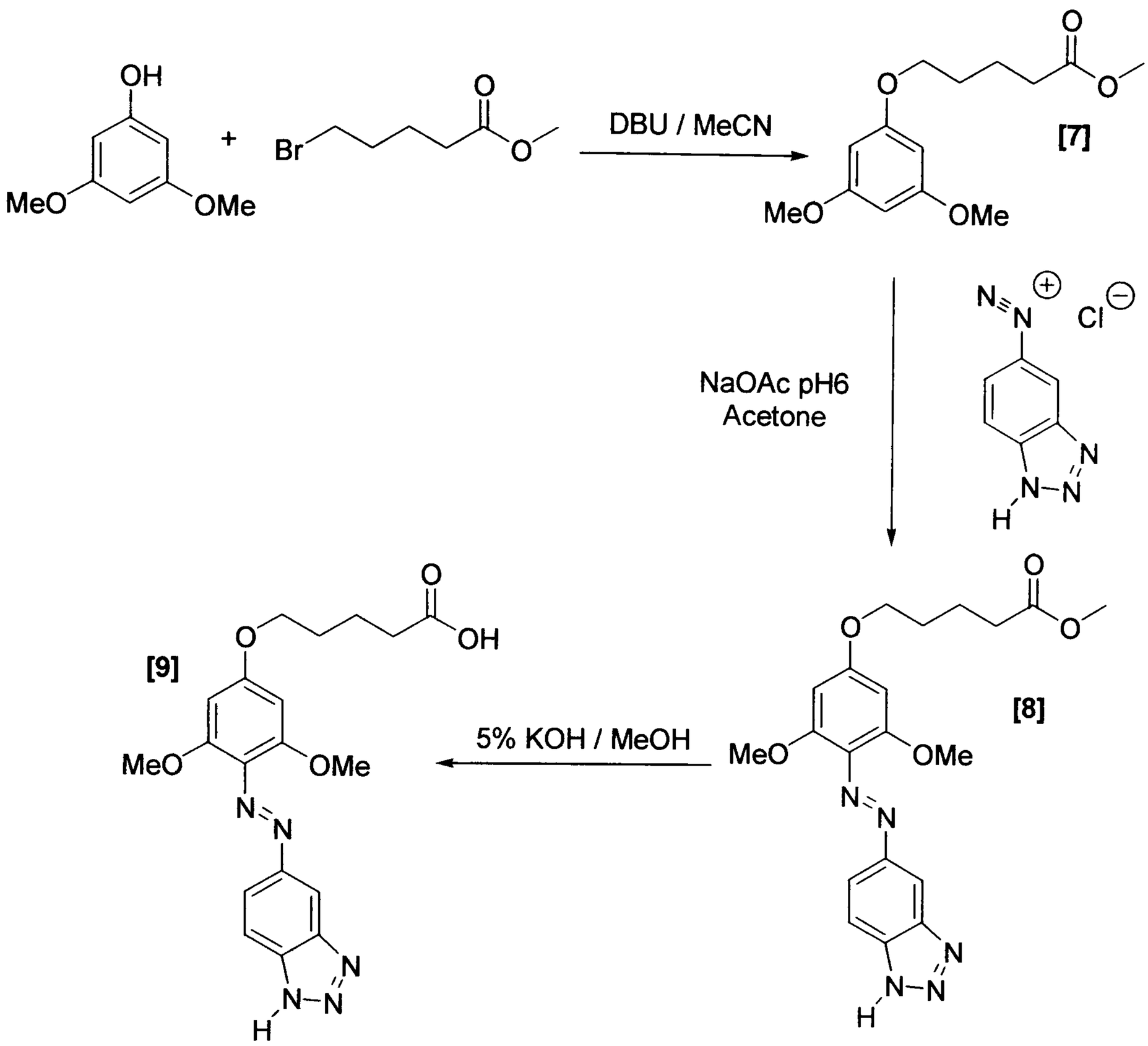


Figure 30. Synthetic route to [3,5-dimethoxy-4-(5'-azobenzotriazoyl) phenoxy]-pentanoic acid.

3,5-Dimethoxyphenol was derivatised by a standard Williamson ether synthesis to introduce the carboxylic acid group, protected as the methyl ester. The reaction was carried out in acetonitrile with DBU, which deprotonated the phenol to allow nucleophilic displacement of bromide from bromovalerate to form compound **[7]** in 82% yield. The diazonium salt of 5-aminobenzotriazole was synthesised as described previously, then coupled to compound [7] in a mixture of sodium acetate buffer (pH 6) and acetone, to give the azo dye **[8]** in 65% yield. However, repeating the synthesis of the azo dye proved problematic, this was attributed to the reduced

activity of the aromatic ring towards attack by an electrophile, on formation of the ether.

To counter this problem, a second route was attempted to obtain a dye with the same chromophore. The strategy was altered from derivatisation of the coupling agent to formation of the azobenzotriazole dye followed by derivatisation (Figure 31).

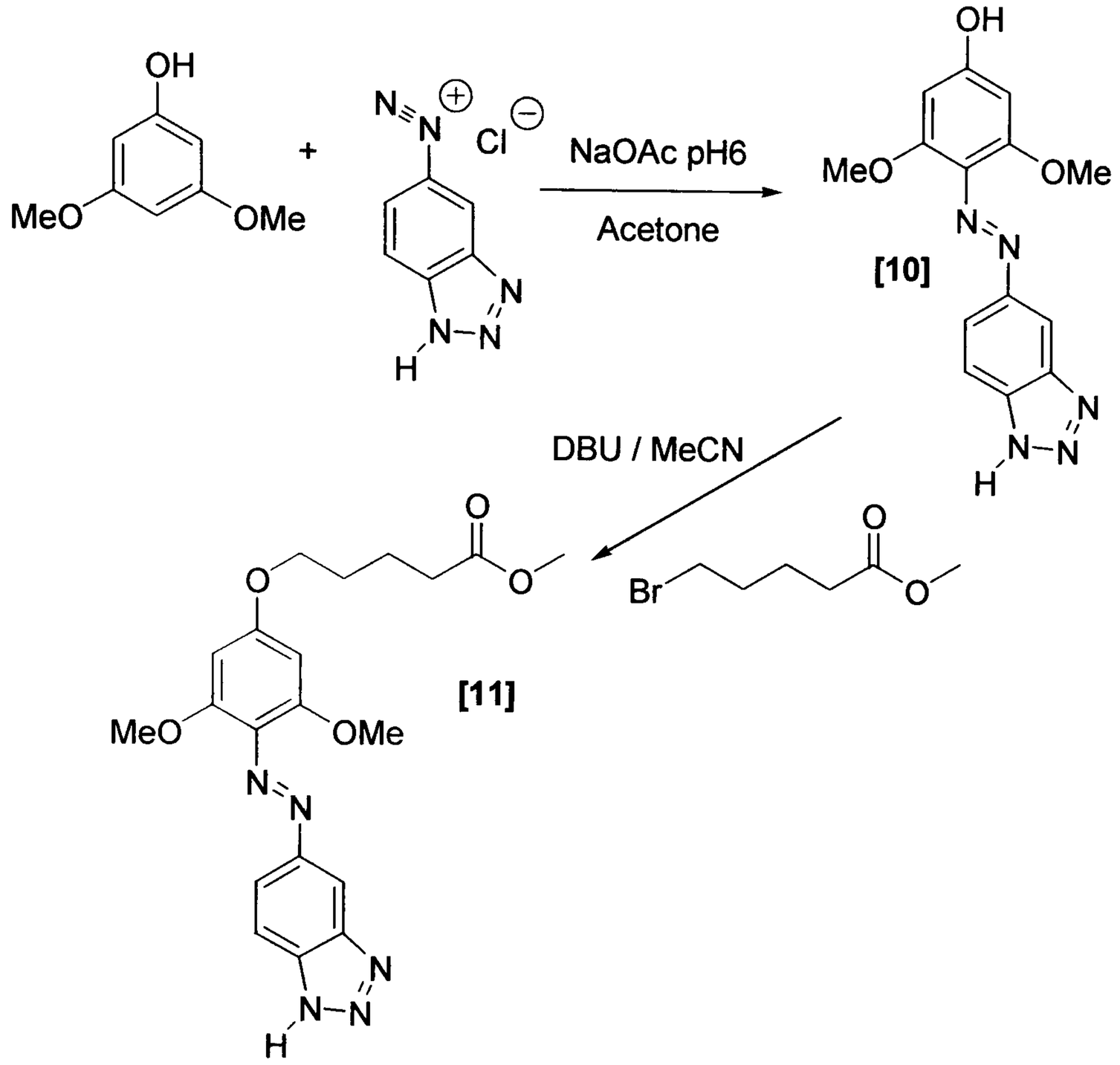


Figure 31. Revised reaction route.

The first step of synthetic pathway was formation of the azobenzotriazole dye using 3,5-dimethoxyphenol as the coupling agent. This reaction was carried out successfully to give the orange solid 4-(5'-azobenzotriazoyl)-3,5-dimethoxy-phenol, **[10]** in 64 % yield.

The final step in the synthesis involved introduction of the carboxylic acid functionality which was carried out by reaction of the phenol with 5-bromovalerate as before. Several products resulted from this reaction due to the base DBU deprotonating both the phenol and the triazole ring resulting in reaction at both sites. Therefore a suitable protecting group was required to protect the triazole ring during the synthesis. Figure 32 shows two protecting groups that were assessed.

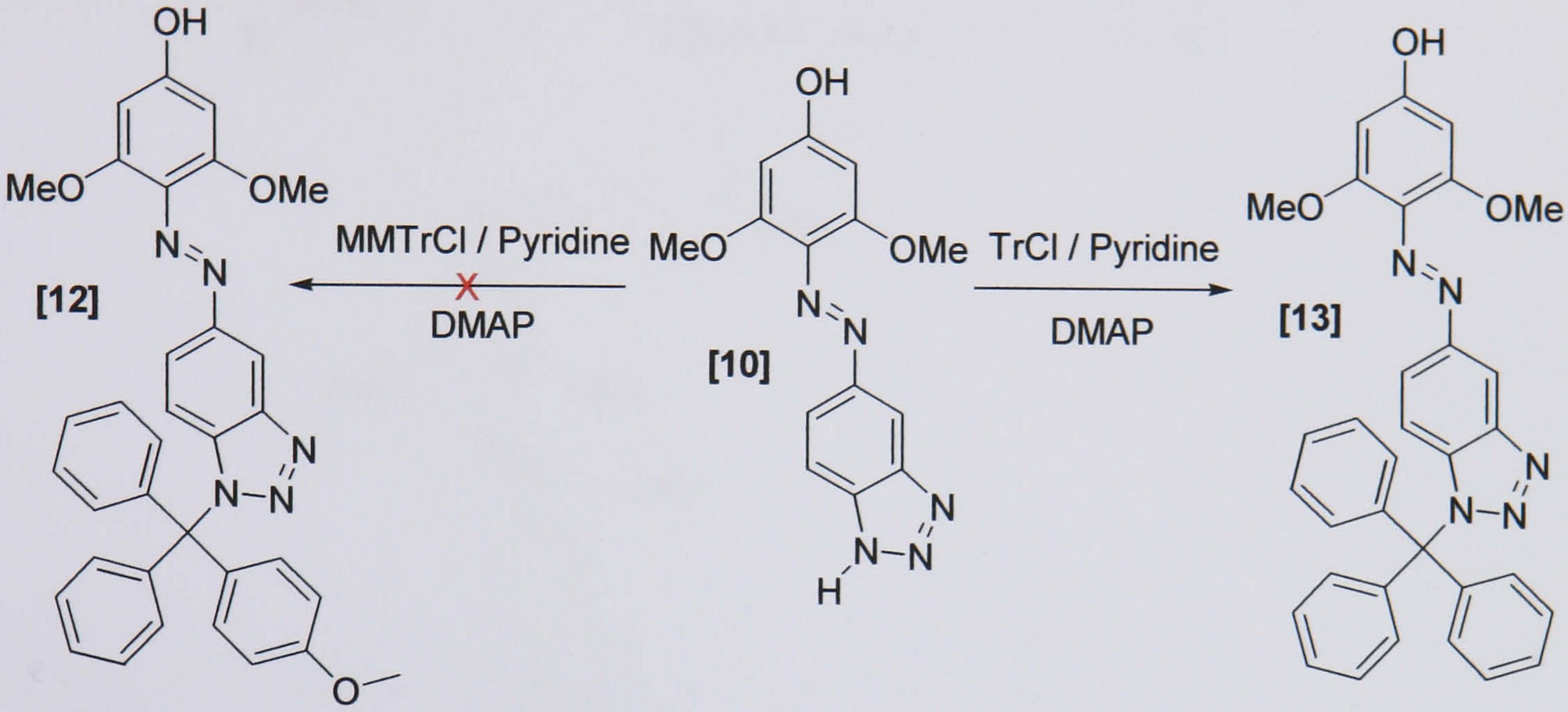


Figure 32. Protection of the triazole ring of 4-(5'-Azobenzotriazoyl)-3,5-dimethoxy-phenol.

The protecting group was chosen to be easily removed after coupling to the DNA sequence. The acid labile monomethoxy trityl group, MMTr, (analogous to the dimethoxy trityl group used as a standard DNA protecting group) was used initially, however, the product readily decomposed on purification. Secondly the less labile trityl protecting group was employed successfully to selectively protect the triazole ring in the presence of the free phenol, forming product **[13]** in 69 % yield. The ether synthesis was repeated using the protected azobenzotriazole dye under the same reaction conditions (Figure 33).

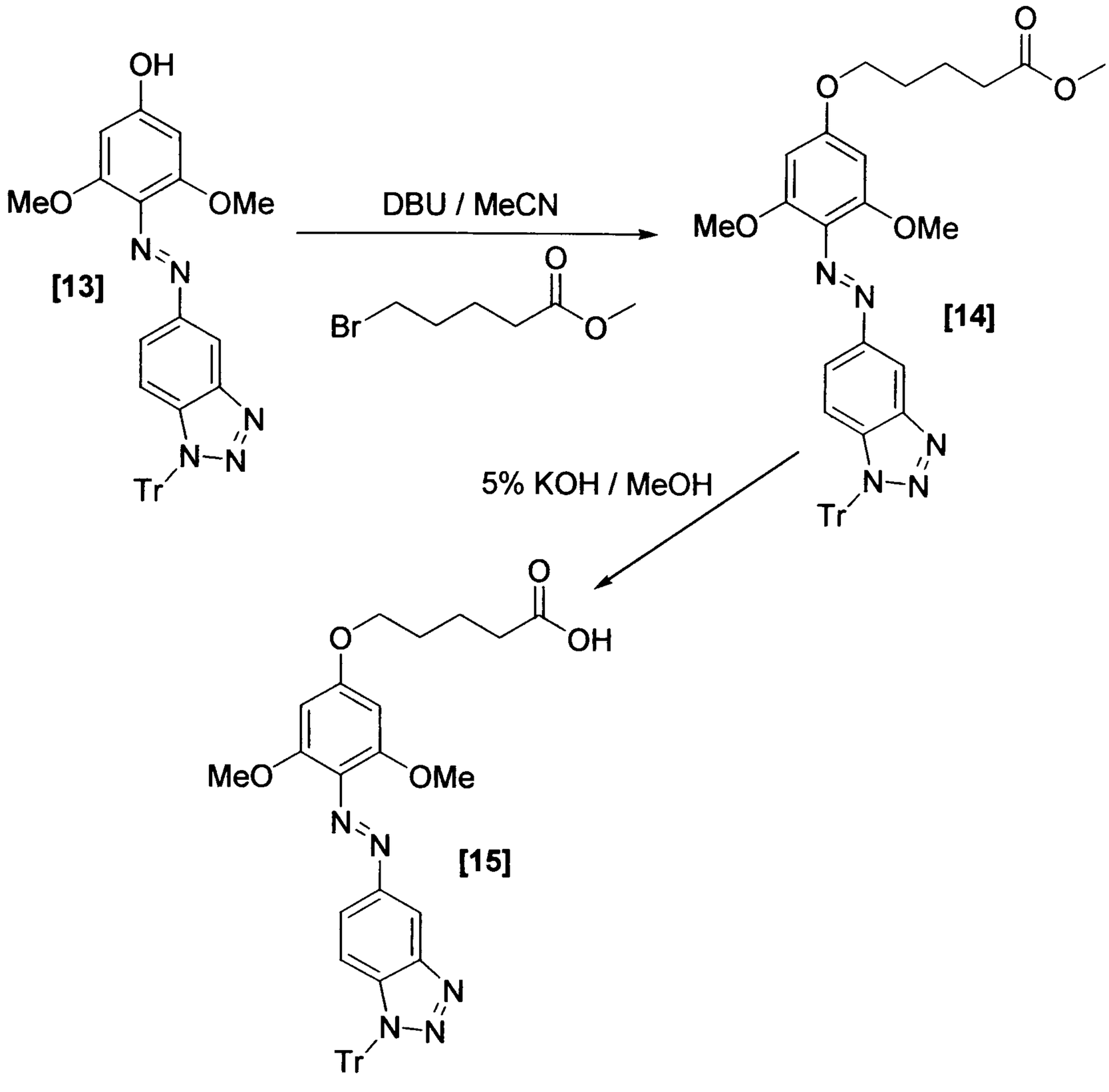


Figure 33. Derivatisation of the trityl protected azobenzotriazole dye.

The ether synthesis was successful, providing compound **[14]** in 96 % yield. The carboxylic acid was obtained by cleavage of the methyl ester by treatment with 5 % methanolic potassium hydroxide under reflux conditions. The desired dye 5-[3,5-dimethoxy-4-(1-trityl-5'-azobenzotriazoyl)-phenoxy]-pentanoic acid, was obtained in 88 % yield.

This gave three dyes capable of addition to an amino-linked oligonucleotide.

2.3.2 Synthesis of a Benzotriazole Rhodamine Dye

To further increase the range of SERRS labels, attempts were made to synthesise a benzotriazole rhodamine dye. This dye should combine the excellent SERRS properties already observed for rhodamine[1], with the surface complexing ability of benzotriazole. Modification of the dye itself (for example the carboxylic acid) was not practical since further functionality was required for addition of the dye to DNA. Figure 34 shows the structure of rhodamine 6G.

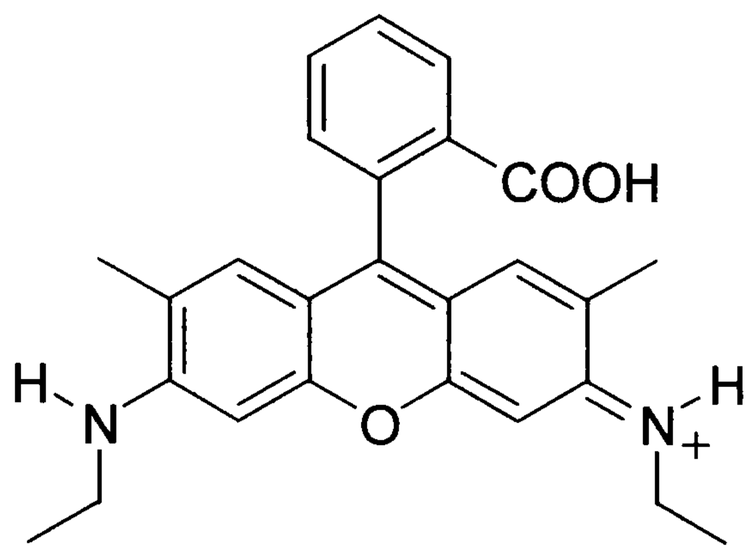


Figure 34. Structure of rhodamine 6G.

It was proposed to incorporate benzotriazole directly into the xanthene ring during synthesis of the dye, however very little literature is available on the synthesis of rhodamine compounds. A method used by Scala-Valéro *et al* [55] was attempted (figure 35).

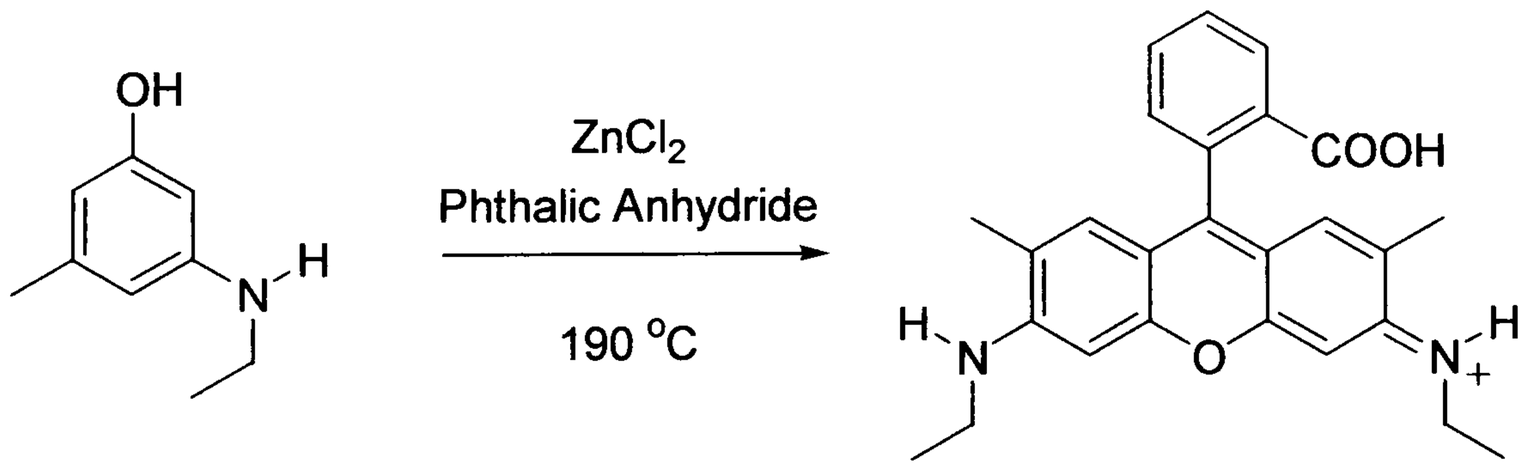


Figure 35. The Scala-Valéro method of rhodamine synthesis.

In order to incorporate benzotriazole into the xanthene ring the following scheme was proposed (figure 36):

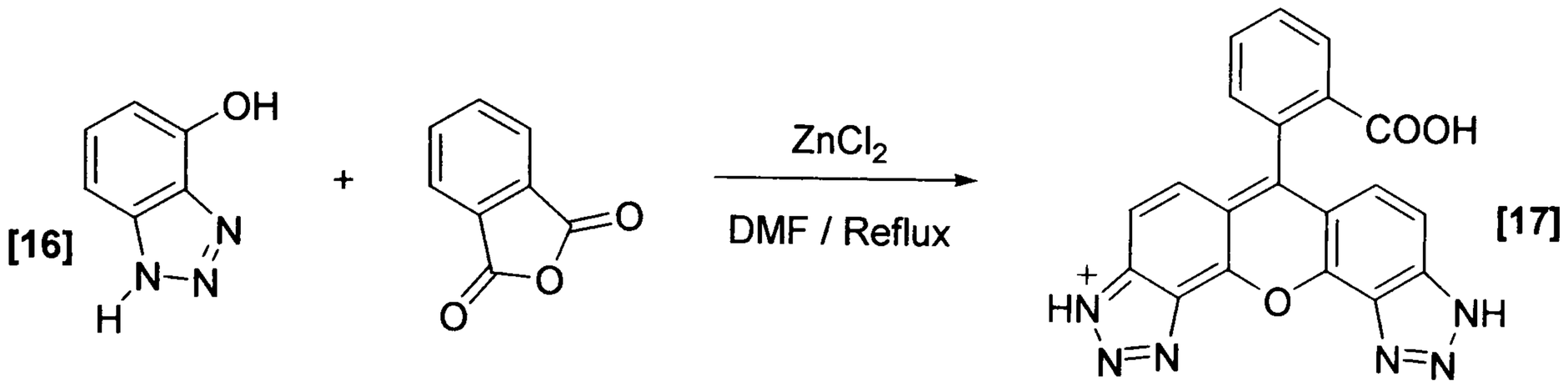


Figure 36. Proposed synthesis of a benzotriazole rhodamine dye.

This synthetic route requires the synthesis of 4-hydroxybenzotriazole **[16]** since this is not a readily available starting material. Compound **[16]** has been synthesised previously using various methods, however a simpler synthesis was carried out using 2,3-diaminophenol.[56] The desired compound was obtained by a ring closure reaction, outlined in figure 37.[57]

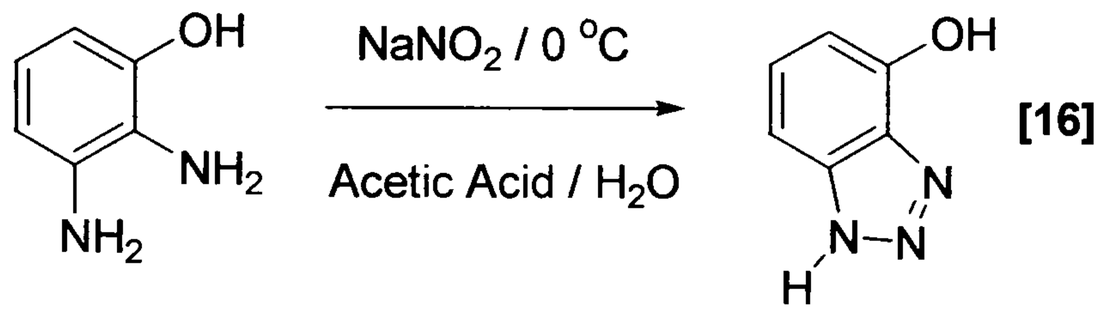


Figure 37. Synthesis of 4-hydroxybenzotriazole.

The synthesis of Bt rhodamine was attempted as described above, however no dye was obtained. The reaction formed a brown polymeric residue. This could be due to one of two factors. Available literature indicates that the phenol of the starting material must be meta to the reacting amine. In the case of 4-hydroxybenzotriazole the phenol is meta to N(1), the requirement may be that the phenol should be meta to the entire triazole ring. In this case the reaction should be repeated with 5-hydroxybenzotriazole.

Another possibility for the failure of the reaction is the ability of benzotriazole to complex to metals in particular the Zn of the catalyst, which could account for the brown polymeric material in the reaction mixture. This problem could be overcome by the protection of the triazole ring. The complex nature of this reaction and the failure to isolate sufficient characterisable products led to this route being abandoned in favour of more fruitful work.

2.4 Coupling of the SERRS Label to DNA Containing an Amino Linker

The final step in the modification of DNA at the 5' hydroxyl *via* an amino linker, is the coupling step. The derivatised label is activated by means of a coupling agent to provide a reactive intermediate that readily reacts with a primary amine thus ensuring maximum coupling efficiency. One such coupling agent is 1,1-carbonyldiimidazole (CDI). Work by Hirokawa *et al*[58] outlined the use of CDI for the activation of a carboxylic acid group in the presence of the benzotriazole moiety, without protection of the triazole ring. Therefore CDI was used as a means of selectively activating the carboxylic acid of the azobenzotriazole dye series before coupling to DNA. Figure 38 shows a schematic of the activation reaction.

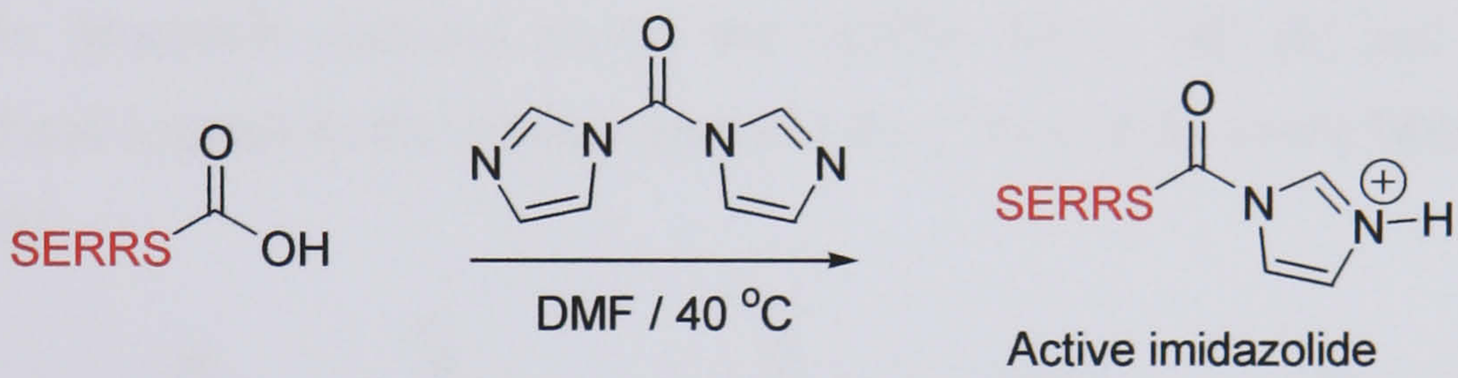


Figure 38. Activation of the SERRS label.

A solution of the SERRS label in DMF was prepared and treated with CDI, the reaction was heated to 40 °C for 15 minutes and allowed to cool to room temperature. The resulting active amide was introduced to the column containing the bound amino-oligonucleotide (outlined in section 2.2 of this chapter). Nucleophillic attack by the free amine at the 5' end of the oligo on the carbonyl of the active intermediate led to the displacement of imidazole, followed by formation of the amide bond. This process was carried out on the solid support to effect maximum efficiency of the coupling reaction, since a large excess of activated dye could be flushed through the column containing the solid support. Another advantage of carrying out the reaction on the solid support was ease of purification of the product since all unreacted dye and impurities could be washed from the column leaving the

pure labelled product bound to the solid support.

The modified oligonucleotide was then cleaved from the solid support and the protecting groups removed using ammonia. The products were purified by reverse phase HPLC and initially de-salted by passing through a Sephadex size exclusion column prior to use.

The following oligonucleotide containing the amino linker was synthesised.

DQA Primer Sequence:

5' **NH_2-C_6-**GTG CTG CAG GTG TAA ACT TGT ACC AG *3'*

Using the procedure outlined above, the SERRS labels **[4]**, **[6]** and **[15]** were activated and coupled to the primary amine at the 5' end of the above DNA sequence (figure 39).

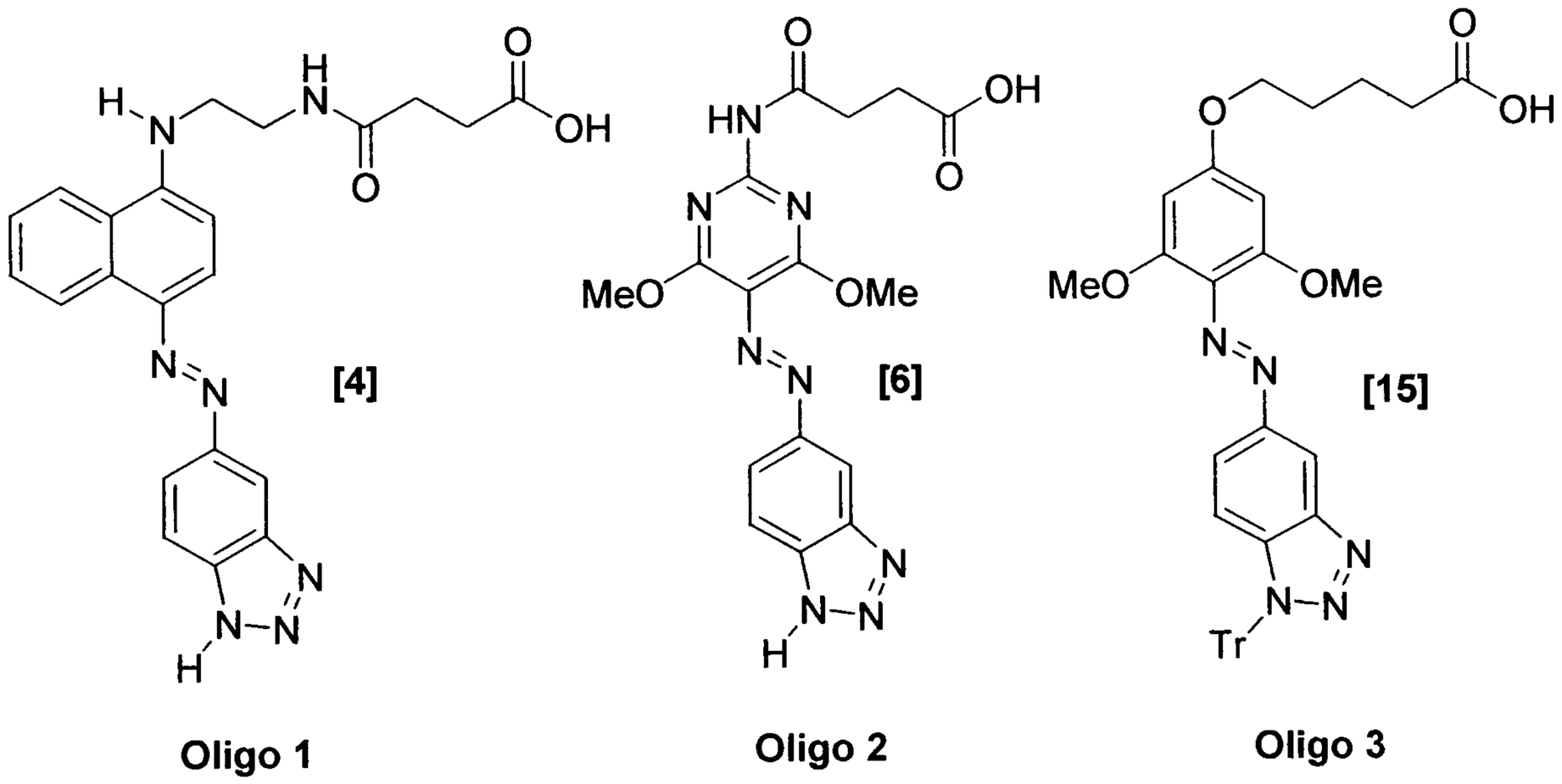


Figure 39. The three SERRS labels coupled to the DQA sequence.

Figure 40 shows the HPLC traces of **Oligo 1** and **Oligo 2**.

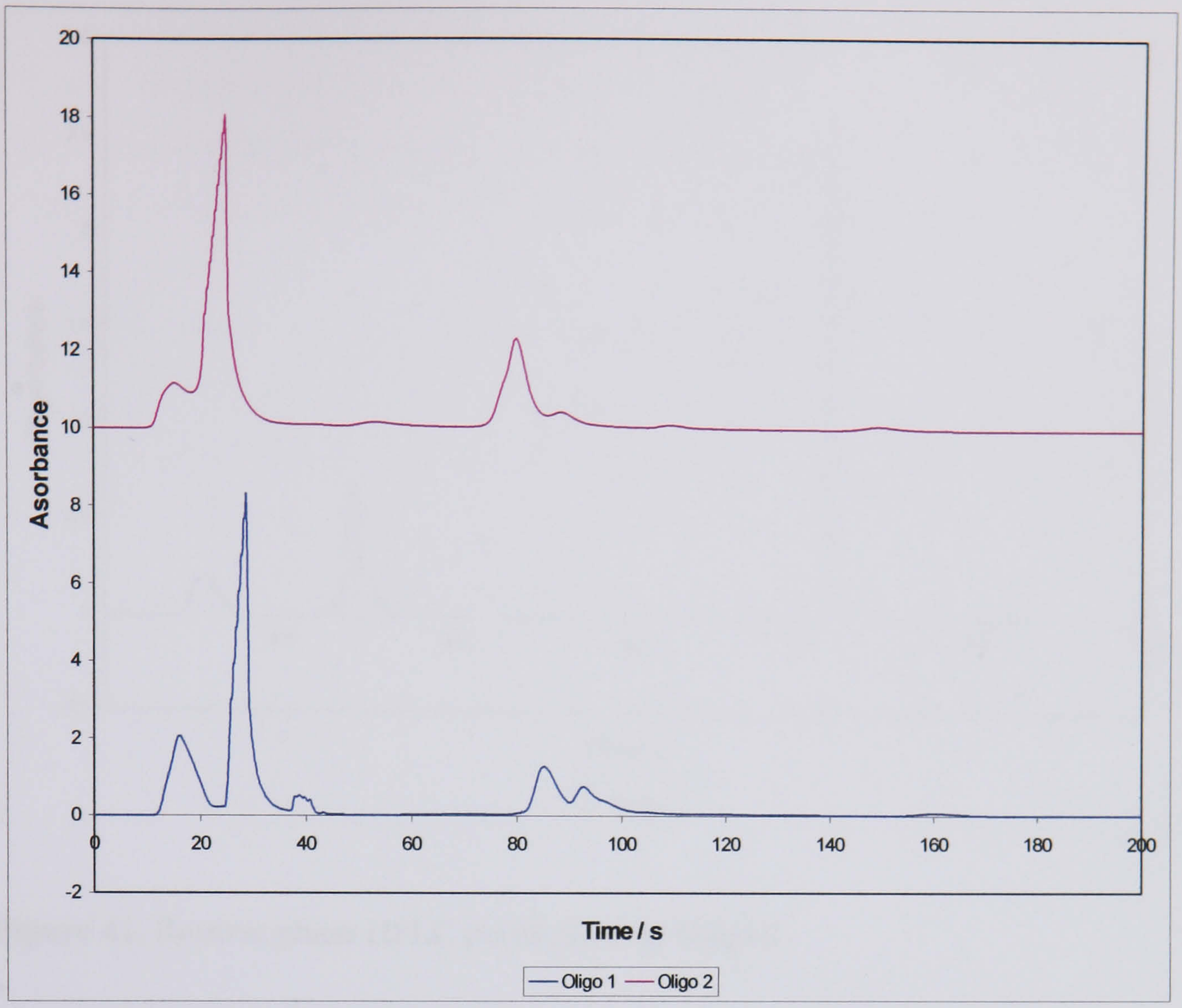


Figure 40. Reverse phase HPLC traces of labelled oligonucleotide purification.

In the case of **Oligo 3** the labelled oligonucleotide was cleaved from the solid support in the usual way. The trityl protecting group was removed during HPLC purification by on-column de-tritylation, this involved a wash step of 5% trifluoroacetic acid. Figure 41 shows the HPLC trace obtained for the on-column de-tritylation.

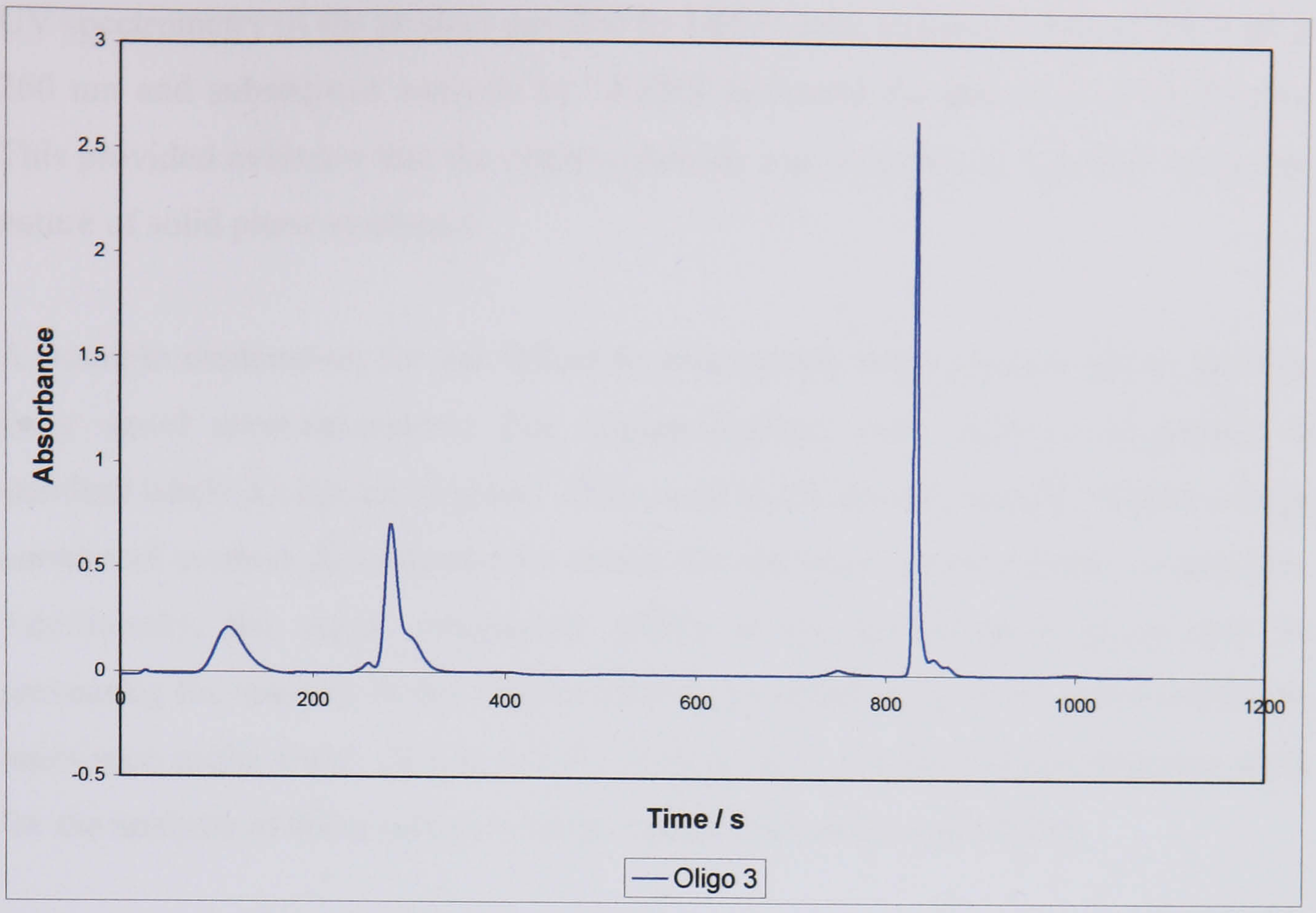


Figure 41. Reverse phase HPLC purification of **Oligo 3**.

MALDI TOF mass spectrometry was attempted to characterise the oligonucleotide products, Table 1 below shows the results obtained for the labelled oligonucleotides.

Oligonucleotide	Calculated Mass	Observed Mass
Oligo 1	8538.92	8586.81
Oligo 2	8617.92	8562.05
Oligo 3	8486.74	1799.96

Table 1. MALDI TOF mass spectrum data for amino-linked oligonucleotides.

The required molecular ions were not found, however the observed molecular ions were only found in low concentrations. If the coupling of the azobenzotriazole dyes had failed, the main product would be the full sequence containing the amino linker minus the azobenzotriazole dye, with a molecular weight of 7359.91 in each case.

UV spectrometry of the product purified by HPLC gave a characteristic DNA peak at 260 nm and subsequent analysis by SERRS indicated the presence of the Bt dye. This provided evidence that the oligonucleotide was probably as expected due to the nature of solid phase synthesis.

A possible explanation for the failure to observe the molecular ion is the fact that these novel azobenzotriazole dye oligonucleotides have different properties to standard labelled oligonucleotides. This could mean that the samples require a large amount of method development to obtain the optimum sample matrix for analysis. Additionally, the metal complexing ability of the benzotriazole group may be preventing the analysis of the oligonucleotide by complexing to the metal within the mass spec probe itself. It was not possible to carry out method development work for the analysis of these samples due to limited instrument availability.

2.5 Conclusion

A series of novel azobenzotriazole dyes were designed and synthesised in order to contain a metal surface attachment group and also chromophore in the visible region to allow detection by SERRS. The carboxylic acid derivatives of three novel azobenzotriazole dyes were synthesised and coupled successfully to an oligonucleotide modified to contain a primary amine at the 5' end. The first labelling of DNA with a specifically designed SERRS label was achieved.[59]

2.6 Experimental

2.6.1 General

Solvents were of laboratory grade except those used for the purification of DNA, which were of HPLC grade. Tetrahydrofuran was distilled from sodium metal and benzophenone; pyridine was distilled from CaH_2; triethylamine and diisopropylethylamine were dried over CaH_2; anhydrous N, N-dimethylformamide was purchased from Aldrich. 5-Aminobenzotriazole was supplied by Lancaster and all other chemicals were supplied by Aldrich or Fluka. Thin layer chromatography (TLC) was carried out on silica gel 60 F_{254}, 0.2 mm layer backed with aluminium (Merck). Flash chromatography was carried out using silica gel 60, mesh 200-300 (Prolabo). The following solvent systems were employed for T.L.C analysis:

A = Hexane – ethyl acetate (1:1, v/v)

B = Ethyl acetate

C = Ethyl acetate – methanol – ammonia (5:1:1, v/v)

D = Dichloromethane – methanol (9:1, v/v)

E = Dichloromethane – methanol (8:2, v/v)

F = Hexane – ethyl acetate (2:1, v/v)

G = Dichloromethane

All samples for analysis were dried over P_2O_5 under high vacuum. Mass Spectra were recorded on a Jeol JMS AX505 mass spectrometer. IR spectra were recorded on a Unicam Mattson 1000 series FTIR spectrometer using NaCl plates. UV spectra were recorded on a Perkin-Elmer Lambda 15 ultraviolet-visible spectrometer operating PECSS2 software. NMR spectra were recorded on a Bruker DPX400 MHz spectrometer.

The synthesis of oligonucleotides was carried out using an Applied Biosystems Expedite 8909 System. All reagents for DNA synthesis were supplied by Applied Biosystems, including the Expedite fast de-protection monomers used for DNA synthesis. The estimated loadings of the columns were 0.2 μmol or 1.0 μmol. HPLC grade solvents were used for the purification of DNA and all buffers were filtered prior to use. Ultra pure water for final dissolution of the synthetic oligonucleotides was purchased from Fischer.

General Procedure 1: Diazotisation of 5-Aminobenzotriazole

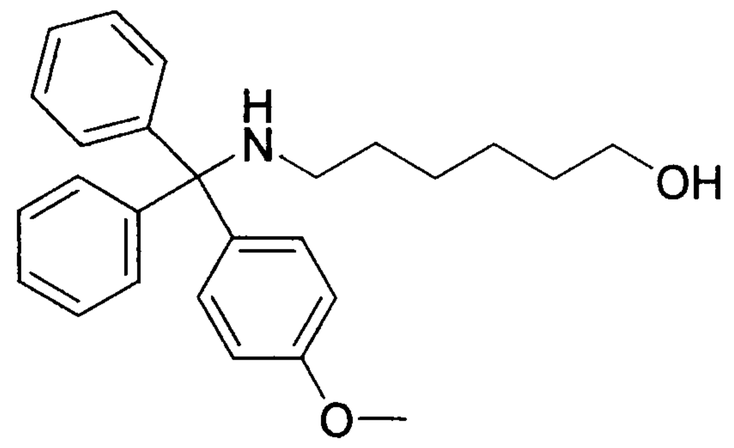
5-Aminobenzotriazole (1.1 eq.) was dissolved in 50% aqueous HCl (5 ml) and diazotised by dropwise addition of sodium nitrite solution (1.2 eq. in 5ml H_2O) at 0°C. The coupling component (1.0 eq.) was dissolved in sodium acetate buffer (1.0 M, pH6) to which the diazonium solution was added dropwise at room temperature. The solution was allowed to stir for 1-24 hours. The resulting azobenzotriazole dye precipitated from solution and was collected by filtration. The pure dye was obtained by wet flash column chromatography.

General Procedure 2: Phosphitylation Reaction

The alcohol was co-evaporated with anhydrous THF (3 x 15 ml) and then dissolved in anhydrous THF (15 ml). To the stirring solution, anhydrous diisopropylethylamine (4 equivalents) was added under nitrogen. 2-Cyanoethyl-N, N-diisopropylchloro-phosphoramidite (1.1 equivalents) was added dropwise and the reaction allowed to stir for one hour. After this time the solvent was removed and the residue redissolved in ethyl acetate (50 ml). The organic layer was washed with saturated potassium chloride (20 ml), dried over anhydrous sodium sulfate and the solvent removed *in vacuo*. The resulting oil was purified by wet flash column chromatography, eluting with 100 % ethyl acetate, using silica pre-equilibrated with 1% triethylamine.

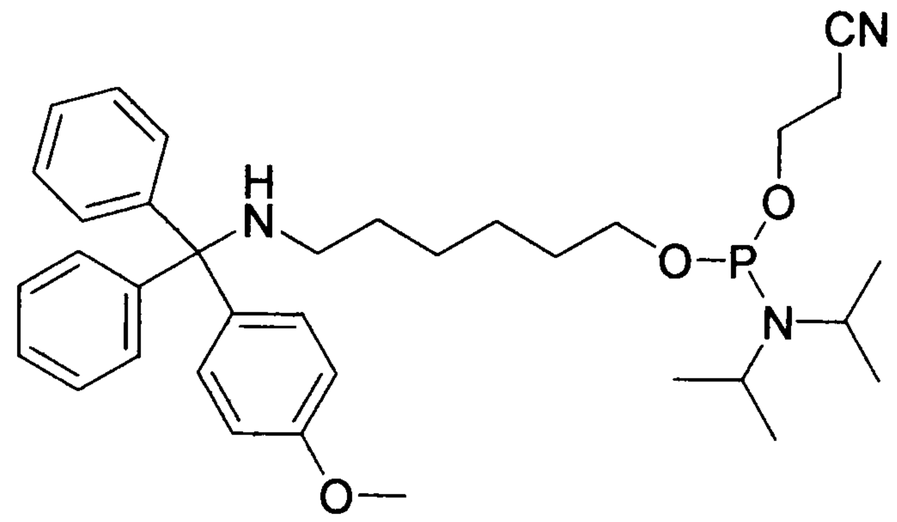
2.6.2 Chemical Synthesis

6-(Monomethoxy trityl)hexan-1-ol [1]



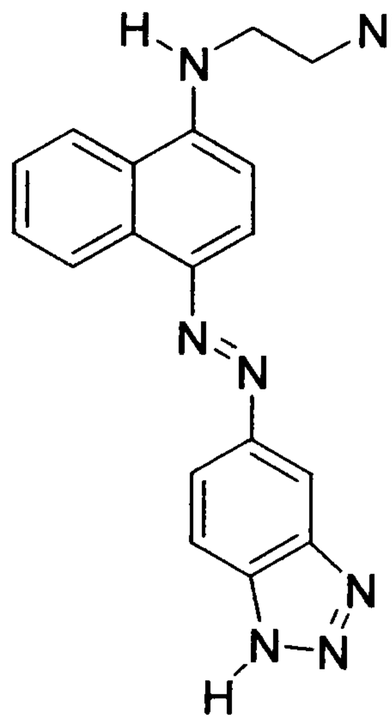
6-Aminohexan-1-ol (0.500 g, 4.27 mmol) was co-evaporated with anhydrous pyridine (3 x 10 ml) and then dissolved in anhydrous pyridine (7 ml) with anhydrous triethylamine (0.2 ml). (4-Methoxyphenyl)-diphenylmethyl chloride (1.251 g, 4.05 mmol, 0.95 eq.) was dissolved in anhydrous pyridine and added in three portions at forty five minute intervals to the stirring amine solution and left for thirty minutes. The reaction was quenched with methanol (3 ml) and the volume reduced *in vacuo*. The residue was re-dissolved in ethyl acetate and extracted with sodium carbonate (20 ml of a saturated solution), washed with water (20 ml) and then with saturated potassium chloride (20 ml). The organic extract was dried over anhydrous sodium sulfate and the solvent removed *in vacuo*. The residue was purified by wet flash column chromatography, eluting with hexane / ethyl acetate in a 1:1 ratio. Silica was pre-equilibrated with 1 % triethylamine. The product was collected and the solvent removed to yield a yellow oil (0.952 g, yield 57.3 %). R_f (A) 0.46, ^{1}H NMR ($CDCl_3$) δ_H 1.23 (4H, m, C*H*$_2$); 1.37 (2H, t, *J* 6.5, C*H*$_2$); 1.42 (2H, t, *J* 6.6, C*H*$_2$); 3.71 (3H, s, O-C*H*$_3$); 4.02 (2H, t, *J* 7.1, N-C*H*$_2$); 4.31 (2H, t, *J* 5.1, O-C*H*$_2$); 6.84 (2H, d, *J* 8.9, Ar*H*); 7.16 (2H, t, *J* 7.4, Ar*H*); 7.27 (6H, t, *J* 7.6, Ar*H*); 7.38 (4H, d, *J* 7.9, Ar*H*). M/z (EI) 389.2363 [C_{26} H_{31} N O_2 (M^+) < 2.1 ppm].

1-(2-Cyanoethyl N,N-diisopropylphosphoramidite)-6-[(monomethoxy trityl]hexane [2]



Phosphitylation of compound **[1]** (was carried out using general procedure 2. Product **[2]** was obtained as a yellow oil (1.283 g, 2.176 mmol, 99.1 % yield). R_f (B) 0.79, ^{31}P NMR, ($CDCl_3$), referenced to phosphoric acid, δ_P 147.90 (s).

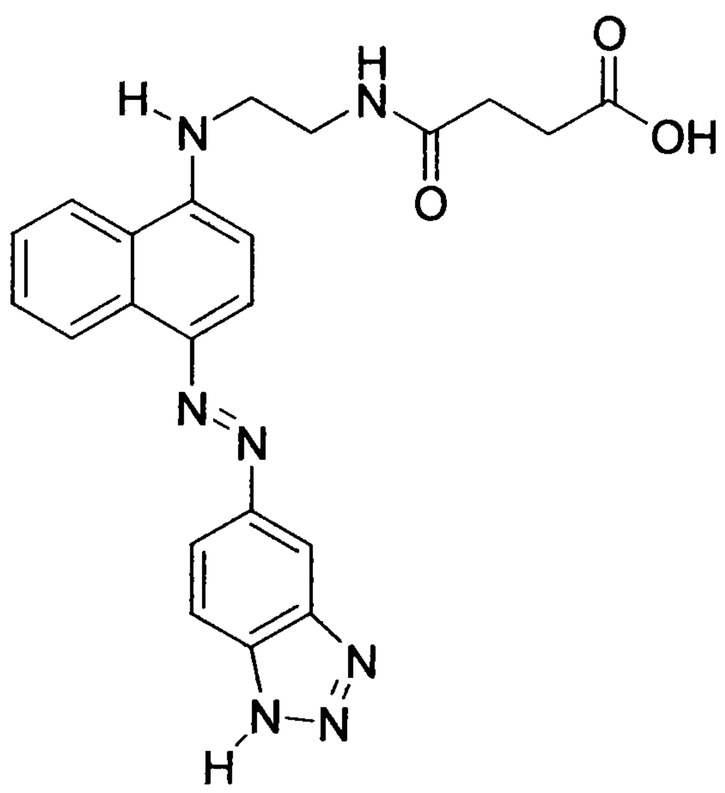
N-[4-(5'-Azobenzotriazoyl)naphthalen-1-yl]ethylenediamine [3]



5-Aminobenzotriazole (0.854 g, 6.37 mmol, 1.1 eq.) was diazotised using standard procedure 2. The diazonium solution was then added dropwise to N-(1-naphthyl)-ethylenediamine (1.500 g, 5.79 mmol, 1.0 eq.) dissolved in sodium acetate buffer (1.0 M, pH 6.0, 60 ml) and acetone (60 ml) and left to stir for 20 minutes. The acetone was then removed *in vacuo* and the resulting orange solid isolated by filtration. Trituration from methanol with diethyl ether yielded an orange solid in

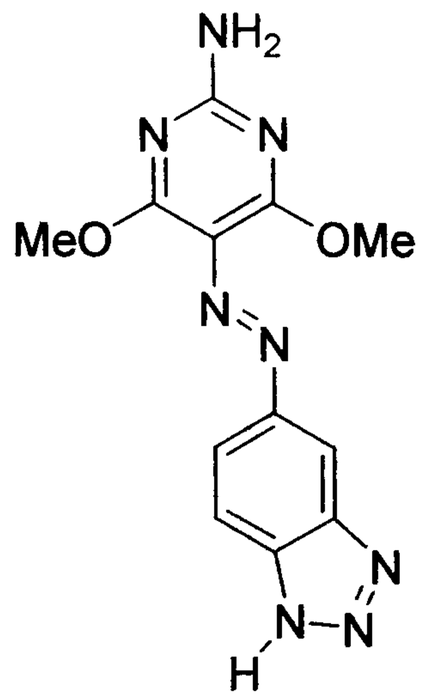
73% yield (1.390 g, 4.20 mmol). R_f (C) 0.13, ^{1}H NMR [CD_3OD] δ_H 2.92 (2H, t, *J* 6.2, C*H*$_2$); 3.37 (2H, t, *J* 5.5, C*H*$_2$); 6.70 (1H, d, *J* 8.7, Ar*H*); 7.52 (1H, t, *J* 7.8, Ar*H*); 7.65 (1H, d, *J* 7.6, Ar*H*); 7.71 (2H, s, Ar*H*); 7.90 (1H, d, *J* 8.6, Ar*H*); 8.18 (1H, s, Ar*H*); 8.32 (1H, d, *J* 8.5, Ar*H*); 8.97 (1H, d, *J* 8.4, Ar*H*); δ_C [CD_3OD] 41.40 (*C*H$_2$); 41.56 (*C*H$_2$); 104.26 (*C*H); 114.93 (*C*H); 115.13 (*C*H); 116.58 (*C*H); 117.47 (*C*H); 122.20 (*C*H); 123.98 (*C*); 124.73 (*C*H); 126.03 (*C*H); 127.91 (*C*H); 134.65 (*C*); 139.91 (*C*); 146.12 (*C*); 146.76 (*C*); 149.00 (*C*); 151.30 (*C*); λ_{max} (MeOH) 448 nm ε_{260} 21.2 mlμmol^{-1}cm^{-1}. M/z (FAB) 332.16339 [$C_{18}H_{18}N_7$ $(M + H)^+$ < 3.1 ppm].

N-[2-(4'-(5''-Azobenzotriazolyl)naphthalen-1yl)-aminoethyl]succinamic acid [4]



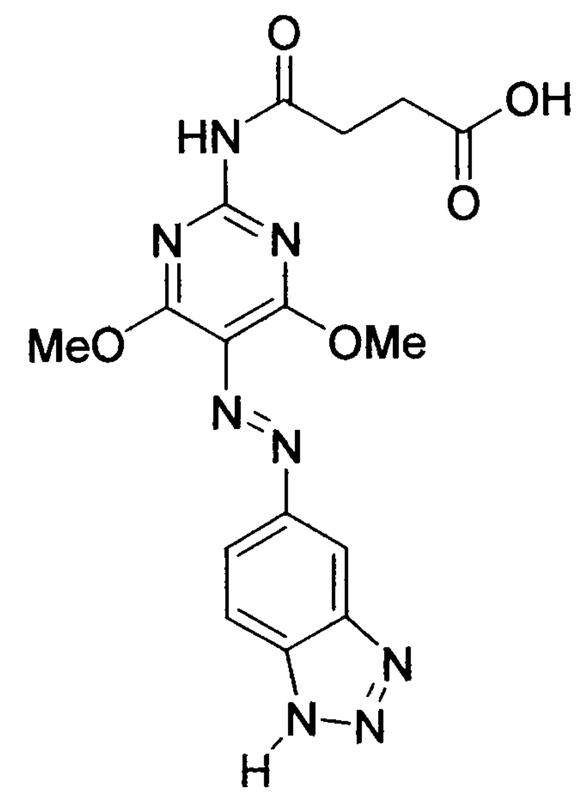
Succinic anhydride (0.363 g, 3.63 mmol, 1.2 eq.) dissolved in acetone (20 ml) was added dropwise to compound **[3]** (1.000 g, 3.02 mmol, 1 eq.) dissolved in dimethylformamide (100 ml) and left to stir for 18 hours. The solvent was removed *in vacuo* and the residue applied directly to the top of a wet flash column. The product was eluted using 5:1:1 ethyl acetate/methanol/conc. ammonia to yield an orange solid in 66% yield (0.856 g, 2.00 mmol). R_f (C) 0.11, H NMR (d_6-DMSO) δ_H 3.20 (2H, t, *J* 6.1, C*H*$_2$); 3.25 (2H, t, *J* 6.6, C*H*$_2$); 4.24 (4H,brs, C*H*$_2$); 7.57 (1H, d, *J* 8.7, Ar*H*); 8.06 (1H, brs, CON*H*); 8.36 (1H, t, *J* 8.1, Ar*H*); 8.49 (1H, t, *J* 7.3, Ar*H*); 8.76 (3H, m, Ar*H*); 9.05 (2H, d, *J* 8.6, Ar*H*); 9.10 (1H, brs). M/z 432.17974 [$C_{22}H_{22}N_7O_3$ $(M + H)^+$ < 3.1ppm].

5-(5'-Azobenzotriazoyl)-4,6-dimethoxy-pyrimidin-2-ylamine [5]



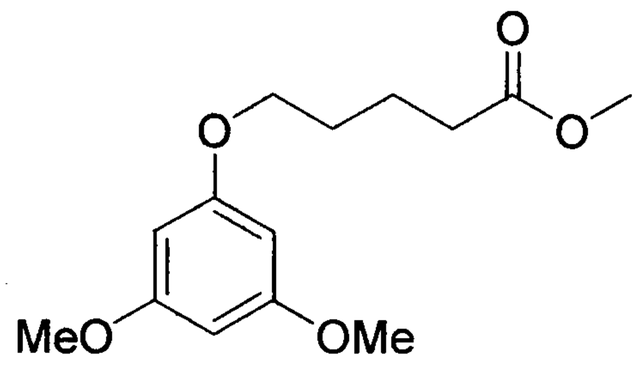
5-Aminobenzotriazole (1.209 g, 9.01 mmol) was diazotised using general procedure 1. The resulting diazonium was added dropwise to a solution of 2-amino-4,6-dimethoxypyrimidine (1.272 g, 8.20 mmol) dissolved in sodium acetate buffer (40 ml, 1M, pH6) with 20 ml of acetone. An orange compound precipitated and was collected by filtration. The pure dye was isolated by wet flash column chromatography, eluting with methanol in DCM (0 – 10%) to give compound **[5]** in 64% yield (1.560 g, 5.20 mmol). R_f (C) 0.25, ^{1}H NMR (d_6-DMSO), δ_H 3.95 (6H, s, O-CH_3); 7.42 (2H, brs, NH_2, peak disappears on addition of D_2O); 7.78 (1H, d, *J* 8.9, Ar*H*); 7.96 (1H, d, *J* 8.9, Ar*H*); 8.02 (1H, s, Ar*H*). M/z (FAB) 301.11694 [$C_{12}H_{13}N_8O_2$ $(M+H)^+$ < 2.6 ppm].

N-[5-(5'-Azobenzotriazoyl)-4,6-dimethoxy-pyrimidin-2-yl]-succinamic acid [6]



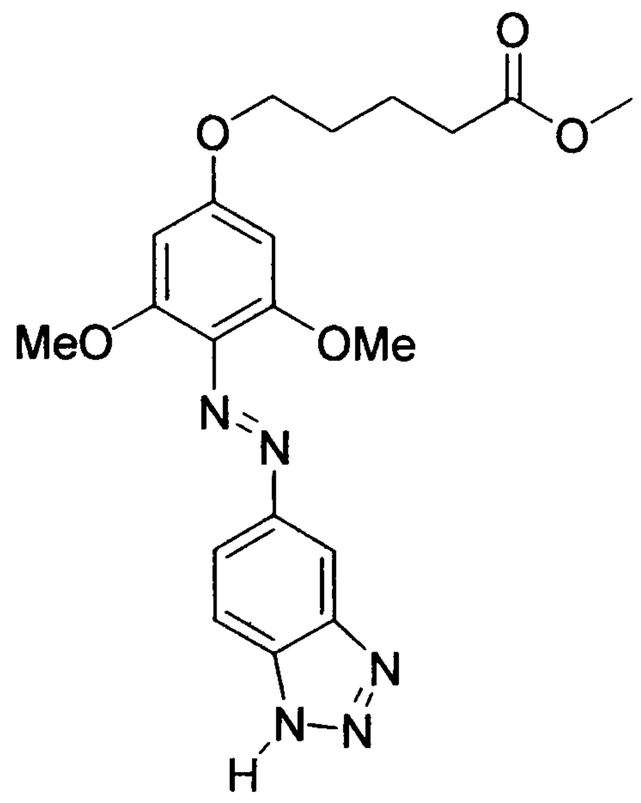
Compound **[5]** (0.117 g, 0.393 mmol) was dissolved in dimethylformamide (50 ml) and DMAP (9.6 mg, 0.079 mmol, 0.2 eq.) added followed by dropwise addition of succinic anhydride (0.047 g, 0.471 mmol, 1.2 eq.) dissolved in acetone (10 ml). The reaction was allowed to stir at room temperature for 5 days. After this time the solvent was removed *in vacuo* and the residue applied directly to the top of a wet flash column. The product was eluted using 5:1:1 ethyl acetate/methanol/conc. ammonia to yield an orange solid in 82% yield (0.127 g, 0.318 mmol). R_f (C) 0.59, H NMR (d_6-DMSO) δ_H 3.31 (4H, m, C*H*$_2$); 7.85 (1H, d, *J* 8.9, Ar*H*); 7.91 (1H, d, *J* 8.9, Ar*H*); 8.11 (1H, s, Ar*H*). M/z (FAB) 401.13406 [$C_{16}H_{17}N_8O_5$ $(M+H)^+$ < 4.7 ppm].

5-(3,5-Dimethoxy-phenoxy)-pentanoic acid methyl ester [7]



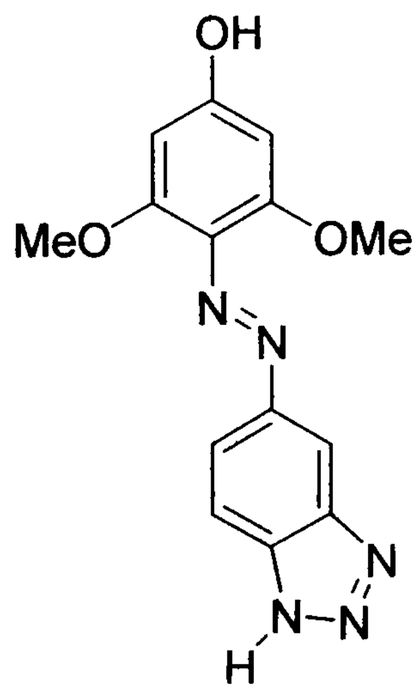
3,5-Dimethoxyphenol (1.000 g, 6.49 mmol) was dissolved in anhydrous acetonitrile (10 ml) and DBU (2.960 g, 19.46 mmol, 2.904 ml, 3 eq.) was added to the stirred solution followed by 5-bromovalerate (1.900 g, 9.73 mmol, 1.39 ml, 1.5 eq.). The reaction was allowed to proceed for 24 hours, after which time the volatiles were removed by evaporation. The residue was re-dissolved in hexane (20 ml) and washed with a sat. sodium carbonate solution (20 ml), followed by water (20 ml) then sat. potassium chloride (20 ml). The combined organic extracts were dried over anhydrous sodium sulfate and the solvent removed *in vacuo* to yield white solid, **[7]** (1.419 g, 5.29 mmol, 81.5%). R_f (A) 0.62, C, H, N; found: C, 62.88; H, 7.45; $C_{14}H_{20}O_5$ requires C, 62.67; H, 7.51. ^{1}H NMR ($CDCl_3$), δ_H 1.82 (4H, m, C*H*$_2$); 2.39 (2H, m, C*H*$_2$-O); 3.68 (3H, s, O-C*H*$_3$); 3.77 (6H, s, O-C*H*$_3$); 3.93 (2H, m, C*H*$_2$-O); 6.08 (3H, d, *J* 4.7, Ar*H*). M/z (EI) 268.12928 [$C_{14}H_{20}O_5$ M^+ < 6.0 ppm].

[3,5-Dimethoxy-4-(5'-azobenzotriazoyl) phenoxy]-pentanoic acid methylester [8]



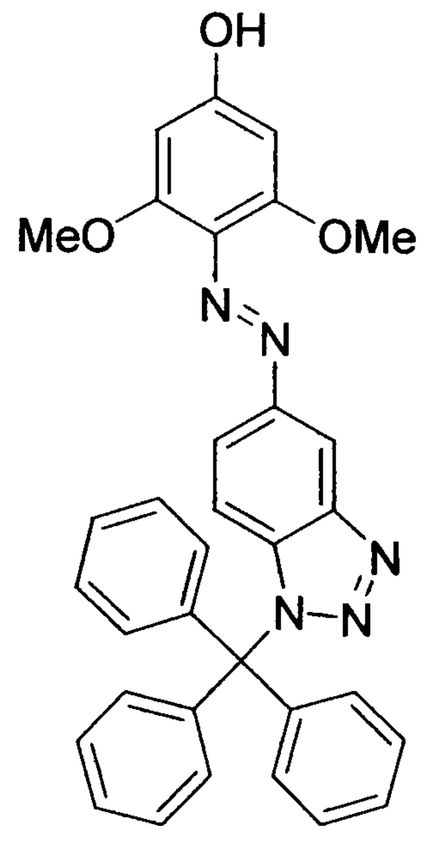
5-Aminobenzotriazole (0.110 g, 0.820 mmol) was diazotised using general procedure 1. The resulting diazonium was added dropwise to a solution of compound **[7]** (0.200 g, 0.745 mmol) dissolved in sodium acetate buffer (20 ml, 1M, pH6) with 10 ml of acetone. An orange / red compound precipitated and was collected by filtration. The pure dye was isolated by wet flash column chromatography, eluting with methanol in DCM (0 – 2%) to give compound **[8]** in 68% yield (0.210 g, 0.508 mmol). R_f (D) 0.75, ^{1}H NMR (d_6-DMSO), δ_H 1.69 (4H, m, C*H*$_2$); 2.26 (2H, t, *J* 6.5, C*H*$_2$-O); 3.84 (3H, s, O-C*H*$_3$); 3.87 (6H, s, O-C*H*$_3$); 4.05 (2H, t, *J* 5.4, C*H*$_2$-O); 6.38 (2H, s, Ar*H*); 7.86 (1H, d, *J* 8.9, Ar*H*); 7.97 (1H, d, *J* 8.7, Ar*H*); 8.20 (1H, s, Ar*H*). M/z (FAB) 414.17599 [$C_{20}H_{24}N_5O_5$ $(M+H)^+$ < 4.2 ppm.

4-(5'-Azobenzotriazoyl)-3,5-dimethoxy-phenol [10]



5-Aminobenzotriazole (1.209 g, 9.01 mmol) was diazotised using general procedure 2. The resulting diazonium was added dropwise to a solution of 3,5-dimethoxy phenol (0.517 g, 3.35 mmol) dissolved in sodium acetate buffer (40 ml, 1M, pH6) with 20 ml of acetone. A red / orange compound precipitated and was collected by filtration and washed with water then diethyl ether. The pure dye was isolated by trituration from warm DMF using water and dried to give compound **[10]** in 64% yield (0.710 g, 2.37 mmol). R_f (C) 0.28, ^{1}H NMR (d_6-DMSO), δ_H 3.79 (6H, s, O-C*H*$_3$); 6.03 (2H, s, Ar*H*); 7.79 (1H, d, *J* 9.2, Ar*H*); 7.99 (1H, d, *J* 9.2, Ar*H*); 8.04 (1H, s, Ar*H*). M/z (FAB) 300.1088 [$C_{14}H_{14}N_5O_3$ $(M+H)^+$ < 2.8 ppm].

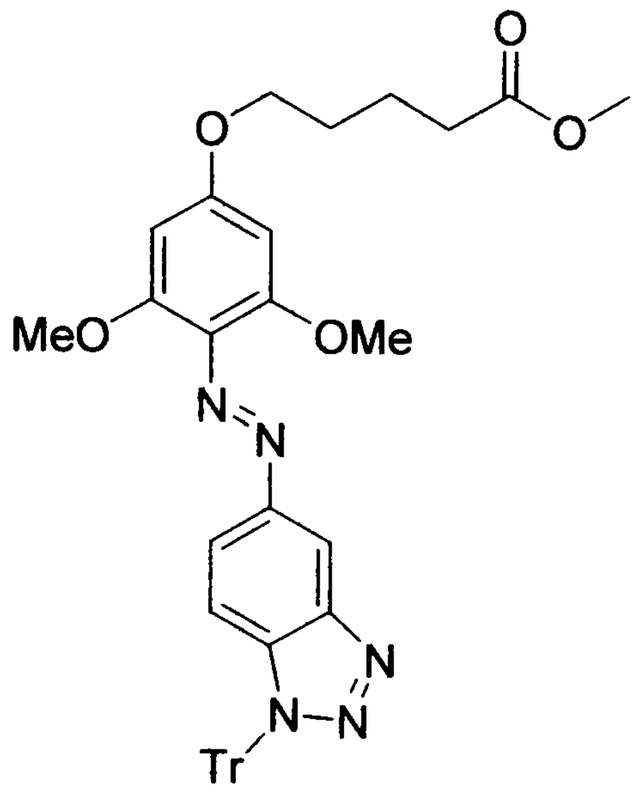
3,5-Dimethoxy-4-(1-trityl-5'-azobenzotriazoyl)-phenol [13]



Dye **[10]** (0.50 g, 1.67 mmol) was co-evaporated with anhydrous pyridine (3 x 30 ml) and dissolved in anhydrous pyridine (30 ml). To the stirring solution was added DMAP (0.0408 g, 0.334 mmol, 0.2 eq.) followed by triphenylmethylene chloride (0.559 g, 2.0 mmol, 1.2 eq.). The reaction was allowed to stir at room temperature for seven days. After this time the solvent was removed in vacuo and the residue re-dissolved in ethyl acetate (20 ml) and washed with sat. KCl (20 ml) followed by water. The combined organic extracts were dried over anhydrous sodium sulfate and the solvent removed in vacuo. The pure product was obtained by washing the

resulting orange solid with diethyl ether and collection by filtration. Compound **[11]** was obtained in 69% yield (0.378 g, 0.696 mmol). R_f (C) 0.48, ^{1}H NMR ($CDCl_3$), δ_H 3.86 (3H, s, O-C*H*$_3$); 3.94 (3H, s, O-C*H*$_3$); 5.75 (2H, d, *J* 2.2, Ar*H*); 6.40 (1H, d, *J* 9.1, Ar*H*); 7.19 (6H, m, Ar*H*); 7.32 (10H, m, Ar*H*); 7.66 (1H, s, Ar*H*); 11.53 (1H, brs, Ar-O*H*). M/z (FAB) 542.2203 [$C_{33}H_{28}N_5O_3$ $(M+H)^+$ < 1.9 ppm].

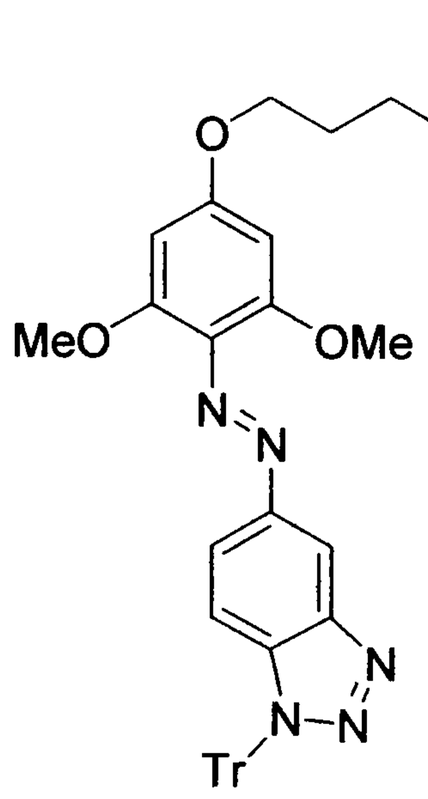
6-[3,5-Dimethoxy-4-(1-trityl-5'-azobenzotriazoyl)-phenoxy]-hexan-2-one[14]



Compound **[11]** (0.100 g, 0.185 mmol) was dissolved in anhydrous acetonitrile (10 ml). DBU (0.084 g, 0.554 mmol, 0.083 ml, 3 eq.) was added to the stirring solution followed by 5-bromovalerate (0.072 g, 0.369 mmol, 0.053 ml, 1.5 eq.). The reaction was allowed to proceed for 24 hours, after which time the volatiles were removed *in vacuo*. The residue was re-dissolved in DCM (20 ml) and washed with sat. potassium chloride (20 ml). The combined organic extracts were dried over anhydrous sodium sulfate and the solvent removed *in vacuo*. The crude product was purified by wet flash column chromatography eluting DCM, using silica pre-equilibrated with 1% triethylamine. Product **[12]** was obtained in 96 % yield (0.116 g, 0.177 mmol). R_f (C) 0.85, ^{1}H NMR (d_6-DMSO), δ_H 2.37 (4H, m, C*H*$_2$); 3.43 (3H, s, O-C*H*$_3$); 3.59 (3H, s, O-C*H*$_3$); 3.75 (3H, s, O-C*H*$_3$); 3.99 (2H, t, *J* 6.5, O-C*H*$_2$); 4.08 (2H, t, *J* 6.5, O-C*H*$_2$); 6.35 (1H, s, Ar*H*); 6.43 (1H, s, Ar*H*); 7.20 (15H, m,

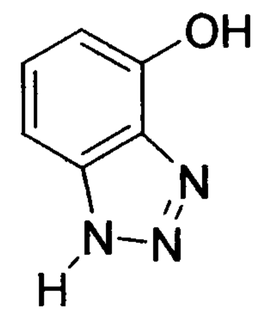
Ar*H*); 7.67 (1H, d, *J* 8.9, Ar*H*); 8.10 (1H, d, *J* 9.0, Ar*H*); 8.35 (1H, s, Ar*H*). M/z (FAB) 656.2901 [$C_{39}H_{38}N_5O_5$ $(M+H)^+$ < 4.2 ppm].

5-[3,5-Dimethoxy-4-(1-trityl-5'-azobenzotriazoyl)-phenoxy]-pentanoic acid [15]



Compound **[12]** (0.983 g, 0.150 mmol) was added to 25ml of a 5 % w/v solution of methanolic potassium hydroxide. The solution was refluxed for 30 minutes, after which time the solvent was removed *in vacuo*. The residue was re-dissolved in DCM and any un-dissolved solid removed by filtration, the solvent was removed and the residue washed with ethyl acetate to remove any traces of starting material. The residue was further treated with 10 % citric acid (20 ml) and the free acid extracted with ethyl acetate. The combined organic extracts were dried over anhydrous sodium sulfate and the solvent removed by evaporation to yield compound **[13]** in 88 % (85.5 mg, 0.133 mmol). R_f (C) 0.15, ^{1}H NMR (d_6-DMSO) δ_H 2.46 (4H, m, C*H*$_2$); 3.89 (3H, s, O-C*H*$_3$); 3.92 (3H, s, O-C*H*$_3$); 4.05 (2H, t, *J* 6.0, O-C*H*$_2$); 4.23 (2H, t, *J* 6.8, O-C*H*$_2$); 6.42 (1H, s, Ar*H*); 6.49 (1H, s, Ar*H*); 7.40 (15H, m, Ar*H*); 8.08 (1H, d, *J* 8.8, Ar*H*); 8.16 (1H, d, *J* 8.9, Ar*H*); 8.45 (1H, s, Ar*H*). M/z (FAB) 642.2715 [$C_{38}H_{36}N_5O_5$, $(M+H)^+$ < 0.3 ppm].

4-Hydroxybenzotriazole [16]



2,3-Diaminophenol (0.500 g, 4.03 mmol) was dissolved in a glacial acetic acid / water mixture (20 ml and 15 ml respectively) and cooled to 0 °C. A solution of sodium nitrite (0.334 g, 4.84 mmol, 1.2 eq., in 10 ml of water) was added dropwise to the diamine and the reaction was allowed to proceed for 24 hours. When the reaction was complete, the mixture was extracted with ethyl acetate (20 ml). After removal of the solvent by rotary evaporation, the residue was applied directly to a wet flash chromatography column, eluting methanol in DCM (0 – 10%) to give 4-hydroxybenzotriazole **[14]** in 83% (0.450 g, 3.33 mmol). R_f (E) 0.56. ^{1}H NMR (d_6-DMSO), δ_H 6.6 (1H, brs, ar); 7.22 (2H, brs, ar); 10.55 (1H, brs, ar-OH); 15.49 (1H, brs, Bt-NH). M/z (EI) 135.0435(M^+) < 1.9 ppm].

2.6.3 Synthesis of an Oligonucleotide Containing an Amino-Linker at the 5' End

A solution of phosphitylated linker **[2]** (60 mg ml $^{-1}$) was placed in monomer port 5 of the instrument. The protocol was set to add the linker as the final monomer of the automated synthesis. Efficient coupling of the monomer was ensured by double delivery to the column, with an extended coupling cycle of 15 minutes for each monomer delivery. The instrument was programmed to remove the final monomethoxytrityl protecting group on completion of the synthesis.

The column containing the oligonucleotide was removed from the instrument to allow addition of the activated SERRS label by manual coupling.

Activation and Coupling of SERRS Label to Amino-linked DNA

The carboxylic acid of the label was dissolved in 2 ml of anhydrous DMF (60 mg / ml) and then activated by reaction with 1,1-carbonyldiimidazole (1.56 eq.). The mixture was warmed to 40 ^{o}C for 5 minutes and then allowed to come to room temperature. The resulting activated acid was loaded into a 1ml syringe and attached to the column containing the oligonucleotide. The solution was passed through the column until it just reached the top frit, then an empty syringe was attached to the other end of the column and the solution pushed through to the vacant syringe. The solution was passed through the column from one syringe to the other four times and then allowed to stand for one hour. This process was repeated once more after which the solution was taken up into one syringe and removed from the column. The column was then washed with several syringe volumes of DMF then acetonitrile and dried with nitrogen.

Deprotection and Removal from the Solid Support

The DNA was removed from the solid support and the protecting groups removed using concentrated aqueous ammonia. 1ml of ammonia was taken up into a syringe and then introduced to the column containing the bound oligonucleotide, again an empty syringe was placed at the opposite end of the column and the solution passed through to the vacant syringe. The solution was passed through the column from one syringe to the other four times, and allowed to stand for two hours in total. After this the solution was taken up into the syringe and removed from the column. The solution containing the de-protected oligonucleotide was then placed in a round bottom flask and evaporated to dryness.

2.6.4 HPLC Purification of Modified Oligonucleotides

Reverse phase HPLC purification of the oligonucleotides was carried out using an Applied Biosystems Biocad Sprint HPLC system running Biocad software. For each DNA sequence, an analytical run was carried out before a full preparative run was attempted. UV detection of eluting products was used in each case, monitoring at 260 nm.

Reverse Phase HPLC Method

Using a Chromolith column. The following reverse phase method was used for the preparative purification of oligos **[1]** and **[2]**.

Flowrate = 4 ml / min

Buffer A = 100mM Triethyl ammonium acetate pH 8.0

Buffer B = Acetonitrile

Equilibration Block

0.00 - 3.00 min	Step Segment: 100% A.

Load Block

3.00 min	Load sample into 1 ml loop.
3.00 min	Zero UV Detector
3.00 min	Inject sample.

Wash Block

3.00 - 6.00 min	Step Segment: 100% A.
6.00 min	Set Sample Loop to Load Position

Elute Block

6.00 – 9.00 min	Gradient Segment:100% A to 95% A; 5% B.

Elute Block

9.00 – 17.00 min	Gradient Segment: 95% A; 5% B to 75% A; 25% B.

The purified oligonucleotide was collected in fractions and the solvent removed by evaporation. The oligo was then prepared for desalting.

Reverse Phase HPLC Method – On-column De-tritylation

Using a column packed with Oligo R3 media. The following reverse phase method was used for the preparative purification of **oligo 3,4** and **5**.

Flowrate = 5 ml/min

Buffer A = 100mM Triethyl ammonium acetate

Buffer B = Acetonitrile

Buffer C = 5% v/v Trifluoroacetic acid in water.

Equilibration Block

0.00 - 3.00 min	Step Segement: 100% A.

Load Block

3.00 min	Load sample into 1 ml loop.
3.00 min	Zero UV Detector
3.00 min	Inject sample.

Wash Block

3.00 - 6.00 min	Step Segment: 100% A.
6.00 min	Set Sample Loop to Load Position

Elute Block

6.00 – 9.00 min	Gradient Segment: 95% A; 5% B.

Wash Block

9.00 – 13.00 min	Step Segment: 100% C.
13.00 – 15.00 min	Step Segment: 95% A; 5% B.

Elute Block

15.00 – 21.00 min	Gradient Segment: 95% A; 5% B to 75% A; 25% B.

DNA De-salting Procedure

The de-salting step was carried by passing the oligonucleotide through a size exclusion column. The pure DNA sample obtained from HPLC purification was dissolved in 1.0 ml of distilled water and passed through a size exclusion column, Pharmacia NAP-10 column containing G25 Sephadex. The column was equilibrated with 3 column volumes of distilled water. The DNA sample was added to the column and allowed to soak completely into the gel bed. 1.5 ml of distilled water was added to the column to elute the pure DNA. The eluted product was collected in an eppendorf tube and then lyophilised to yield the pure oligo as a white solid.

A solution of the modified oligonucleotide was obtained by dissolution in ultra-pure distilled water.

Chapter 3

Attachment of a SERRS Label to DNA *via* the Phosphoramidite

3.0 Attachment of a SERRS Label to DNA *via* a Phosphoramidite

3.1 Introduction

The second method used for the introduction of a SERRS label to the 5' hydroxyl of DNA was using the phosphoramidite approach. This method involved the synthesis of the phosphoramidite of the SERRS label and subsequent addition as the final monomer during the synthesis of the oligonucleotide. The linker (discussed in chapter 1 section 1.2.4) containing a primary hydroxyl, was incorporated into the SERRS label to allow phosphitylation and subsequent coupling to DNA during standard automated synthesis. Figure 42 shows this methodology.

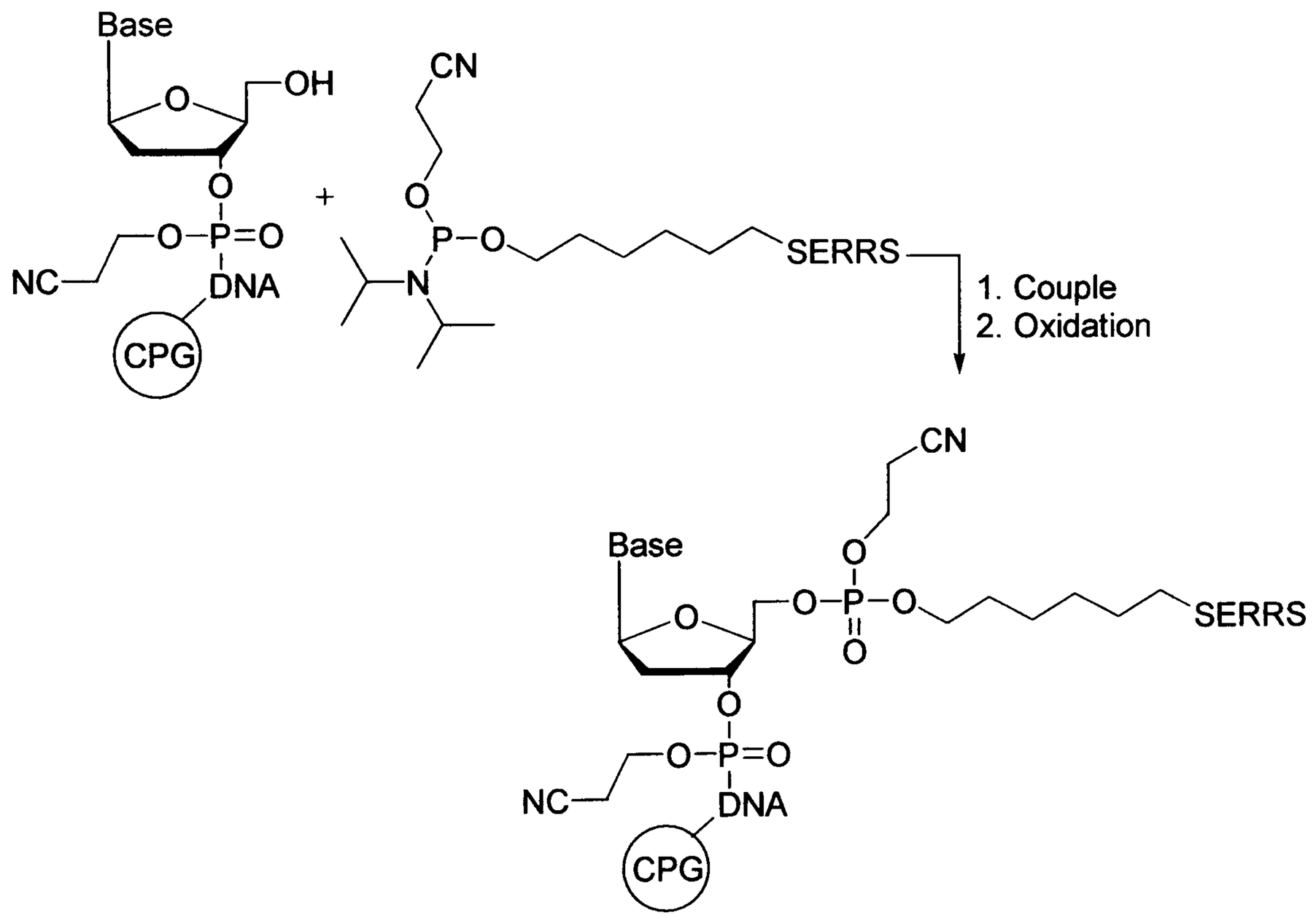


Figure 42. Introduction of the SERRS label *via* the phosphoramidite approach.

The parent chromophores of the azobenzotriazole dyes **[4]**, **[6]** and **[15]** (outlined in chapter 2) were chosen as the SERRS labels. Incorporation of these dyes requires the synthesis of a benzotriazole phosphoramidite.

Due to the poor solubility of azobenzotriazole dyes, a methodology for the synthesis of a benzotriazole phosphoramidite was optimised first using benzotriazole-5-carboxylic acid as the starting material.

3.2 Synthesis of a Benzotriazole Phosphoramidite

Starting from benzotriazole-5-carboxylic acid, the first step in the synthesis was the introduction of the primary alcohol in order to form the phosphoramidite necessary to couple during DNA synthesis. The linker, 6-aminohexan-1-ol was used again for this purpose. The primary alcohol required to be protected throughout the synthesis and this was achieved by protection as the tert-butyldiphenylsilyl ether. Standard reaction conditions were used to yield the protected linker **[18]** in 98% yield (figure 43).

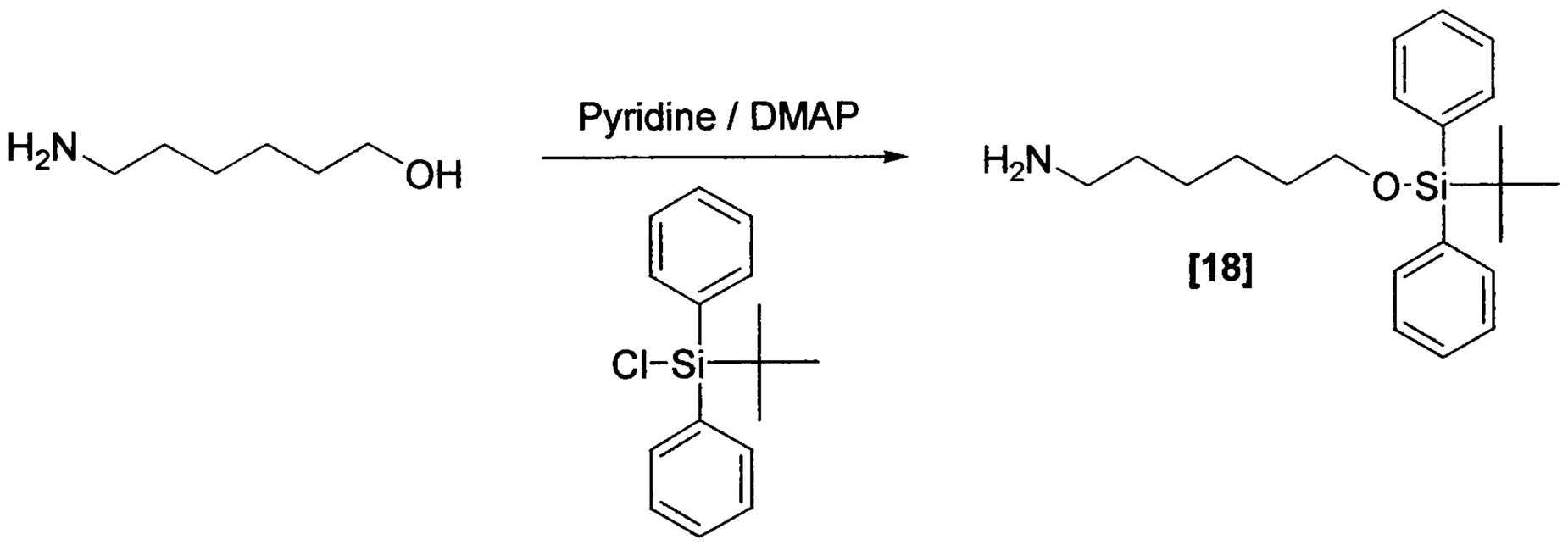


Figure 43. Protection of the linker.

Figure 44 shows the proposed reaction route for the synthesis of the benzotriazole phosphoramidite utilising the protected linker shown above.

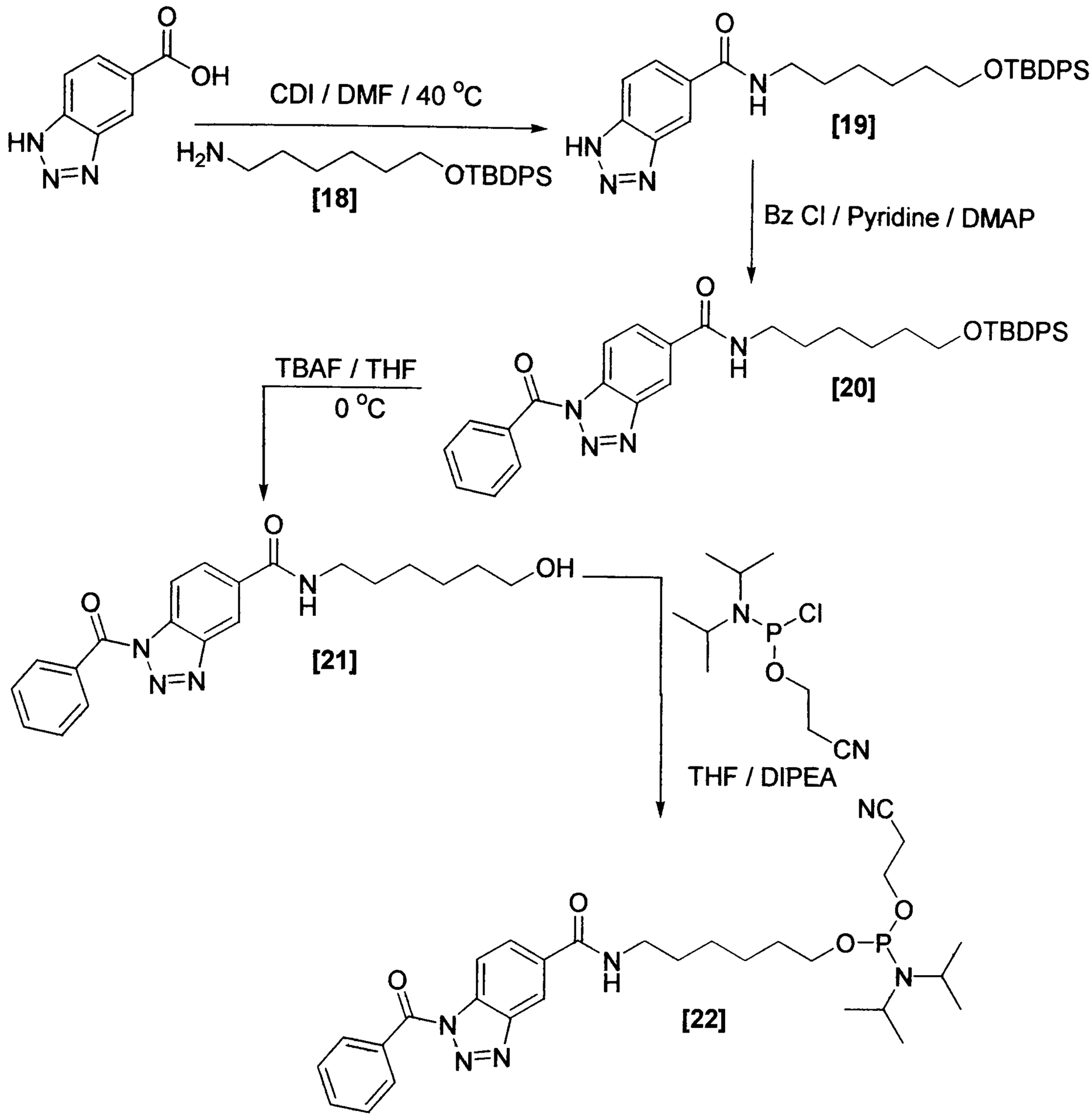


Figure 44. Synthetic route to a benzotriazole phosphoramidite.

The protected linker was then coupled to benzotriazole-5-carboxylic using the coupling agent 1,1-carbonyldiimidazole as outlined in Chapter 2, to give compound **[19]** in 83% yield.[53]

The next step in the synthesis was addition of a protecting group to the triazole ring of the benzotriazole moiety. This group must remain throughout the synthesis of the phosphoramidite and eventual DNA synthesis to prevent side reactions. The benzoyl

protecting group was introduced for this purpose since this base labile protecting group could be removed by ammonia deprotection of the final labelled oligonucleotide. The reaction was carried out in pyridine at 0 °C and following addition of benzoyl chloride, compound **[20]** was obtained in 90% yield. In order to carry out the phosphitylation of the alcohol, the TBDPS protecting group was removed. This was achieved by reaction with a 1M TBAF solution in acetonitrile at 0 °C.[55] The free alcohol **[21]** was obtained in 93% yield.

The final step of the synthesis was the phosphitylation of alcohol **[21]**, and was carried out using 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite.[52] This reaction was carried out in a dry, inert atmosphere and great care was taken during the work-up to prevent decomposition of the sensitive phosphorous (III) product. Despite this, product **[22]** was obtained in low yield (18%). The procedure was repeated several times, but the yield of the phosphoramidite remained low. This was attributed to the instability of the benzoyl-protected benzotriazole resulting in the decomposition of the desired product on further reaction.

To overcome this problem a second protecting group was employed for the protection of the triazole ring. The acid labile monomethoxytrityl (MMTr) group was chosen (outlined in chapter 2 for the protection of the amino linker), which sterically protects the triazole ring from attack. The route was continued as outlined in Figure 45.

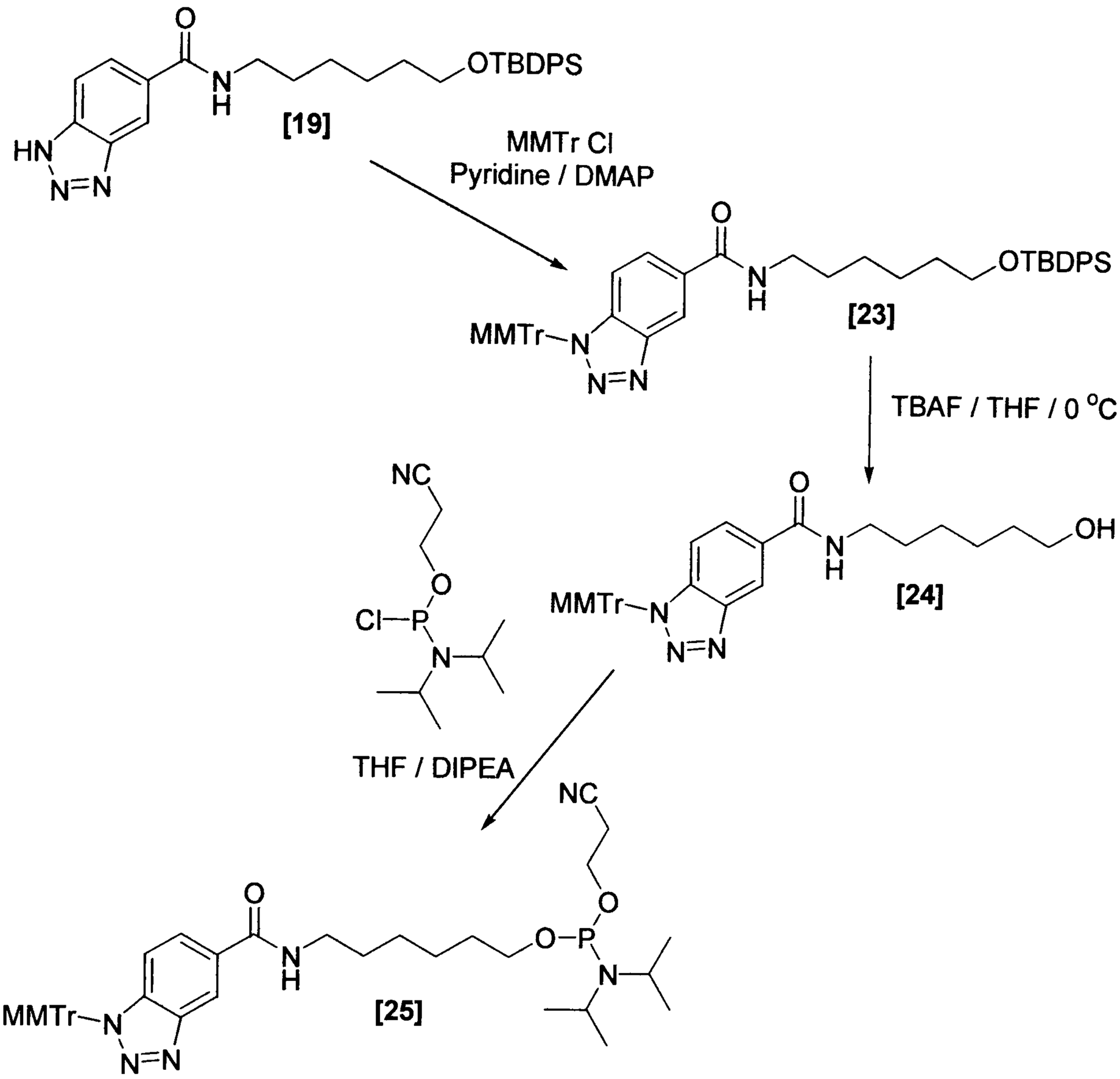


Figure 45. Synthesis of a benzotriazole phosphoramidite using MMTr protection.

The monomethoxytrityl protecting group was introduced to compound **[19]** by reaction with monomethoxytrityl chloride in pyridine, with catalytic amounts of N,N-dimethylaminopyridine. Compound **[23]** was obtained in 74% yield. Removal of the TBDPS protecting group was carried out as before using TBAF to yield alcohol **[24]** in 92%. Finally, the phosphitylation step was carried out using reaction conditions as before to yield the benzotriazole phosphoramidite **[25]** in 84% yield.

To determine if the phosphoramidite would couple to DNA, a test DNA synthesis was carried out with compound **[25]** as the final monomer during synthesis. The

synthesiser was set to remove the final MMTr protecting group from the triazole ring. Since the $MMTr^+$ by-product is yellow in colour, UV monitoring can be used to confirm the coupling of the monomer.

To optimise the purification of the benzotriazole oligo the following sequence of DNA was synthesised with the benzotriazole phosphoramidite as the final monomer. The final MMTr protecting group was retained at the end of the synthesis to allow purification by reverse phase HPLC. The following oligonucleotide was synthesised:

OLIGO 4 *5'* **[25]** ATA GGA AAC ACC *3'*

The sequence was deprotected using ammonia and purified using an Oligo R3 Perfusion Chromatography column allowing on column de-tritylation of the pure oligonucleotide. The MMTr protecting group was removed on the column by washing with 2% trifluoroaceticacid w/v. Elution of the pure oligonucleotide was accomplished by an acetonitrile wash step (figure 46). From the HPLC trace, the coupling efficiency of the benzotriazole phosphoramidite was estimated to be >80%.

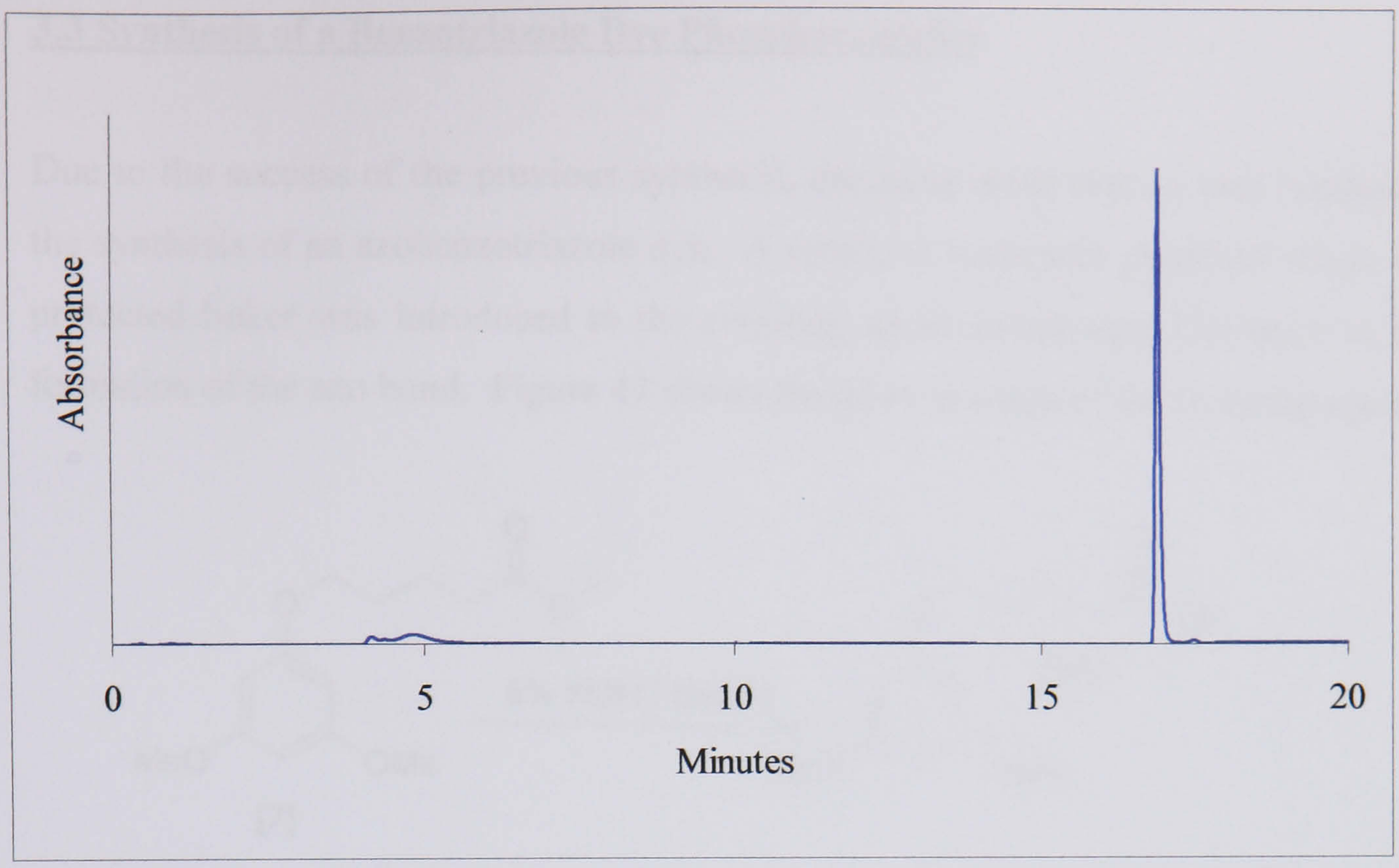


Figure 46. HPLC purification of **Oligo 4**.

Analysis of the labelled oligonucleotide by electrospray MS confirmed that addition of the benzotriazole phosphoramidite to the 5' hydroxyl has been achieved Electrospray MS found 3971.0, calculated 3970.0.[62]

3.3 Synthesis of a Benzotriazole Dye Phosphoramidite

Due to the success of the previous synthesis, the same methodology was applied to the synthesis of an azobenzotriazole dye. A synthetic route was proposed where the protected linker was introduced to the coupling agent (compound **[7]**) prior to the formation of the azo bond. Figure 47 shows the derivatisation of the coupling agent.

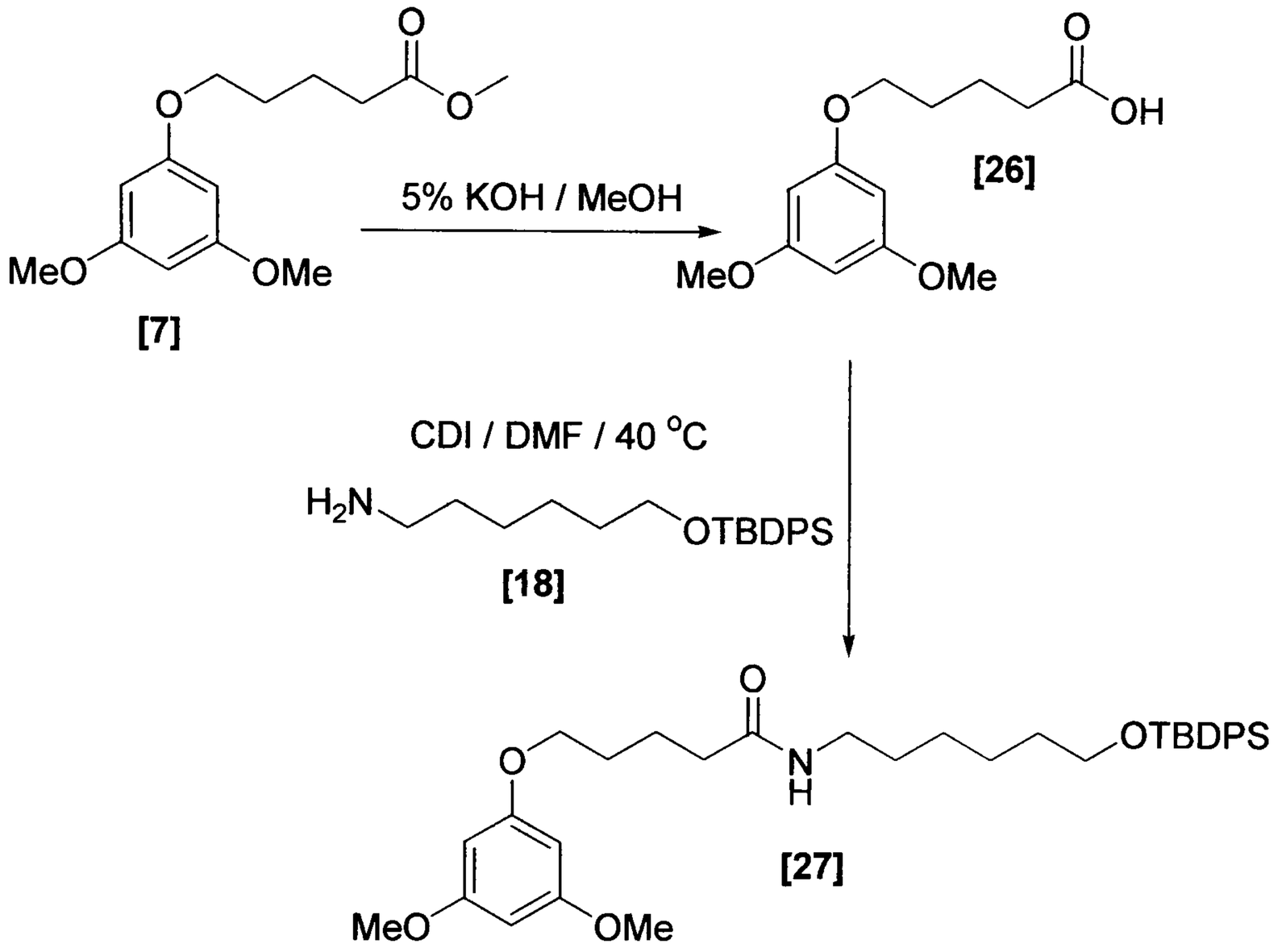


Figure 47. Incorporation of the linker into the coupling agent.

The synthesis of compound **[7]** was outlined in Chapter 2. The second step of the synthesis was cleavage of the methyl ester and was carried out by reaction with 5 % methanolic potassium hydroxide under reflux conditions for 30 minutes. 5-(3,5-Dimethoxy-phenoxy)-pentanoic acid **[26]** was obtained in 86 % yield. The free acid provided the means to couple the protected linker **[18]** (using conditions outlined in section 3.2) and produced compound **[27]** in 67 % yield.

The next stage in the route was synthesis of the azobenzotriazole dye using compound **[27]** as the coupling agent. The benzotriazole diazonium salt was prepared as outlined in the previous chapter and added dropwise to a buffered solution containing the coupling agent. The azobenzotriazole dye **[28]** was obtained in 42 % yield (figure 48).

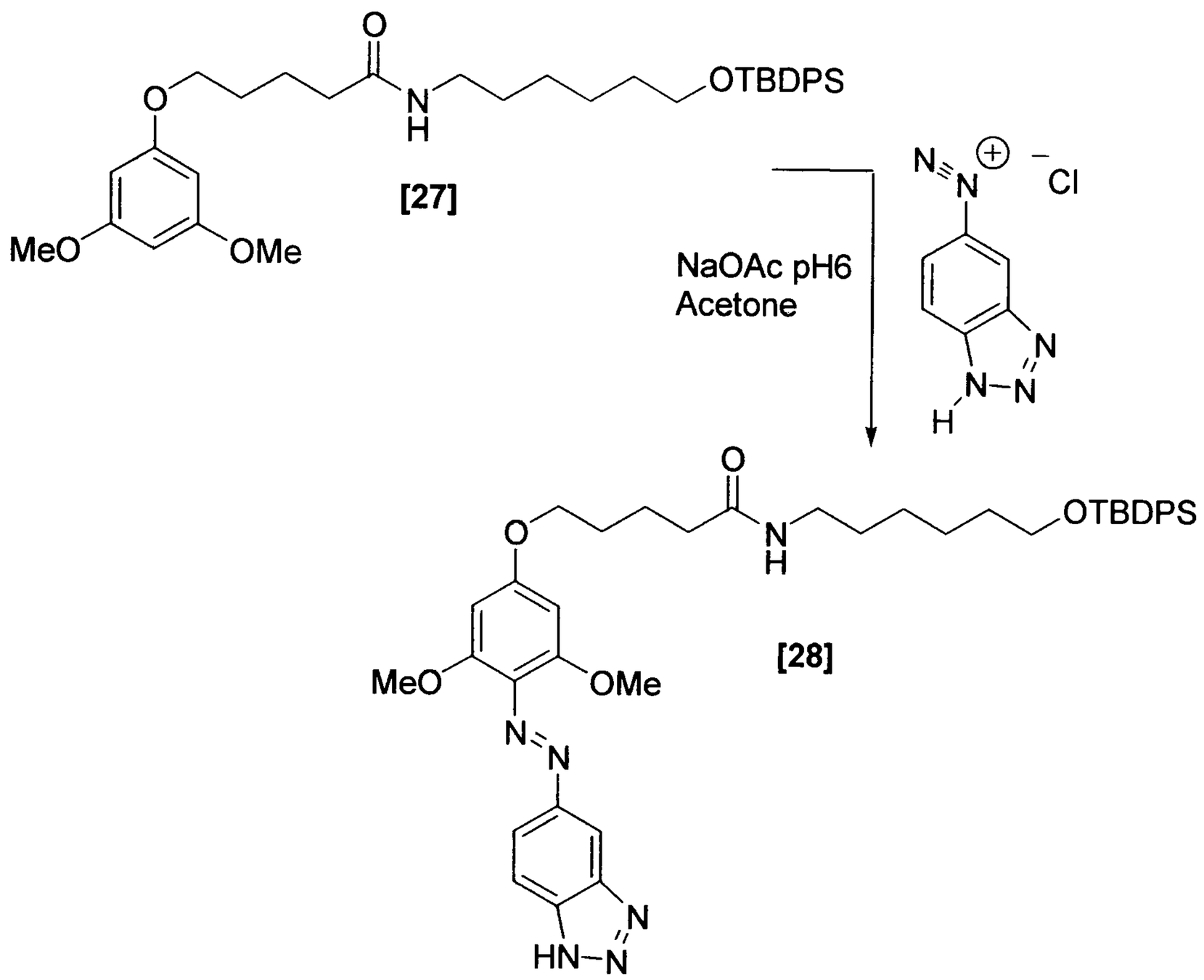


Figure 48. Formation of the azobenzotriazole dye.

The poor yield of the coupling reaction was attributed to the reduced activity of the coupling agent towards electrophilic attack. The alkylated phenol cannot ionise and therefore donate a large amount of electron density into the ring and is thus less activating.

Before the formation of the phosphoramidite could be carried out the triazole moiety was protected as before using the monomethoxy trityl group (figure 49).

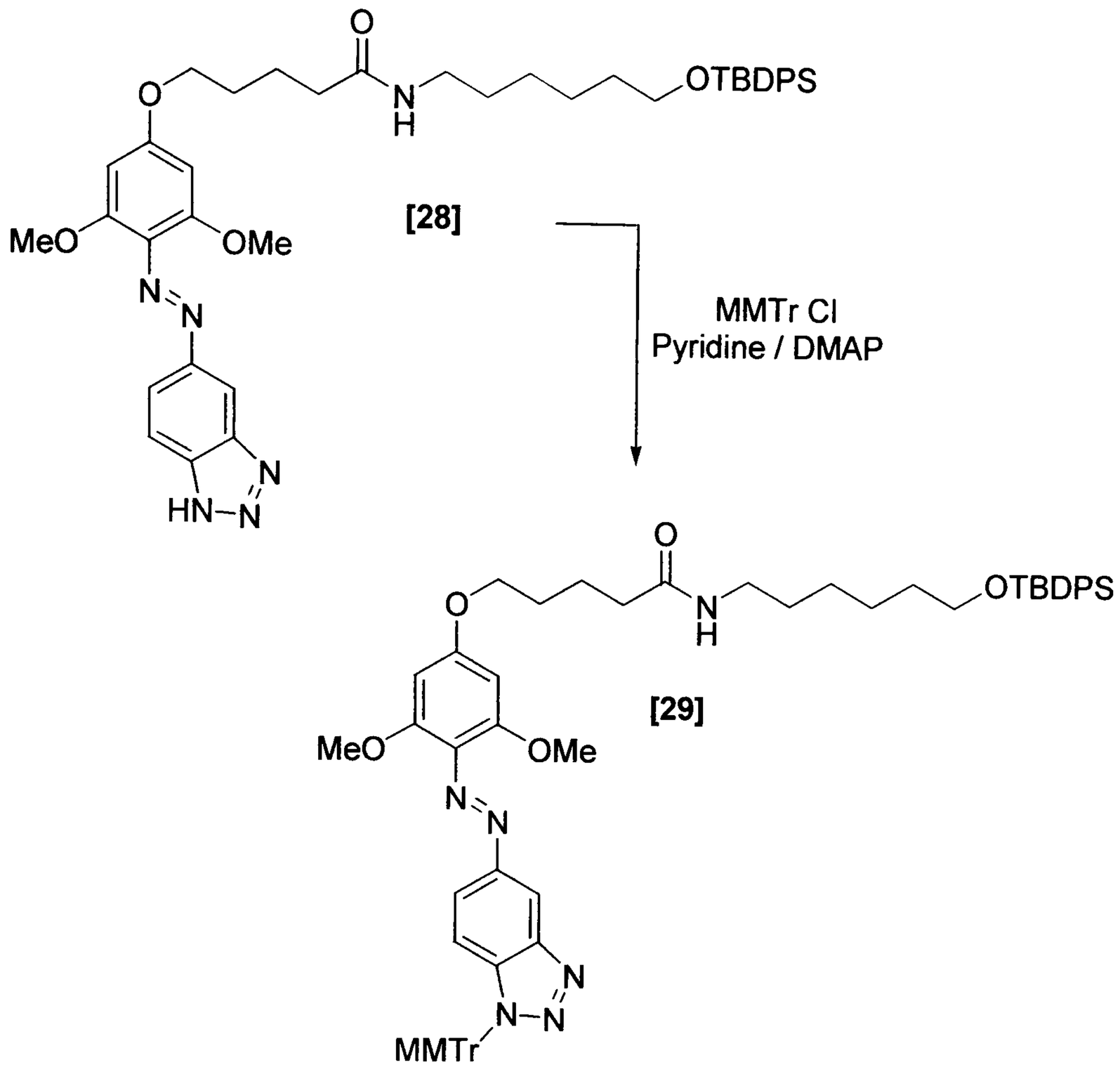


Figure 49. MMTr protection of the azobenzotriazole dye.

The protecting group was introduced by reaction of compound **[28]** with monomethoxy trityl chloride in pyridine with catalytic amounts of DMAP, however the product formed was not stable and readily decomposed on purification. To overcome this problem the less labile trityl (Tr) protecting group could be used. However the poor reaction yields observed for the formation of the azobenzotriazole dye, led to a more successful synthetic route being sought. This reaction route may be used as a general procedure for the phosphoramidite synthesis of a wider range of activated substrates.

The reaction route was revised in a similar manner to that in Chapter 2 (pages 37 to 40) for the synthesis of the trityl protected, alkylated phenol azobenzotriazole dye **[15]**, to the simpler scheme outlined in figure 50.

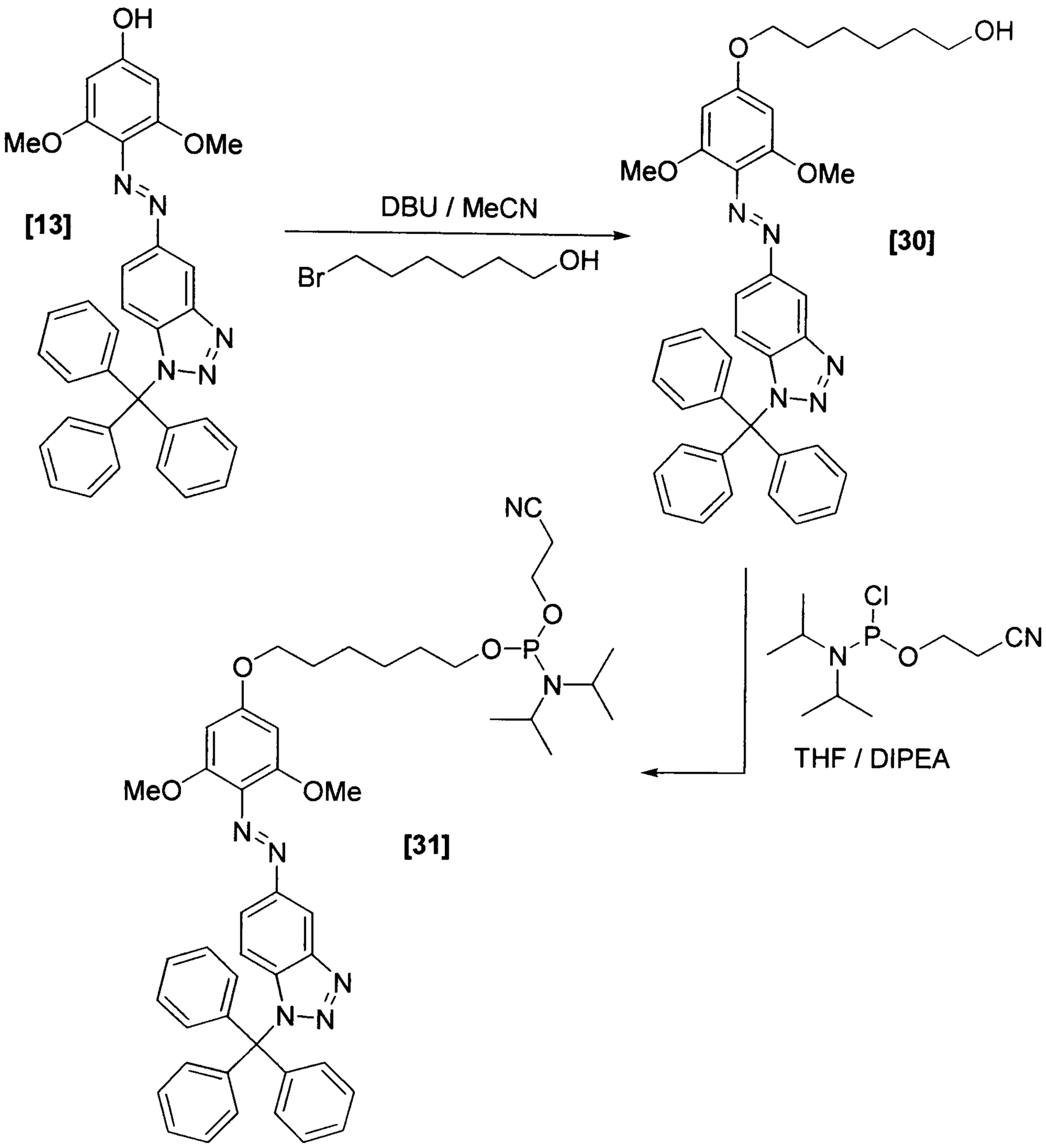


Figure 50. Revised synthetic route

The rationale for this strategy follows on from the successful synthesis of the azobenzotriazole dye, **[14],** in the previous chapter. Since derivatisation of the coupling agent forming the ether linkage deactivates the system towards

electrophillic attack, the azobenzotriazole dye was synthesised initially and the derivatisation performed subsequently.

The triazole ring of 4-(5'-azobenzotriazoyl)-3,5-dimethoxy-phenol **[10]** was selectively protected as outlined in chapter 2 to give the protected dye **[13]**. The linker containing the primary alcohol was introduced by reaction of the substrate with 6-bromohexan-1-ol in acetonitrile with the base DBU. Compound **[30]** was obtained in 73 % yield.

The final procedure was the phosphitylation of the primary alcohol of compound **[30]** to give the desired azobenzotriazole dye phosphoramidite. The phosphitylation was carried out under standard conditions to give the azobenzotriazole dye phosphoramidite **[31]** in 96% yield.

To determine if the phosphoramidite would couple to DNA, an oligonucleotide was synthesised with the azobenzotriazole dye phosphoramidite **[31]** as the final monomer. The final Tr protecting group was removed at the end of the synthesis. The following oligonucleotide was synthesised:

Oligo 5 *5'* **[31]** GAA TCA CGA ATA TCA ATT TGT AGC *3'*

The sequence was deprotected using ammonia and purified using reverse phase HPLC. From the HPLC trace, the coupling efficiency of the azobenzotriazole dye phosphoramidite was estimated to be >90%. Figure 51 shows the HPLC trace obtained for the on-column de-tritylation and purification of **Oligo 5**.

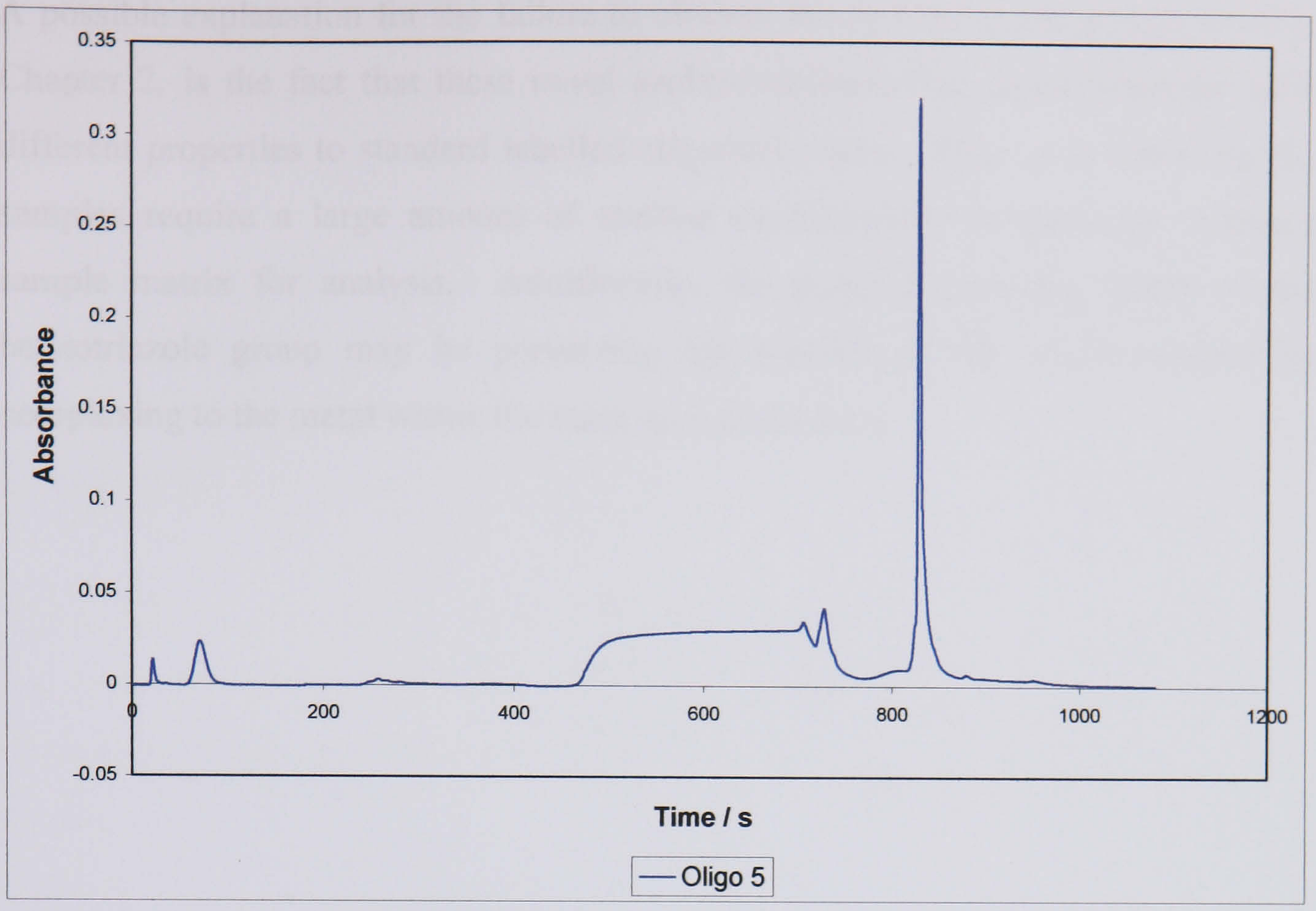


Figure 51. HPLC purification of **Oligo 5** with on-column de-tritylation.

Analysis of the labelled oligonucleotide was attempted by MALDI TOF mass spectrometry however the required molecular ion of 7820.32 was not observed and only low concentrations of low molecular weight fragments were found. If the coupling of the azobenzotriazole dye phosphoramidite had failed, the main product would be the full sequence minus the azobenzotriazole dye, with a molecular weight of 7359.91. UV spectrometry gave a characteristic peak at 428 nm and subsequent analysis by SERRS indicated the presence of the Bt dye. This provided evidence that the oligonucleotide was probably as expected due to the nature of solid phase synthesis.

A possible explanation for the failure to observe the molecular ion, as described in Chapter 2, is the fact that these novel azobenzotriazole dye oligonucleotides have different properties to standard labelled oligonucleotides. This could mean that the samples require a large amount of method development to obtain the optimum sample matrix for analysis. Additionally, the metal complexing ability of the benzotriazole group may be preventing the analysis of the oligonucleotide by complexing to the metal within the mass spec probe itself.

3.4 Development of a General Procedure for the Phosphoramidite Synthesis of Azobenzotriazole Dyes Containing a Primary Amine

An attempt was made to devise a general procedure for the derivatisation of azobenzotriazole dyes. In previous sections the derivatisation of carboxylic acids and phenols have been described, however, many coupling agents with the necessary activation towards the diazonium coupling reaction contain amines, therefore a method for the modification of amine dyes to the phosphoramidite was attempted.

The coupling agent 2-amino-4,6-dimethoxypyrimidine (outlined in chapter 2 for the synthesis of a SERRS label for addition to DNA *via* an amino linker) was used to develop a phosphoramidite of the label. Figure 52 details the proposed synthetic strategy.

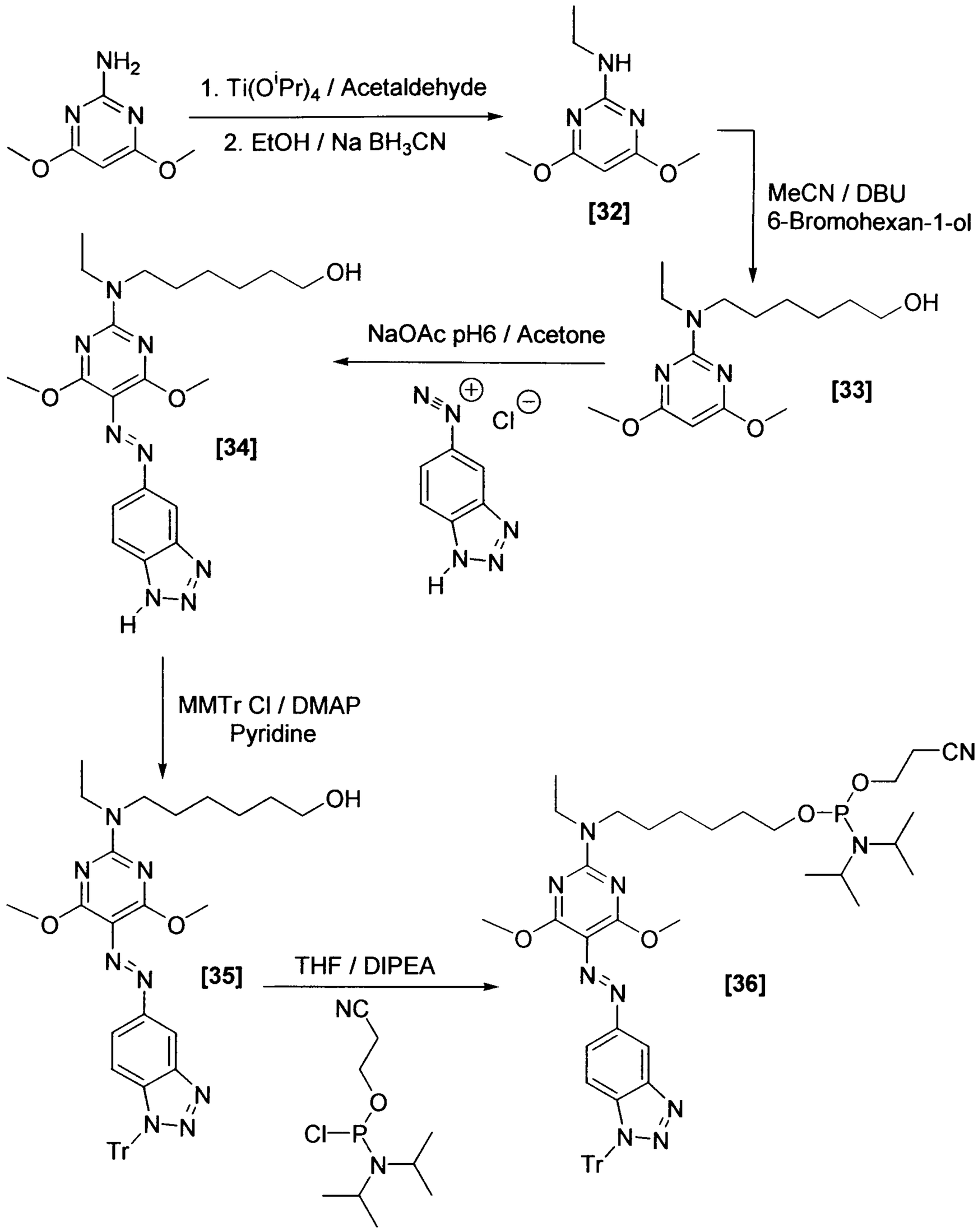


Figure 52. Derivatisation of an aromatic primary amine to give the dye phosphoramidite.

In order to derivatise the primary amine, the linker containing the primary alcohol was introduced by an N-alkylation reaction.

To ensure that only mono-alkylation occurred, the primary amine was first converted to a secondary amine by reaction with acetaldehyde by reductive amination. This method was chosen since the reaction is carried out easily under mild conditions. This first step was carried out successfully to produce compound **[32]** in 73 % yield.

The alkylation step was then attempted. This was carried out by reaction of compound **[32]** with bromohexan-1-ol and triethylamine in acetonitrile under reflux conditions for 18 hours. Unfortunately no reaction was observed. Since the first two routes outlined in this chapter were successful and could be used to provide the labelled oligos to meet the objectives of this thesis, this reaction scheme was not studied further.

3.5 Conclusion

The first methodology for the synthesis of a benzotriazole phosphoramidite was successfully developed and used to introduce the benzotriazole moiety to the 5' end of DNA. The coupling efficiency of the benzotriazole phosphoramidite was >80%.

This strategy was extended to the synthesis of an azobenzotriazole dye phosphoramidite with careful selection of the protecting group strategy. The coupling efficiency of the azobenzotriazole dye phosphoramidite was also >80%. This result constitutes the first labelling of DNA with a SERRS active label *via* the phosphoramidite approach and enabled the SERRS detection of DNA with these specifically designed labels.

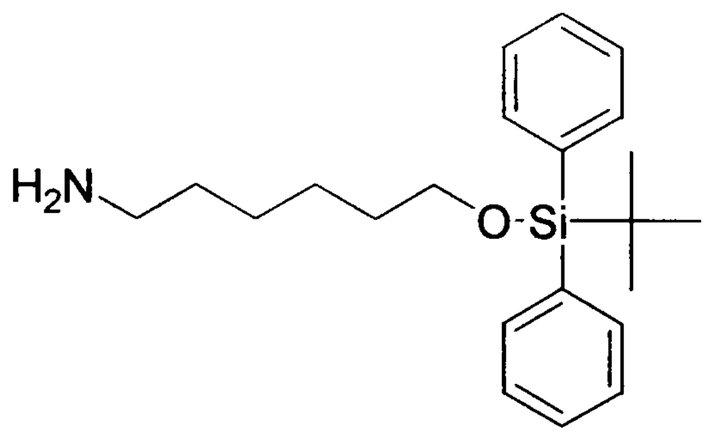
3.6 Experimental

3.6.1 General

As described in Chapter 2 section 2.6.1.

3.6.2 Chemical Synthesis

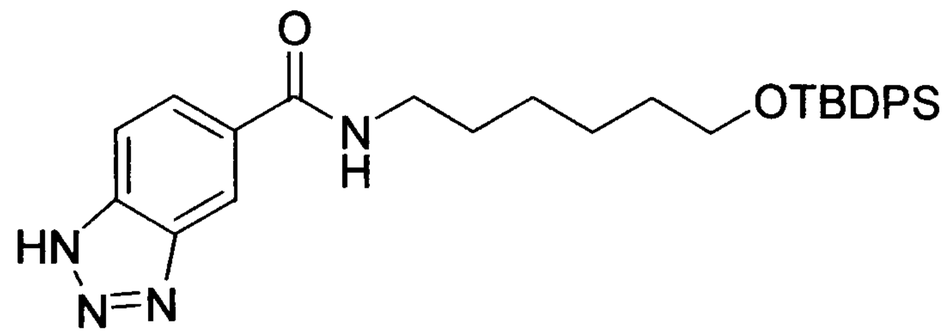
1-(^{t}Butyldiphenylsilylether)-6-aminohexane [18]



6-Aminohexan-1-ol (8.000 g, 0.068 mol) was co-evaporated with anhydrous pyridine (3 x 15 ml) then dissolved in anhydrous pyridine (15 ml). To the stirring solution was added dimethylaminopyridine (1.500 g, 0.01 mol, 1.5 eq.) followed by ^{t}butyl chlorodiphenyl silane (28.150 g, 0.10 mol, 26.63 ml, 1.5 eq.). The reaction was allowed to proceed for 48 hours, then quenched with methanol (20 ml) and the solvents removed *in vacuo*. The residue was redissolved in ethyl acetate and extracted with a saturated KCl solution (2 x 20 ml), the organic extract was dried over anhydrous sodium sulfate and the solvents removed *in vacuo* to yield a crude yellow oil. The residue was purified by wet flash column chromatography, eluting methanol in DCM (0 – 5%). Silica was pre-equilibrated with 1% triethylamine. The product was collected and the solvent removed to yield a yellow oil (21.730 g, 98%). R_f (E) 0.21, ^{1}H NMR, ($CDCl_3$), δ_H 1.07 (9H, s, ^{t}Bu); 1.41 (4H, m, C*H*$_2$); 1.58 (2H, t, *J* 6.8, C*H*$_2$); 1.69 (2H, t, *J* 7.0, C*H*$_2$); 2.89 (2H, t, *J* 7.2, O-C*H*$_2$); 3.67 (2H, t, *J* 6.4, N-C*H*$_2$); 6.24 (2H, brs, N*H*$_2$); 7.36 (6H, m, Ar*H*); 7.72 (4H, m, Ar*H*). ^{13}C NMR, ($CDCl_3$), δ_C 19.37 (*C*H$_2$); 25.50 (*C*H$_2$); 26.59 (*C*H$_2$); 27.04 (*C*H$_3$); 32.52 (*C*H$_2$);

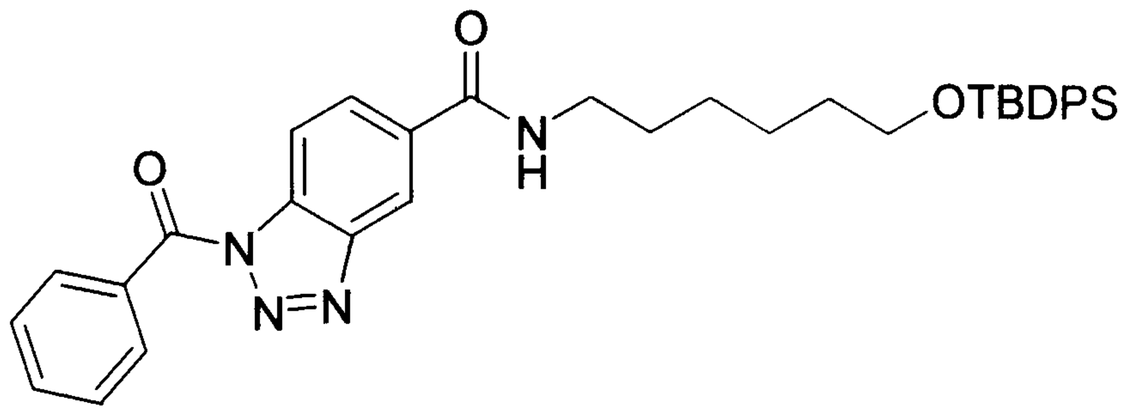
40.61 (*C*); 63.86 (*C*H_2); 127.78 (*C*H); 134.19 (*C*); 135.72 (*C*H). M/z (FAB) 356.2379 [$C_{22}H_{34}NOSi$ $(M+H)^+$ < 8.5 ppm].

1-(^{t}Butyldiphenylsilylether)-6-(5'-amido benzotriazoyl)hexane [19]



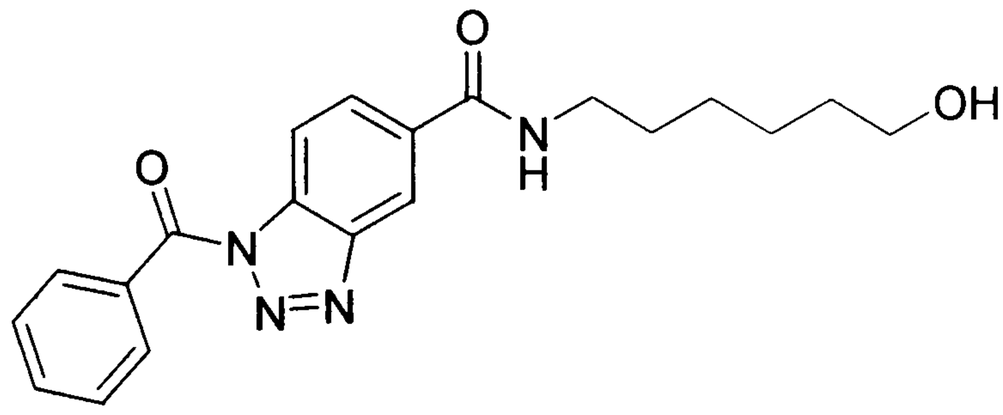
Benzotriazole-5-carboxylic acid (3.147 g, 0.019 moles) was dissolved in anhydrous DMF (10 ml) in a round bottom flask fitted with a condenser and a drying tube. 1,1-Carbonyldiimidazole (1.56 equivalents) was added to the stirring solution. The resulting mixture was heated to 40 ^{0}C for five minutes, until gas evolution had ceased. The reaction mixture was allowed to cool to room temperature, when the amine, compound **[18]** (21.813 g, 0.06 moles, 3 eq.), was added and stirring continued for a further fifteen minutes. After this time, the reaction was quenched with water (7 ml). The solvent was removed under reduced pressure and to the residue, sodium carbonate (50 ml of a 1 M aqueous solution) was added. The mixture was extracted with ethyl acetate (2 x 80 ml), and the combined extracts washed with water (80 ml), brine (80 ml) then dried over sodium sulfate. The crude material was purified by wet flash column chromatography, eluting methanol in DCM (0 – 5%). Silica was pre- equilibrated with 1% triethylamine. Product **[19]** was obtained as a tan foam (8.319 g, 0.02 moles, 86.1% yield). R_f (C) 0.41, C, H, N; found C, 69.37; H, 7.38; N, 10.92, $C_{29}H_{34}N_4O_2Si$ requires C, 69.56; H, 7.25; N, 11.19. ^{1}H NMR ($CDCl_3$) δ_H 1.19 (9H, s, ^{t}Bu); 1.41 (4H, m, CH_2); 1.53 (2H, t, *J* 6.9, CH_2); 1.65 (2H, t, *J* 6.9, CH_2); 3.5 (2H, t, *J* 6.4, O-CH_2); 3.66 (2H, t, *J* 6.5, N-CH_2); 7.39 (6H, m, Ar*H*); 7.66 (4H, m, Ar*H*) 7.79 (1H, d, *J* 8.6, Ar*H*) 7.86 (1H, d, *J* 8.6, Ar*H*); 8.37 (1H, s, Ar*H*). Found: C, 69.37; H, 7.38; N, 10.92. M/z (FAB) 501.26945 [$C_{29}H_{37}N_4O_2Si$ $(M+H)^+$ < 1.7 ppm].

1-(^{t}Butyldiphenylsilylether)-6-[1'-benzoyl-5'-amido-benzotriazoyl)hexane [20]



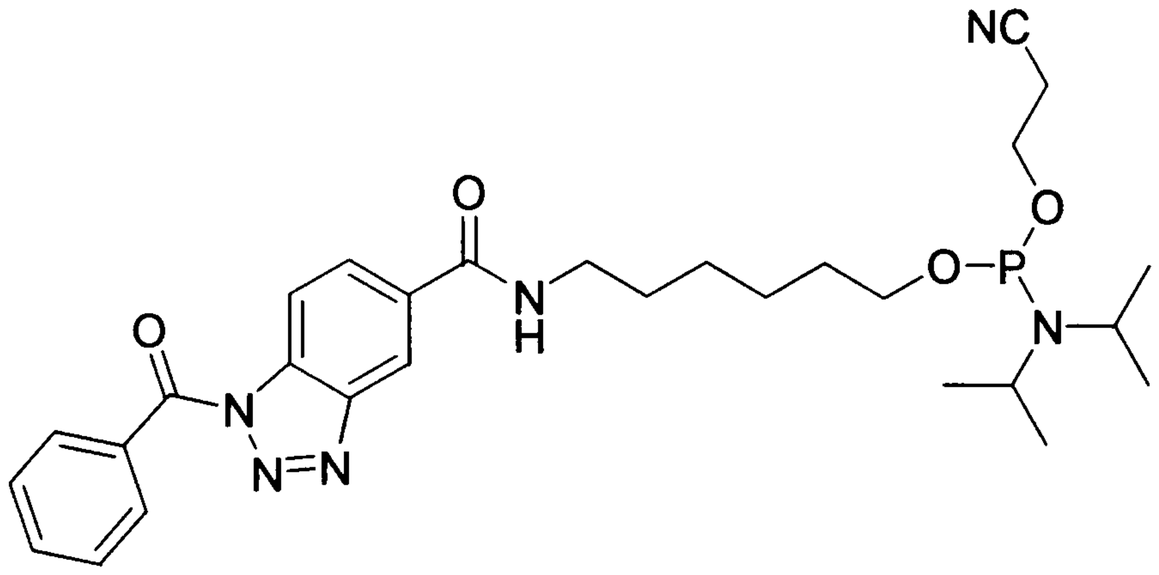
Compound **[19]** (5.008 g, 0.01 moles) was co-evaporated with anhydrous pyridine (3 x 10 ml) and then dissolved in anhydrous pyridine (10 ml). The mixture was cooled to 0 °C and benzoyl chloride (7.030 g, 0.05 mol, 5.80 ml, 5 eq.) was added with stirring. The reaction was allowed to come to room temperature, whilst stirring, for a period of two hours. The reaction was quenched with methanol (20 ml) and the solvent removed *in vacuo*. The residue was re-dissolved in ethyl acetate and extracted with saturated potassium chloride (3 x 20 ml), the combined organic extracts were dried over anhydrous sodium sulfate and the solvent removed *in vacuo*. The crude material was purified by wet flash column chromatography, eluting methanol in DCM (0 – 2%), using silica pre-equilibrated with 1% triethylamine. The product was obtained as a white solid (5.452 g, 9.015 mmol, 90.2% yield). R_f (D) 0.57, C, H, N; found: C, 71.07; H, 6.65; N, 9.33. $C_{36}H_{40}N_4O_3Si$ requires C, 71.49; H, 6.67; N, 9.26. ^{1}H NMR (d_6 acetone) δ_H 1.05 (9H, s, ^{t}Bu); 1.46 (4H, m, C*H*$_2$); 1.62 (2H, t, *J* 7.0, C*H*$_2$); 1.83 (2H, t, *J* 7.2, C*H*$_2$); 3.71 (2H, t, *J* 6.3, N-C*H*$_2$); 4.04 (2H, t, *J* 7.5, O-C*H*$_2$); 7.18 (2H, d, *J* 7.6, Ar*H*) 7.23 (1H, d, *J* 7.1, Ar*H*); 7.44 (6H, m, Ar*H*); 7.48 (2H, d, *J* 8.4, Ar*H*); 7.57 (1H, d, *J* 8.6, Ar*H*); 7.69 (4H, m, Ar*H*); 7.73 (1H, d, *J* 8.6, Ar*H*); 8.06 (1H, s, Ar*H*). M/z (FAB) 548.2209 [$C_{36}H_{40}N_4O_3Si$ $(M+H)^+$ < 6.3 ppm].

6-(1'-Benzoyl-5'- amidobenzotriazoyl)hexan-1-ol [21]



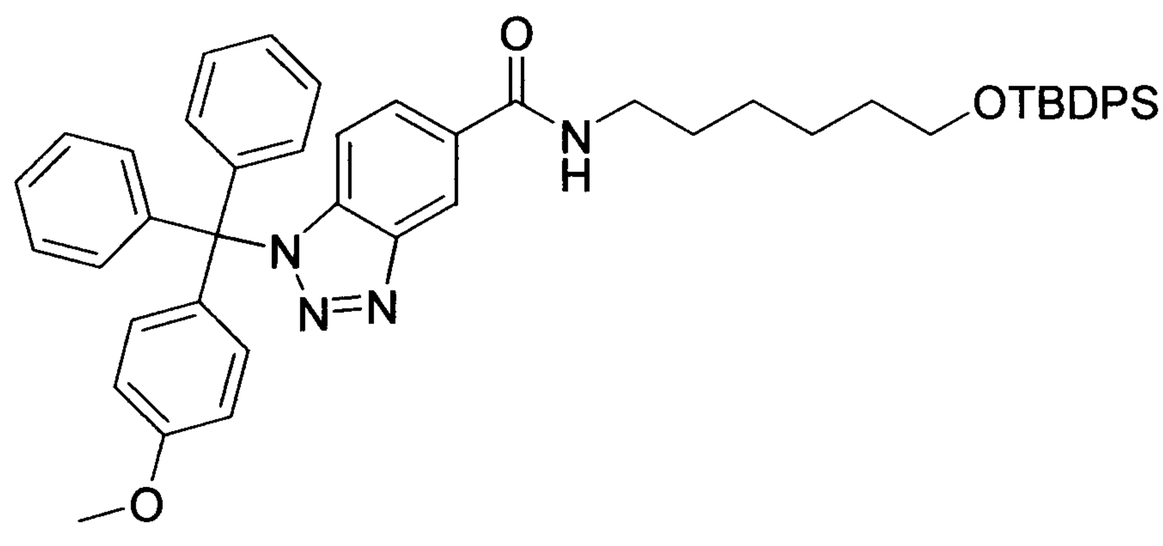
Compound **[20]** (1.558 g, 2.58 mmol) was dissolved in anhydrous THF (10 ml). The solution was cooled to 0 °C and tetrabutyl ammonium fluoride added (5.15 ml of a 1M solution in THF, 5.15 mmol, 2 eq.) dropwise. The reaction was allowed to warm to room temperature overnight. The solvent was removed and the residue dissolved in dichloromethane, excess H^+ Dowex ion exchange resin was added and the mixture allowed to stir for 30 minutes. After this time the solution was filtered and the solvent removed. The residue was purified by wet flash column chromatography, eluting methanol in DCM (0 – 5%). Silica was pre-equilibrated with 1% triethylamine. The product was collected and the solvent removed to yield a yellow oil (0.775 g, 2.12 mmol, 82% yield). R_f (D) 0.34, ^{1}H NMR (d_6 acetone) δ_H 1.43 (4H, m, CH_2); 1.52 (2H, t, *J* 6.7, CH_2); 1.85 (2H, t, *J* 7.4, CH_2); 3.53 (2H, t, *J* 6.3, N-CH_2); 4.04 (2H, t, *J* 7.5, O-CH_2); 7.21 (3H, m, Ar*H*); 7.48 (2H, m, Ar*H*); 7.58 (1H, d, *J* 8.6, Ar*H*); 7.74 (1H, d, *J* 8.6, Ar*H*) 8.06 (1H, s, Ar*H*). M/z (EI) 366.1710 [$C_{20}H_{22}N_4O_2$ (M^+) < 1.3 ppm].

1-(2-Cyanoethyl-N,N-diisopropylphosphoramidite)-6-(1'benzoyl-5'-amido benzotriazoyl)hexane [22]



The phosphitylation of alcohol **[21]** (100 mg, 0.27 mmol) was carried out using general procedure 2. Phosphoramidite **[22]** was obtained as a yellow oil (27.8 mg, 0.01 mmol, 18.0% yield). R_f (B) 0.77, ^{31}P NMR, ($CDCl_3$), referenced to phosphoric acid, δ_P 148.77 (s).

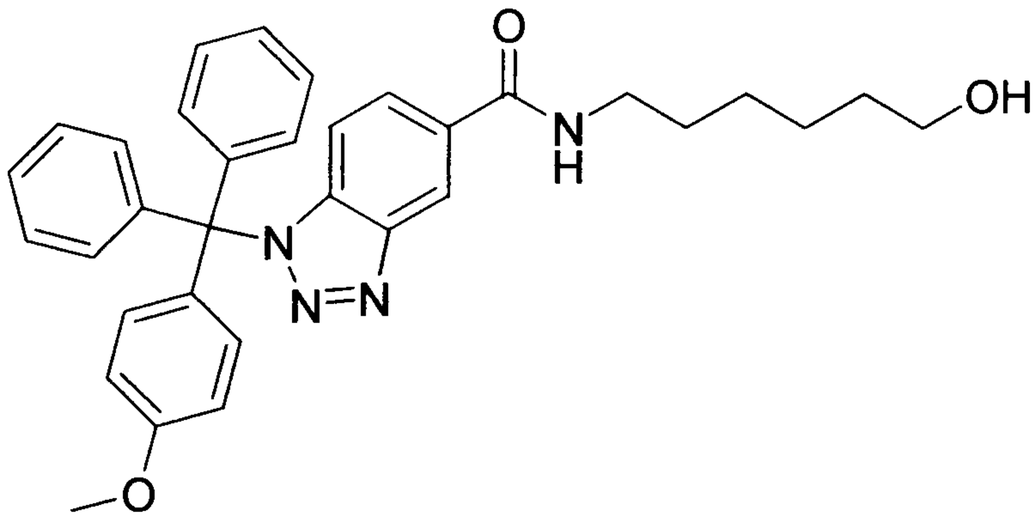
1-(^{t}Butyldiphenylsilylether)-6-[1'-(monomethoxy trityl-5'-amido-benzotriazoyl)hexane [23]



1-(^{t}Butyldiphenylsilylether)-6-(5'-amido benzotriazoyl) hexane **[19]** (1.016 g, 3.03 mmol) was co-evaporated with anhydrous pyridine (3 x 10 ml) and then dissolved in anhydrous pyridine (7 ml) with anhydrous triethylamine (0.2 ml) and DMAP (0.047 g, 0.41 mmol, 0.2 eq.). (4-Methoxyphenyl)-diphenylmethyl chloride (0.752 g, 2.43 mmol, 1.2 eq.) was dissolved in anhydrous pyridine and added in three portions at forty five minute intervals to the stirring amine solution and left for thirty minutes.

The reaction was quenched with methanol (3 ml) and the volume reduced *in vacuo*. The residue was re-dissolved in ethyl acetate and extracted with sodium carbonate (20 ml of a saturated solution), washed with water (20 ml) and then with saturated potassium chloride (20 ml). The organic extract was dried over anhydrous sodium sulfate and the solvent removed *in vacuo*. The residue was purified by wet flash column chromatography, eluting hexane / ethyl acetate in a 1:1 ratio. Silica was pre-equilibrated with 1% triethylamine. The product was collected and the solvent removed to yield a yellow oil (0.952 g, yield 57.3%). R_f (E) 0.48, C, H, N; found: C, 75.95; H, 6.83; N, 6.56. $C_{49}H_{53}N_4O_3Si$ requires C, 76.13; H, 6.78; N, 7.25. ^{1}H NMR ($CDCl_3$) δ_H 1.05 (9H, s, ^{t}Bu); 1.28 (4H, m, CH_2); 1.39 (4H, m, CH_2); 3.46 (2H, t, *J* 6.6, N-CH_2); 3.67 (2H, t, *J* 6.9, O-CH_2); 3.81 (3H, s, O-CH_3); 6.76 (2H, m, Ar*H*); 7.14 (6H, m, Ar*H*); 7.32 (6H, m, Ar*H*); 7.40 (6H, m, Ar*H*); 7.69 (5H, m, Ar*H*); 8.07 (1H, d, *J* 8.6, Ar*H*,); 8.59 (1H, s, Ar*H*). M/z (FAB) 773.3828 [$C_{49}H_{53}N_4O_3Si$ $(M+H)^+$ < 7.7 ppm].

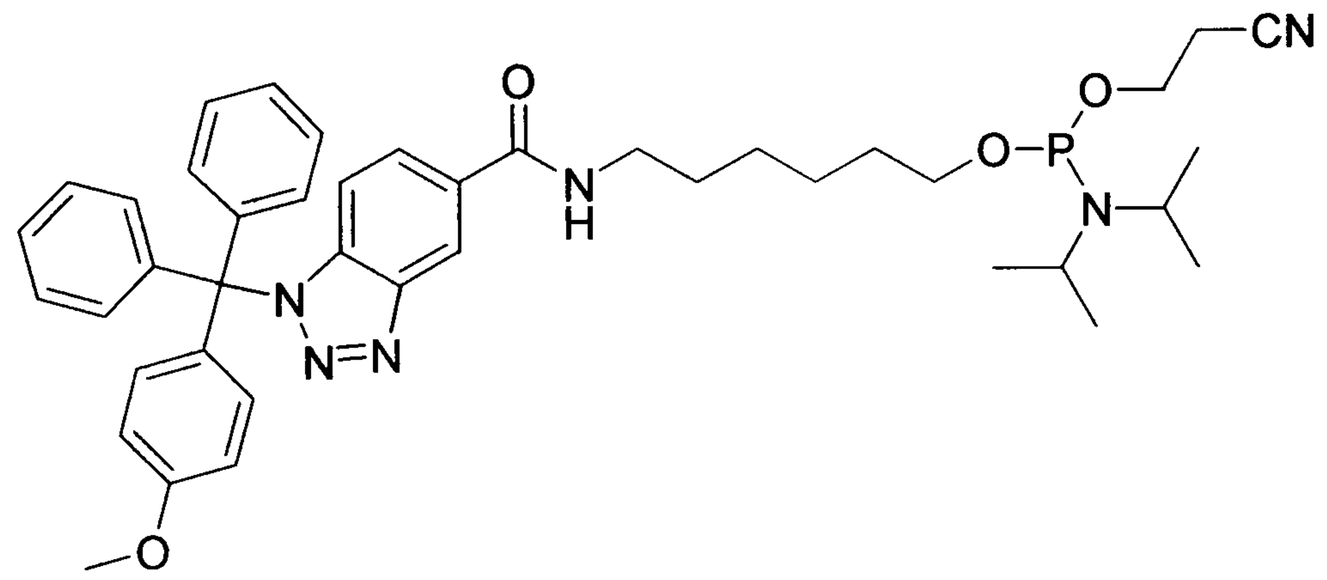
6-[1'-(Monomethoxy trityl)-5'-amido-benzotriazoyl)hexan-1-ol [24]



Compound **[23]** (0.500 g, 0.65 mmol) was dissolved in anhydrous THF (10 ml). The solution was cooled to 0 °C and tetrabutyl ammonium fluoride added (1.29 ml of a 1M solution in THF, 1.29 mmol, 2 eq.) dropwise. The reaction was allowed to warm to room temperature overnight. The solvent was removed and the residue purified by wet flash column chromatography, eluting dichloromethane / methanol with a solvent gradient starting from 2% methanol. The product was isolated and dissolved in dichloromethane, excess $^+$H Dowex ion exchange resin was added the mixture allowed to stir for 30 minutes. After this time the solution was filtered and the

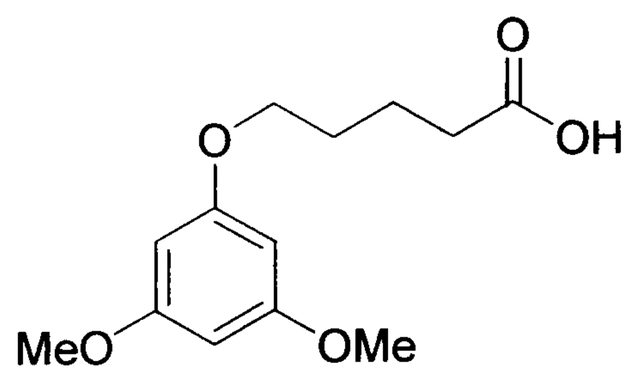
residue purified by wet flash column chromatography, eluting hexane / ethyl acetate in a 1:1 ratio. Silica was pre-equilibrated with 1% triethylamine. The solvent was removed to yield a yellow foam (0.3191 g, 0.60 mmol, 92% yield). R_f (D) 0.43, ^1H NMR ($CDCl_3$) δ_H 1.36 (4H, m, C*H*$_2$); 1.55 (4H, m, C*H*$_2$); 3.34 (2H, t, *J* 6.8, N-C*H*$_2$); 3.47 (2H, t, *J* 6.8, O-C*H*$_2$); 3.80 (3H, s, O-C*H*$_3$); 6.72 (2H, m, Ar*H*); 7.11 (8H, m, Ar*H*); 7.30 (4H, m, Ar*H*); 7.66 (2H, d, *J* 8.6, Ar*H*); 8.06 (1H, d, *J* 8.9, Ar*H*); 8.54 (1H, s, Ar*H*). M/z (EI) 534.2631 [$C_{33}H_{34}N_4O_3$ (M^+) < 0.0 ppm].

1-(2-Cyanoethyl-N,N-diisopropylphosphoramidite)-6-[1-monomethoxy trityl-5'-amido benzotriazoyl)hexane [25]



Compound **[24]** (180 mg, 0.34 mmol) was carried out using general procedure 2. The product was obtained as a yellow oil (202.0 mg, 0.28 mmol, 82.9% yield). R_f (B) 0.68, ^{31}P NMR, ($CDCl_3$), referenced to phosphoric acid, δ_P 148.78 (s).

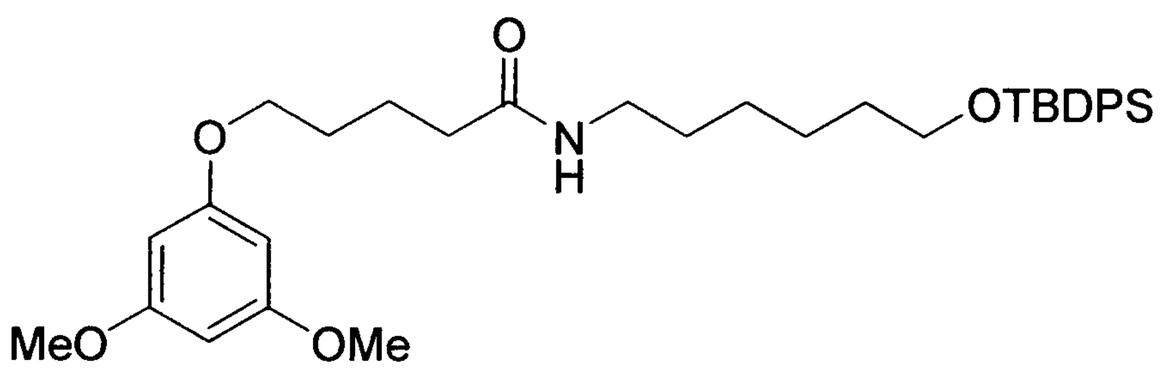
5-(3,5-Dimethoxy-phenoxy)-pentanoic acid [26]



Compound **[7]** (0.257 g, 0.96 mmol) was added to 25ml of a 10% w/v solution of methanolic potassium hydroxide. The solution was refluxed for 1 hour, after which

time the solvent was removed *in vacuo*. The residue was re-dissolved in water and neutralised with 20ml of a 10% w/v citric acid solution, then extracted with ethyl acetate. The combined organic extracts were dried over anhydrous sodium sulfate and the solvent removed by evaporation to yield compound **[26]**, (0.211 g, 0.83 mmol, 86.4%). R_f (E) 0.81, C, H, N; found: C, 61.26; H, 7.42; $C_{13}H_{18}O_5$ requires C, 61.41; H, 7.13. ^{1}H NMR (d6-DMSO) δ_H 1.66 (4H, m, C*H*$_2$); 2.26 (2H, t, *J* 6.8, C*H*$_2$); 3.70 (6H, s, O-C*H*$_3$); 3.91 (2H, t, *J* 6.0, O-C*H*$_2$); 6.08 (3H, s, Ar*H*). ^{13}C NMR (d6, DMSO) δ_C 21.69 (*C*H$_2$); 28.51 (*C*H$_2$); 33.75 (*C*H$_2$); 45.89 (*C*H$_2$); 55.51 (*C*H$_3$); (*C*H$_2$); 67.54 (C); 93.25 (*C*H); 93.62 (*C*H); 160.92 (*C*); 161.53 (*C*OOH). M/z (EI) 254.1158 [$C_{13}H_{18}O_5$ (M^+) < 1.3 ppm].

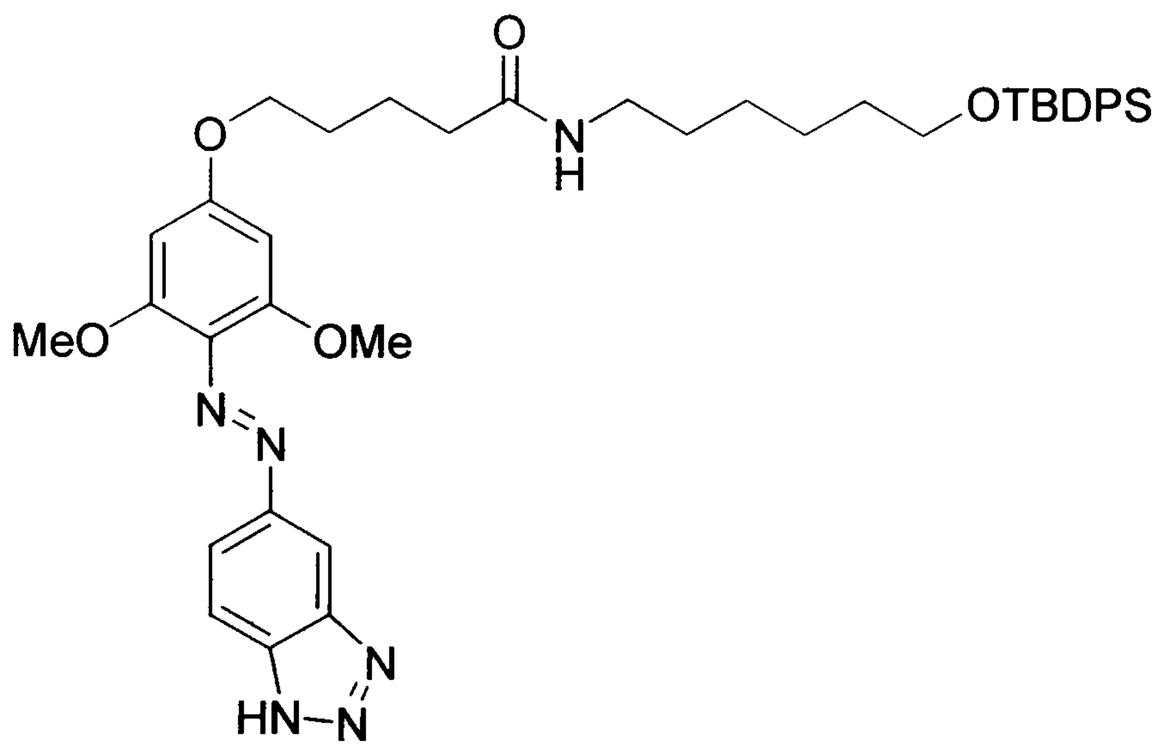
5-(3,5-Dimethoxy-phenoxy)-pentanoic acid [6-(tert-butyl-diphenyl-silanyloxy)-hexyl]-amide [27]



Carboxylic acid **[26]** (0.200 g, 0.79 mmol) was dissolved in anhydrous DMF (10 ml) in a round bottom flask fitted with a condenser and a drying tube. 1,1-carbonyldiimidazole (1.56 equivalents) was added to the stirring solution. The resulting mixture was heated to 40 ^{0}C for five minutes, until gas evolution had ceased. The reaction mixture was allowed to cool to room temperature, when the amine, compound **[18]** (2.360 g, 0.84 mmol, 3 eq.), was added and stirring continued for a further fifteen minutes. After this time, the reaction was quenched with water (7 ml) and the solvent removed under reduced pressure. Sodium carbonate (50 ml of a 1 M aqueous solution) was added to the residue and the mixture extracted with ethyl acetate (2 x 80 ml). The combined organic extracts were washed with water (80 ml), brine (80 ml) then dried over anhydrous sodium sulfate. The crude material was purified by wet flash column chromatography, eluting methanol in DCM (0 –

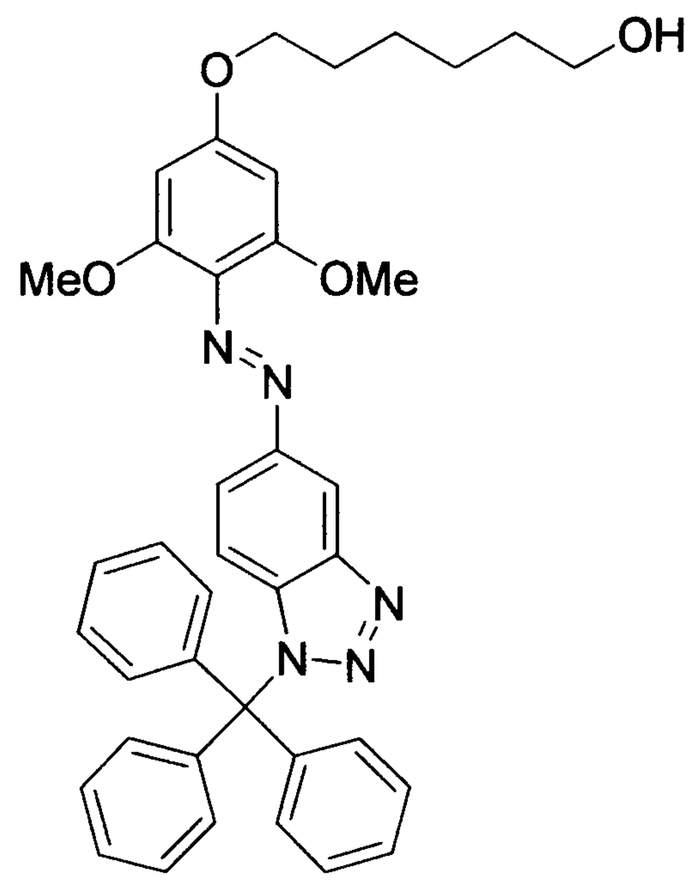
5%). Silica was pre- equilibrated with 1% triethylamine. Product **[27]** was obtained as a yellow oil (0.310 g, 0.52 mmol, 67% yield). R_f (A) 0.33, ^{1}H NMR ($CDCl_3$) δ_H 0.99 (9H, s, ^{t}Bu); 1.32 (2H, m, C*H*$_2$); 1.34 (4H, m, C*H*$_2$); 1.51 (2H, m, C*H*$_2$); 3.00 (4H, t, J = 6.2 Hz, C*H*$_2$); 3.63 (2H, t, J = 6.4 Hz, C*H*$_2$); 3.69 (6H, s, O-C*H*$_3$); 3.90 (2H, t, J = 6.4 Hz, C*H*$_2$); 6.07 (3H, s, Ar*H*); 7.44 (6H, m, Ar*H*); 7.60 (4H, m, Ar*H*). M/z (FAB) 592.3464 [$C_{35}H_{50}NO_5Si$ $(M+H)^+$ < 1.0 ppm].

5-[4-(5'Azobenzotriazoyl)-3,5-dimethoxy-phenoxy]-pentanoic acid [6-(tert-butyl-diphenyl-silanyloxy)-hexyl]-amide [28]



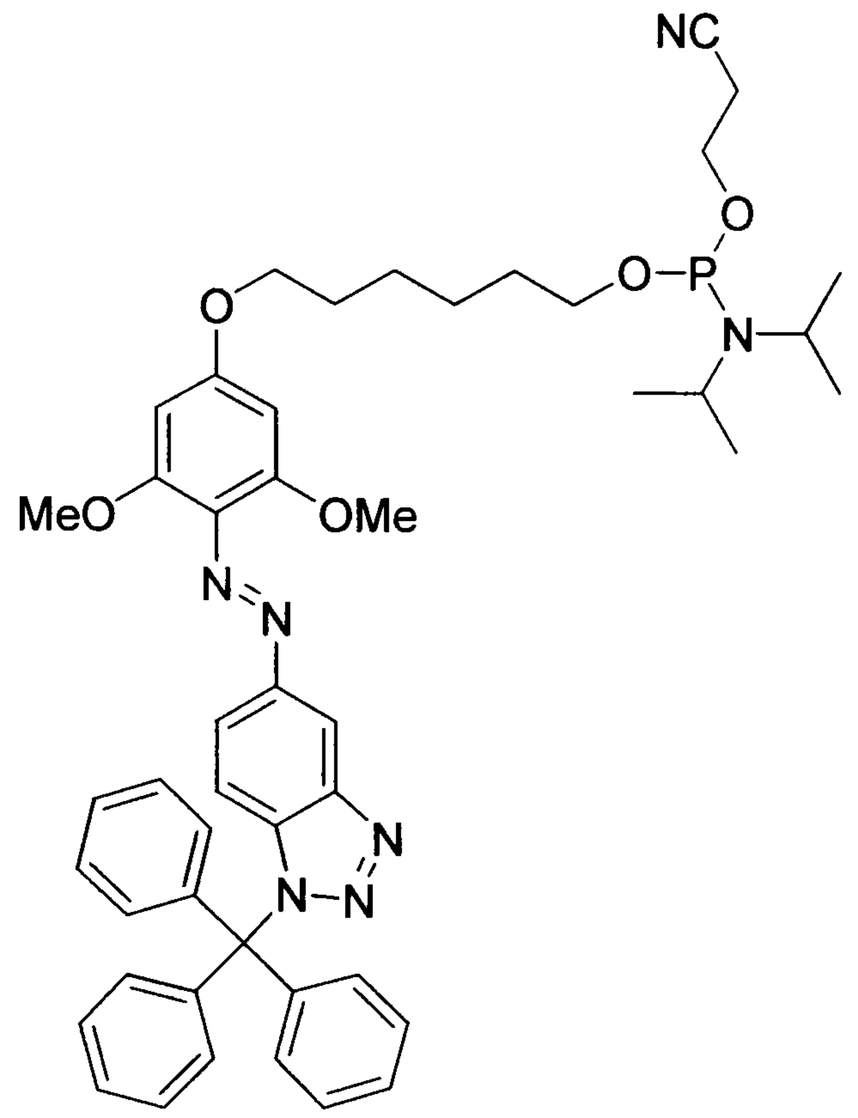
5-Aminobenzotriazole (0.159 g, 1.19 mmol, 1.1 eq.) was diazotised using standard procedure 2 (outlined in chapter 1). The diazonium solution was then added dropwise to compound **[27]** (0.640 g, 1.08 mmol, 1.0 eq.) dissolved in sodium bicarbonate buffer (1.0 M, pH 8.0, 30 ml) and acetone (10 ml) and left to stir for 2 hours. After this time the solvents were removed *in vacuo* and the residue applied directly to the top of a wet flash column. The product was eluted using 5:1:1 ethyl acetate/methanol/conc. ammonia to yield a red solid in 42% yield (0.372 g, 0.504 mmol). R_f (C) 0.26, H NMR (d_6-DMSO) δ_H 0.8 (9H, s, ^{t}Bu); 1.28 (8H, m, C*H*$_2$); 1.66 (4H, m, C*H*$_2$); 3.30 (4H, m, C*H*$_2$); 3.88 (4H, m, C*H*$_2$); 3.96 (6H, s, O-C*H*$_3$); 6.44 (2H, s, Ar*H*); 7.27 (6H, m, Ar*H*); 7.45 (4H, m, Ar*H*); 8.15 (1H, d, *J* 9.0, Ar*H*); 8.32 (1H, d, *J* 8.9, Ar*H*) 8.46 (1H, s, Ar*H*). M/z (FAB) 737.38405 [$C_{49}H_{53}N_6O_5Si$ $(M+H)^+$ < 0.9 ppm].

6-[3,5-Dimethoxy-4-(1-trityl-5'-azobenzotriazoyl)-phenoxy]-hexan-1-ol [30]



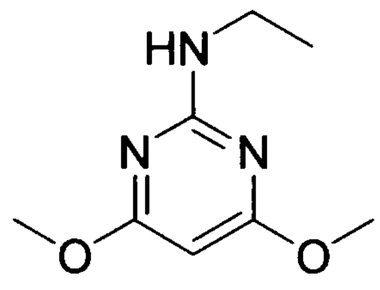
Compound **[13]** (0.200 g, 0.369 mmol) was dissolved in dry acetonitrile (20 ml) to which DBU (0.169 g, 0.166 ml, 1.108 mmol, 3 eq.) and bromohexanol (0.134 g, 0.097 ml, 0.739 mmol, 2 eq.) were added with stirring. The reaction was allowed to stir at room temperature for 7 days. After this time the solvents were removed *in vacuo* and the residue re-dissolved in DCM (20 ml) and washed with sat. KCl (20 ml). The pure product was obtained by column chromatography, eluting DCM and using silica pre-equilibrated with 1% triethylamine. Compound **[30]** was obtained in 73% yield (0.172 g, 0.268 mmol). R_f (C) 0.36, ^{1}H NMR (d_6-acetone) δ_H 1.48 (4H, m, C*H*$_2$); 1.81 (4H, m, C*H*$_2$); 3.78 (3H, s, O-C*H*$_3$); 3.83 (3H, s, O-C*H*$_3$); 3.96 (2H, t, J 6.5, C*H*$_2$); 3.99 (2H, t, J 6.5, C*H*$_2$); 6.33 (1H, s, Ar*H*); 6.38 (1H, s, Ar*H*); 7.32 (15H, m, Ar*H*); 7.78 (1H, d, *J* 8.9, Ar*H*); 8.8.08 (1H, d, *J* 9.0, Ar*H*); 8.37 (1H, s, Ar*H*). M/z (FAB) 642.30968 [$C_{39}H_{40}N_5O_4$ $(M+H)^+$ < 2.6 ppm].

1-(2-Cyanoethyl-N,N-diisopropylphosphoramidite)- 6-[3,5-dimethoxy-4-(1-trityl-5'-azo-benzotriazoyl)-phenoxy]-hexane [31]



Compound **[30]** (0.155 g, 0.242 mmol) was co-evaporated with anhydrous THF (3 x 15 ml) and then dissolved in anhydrous THF (15 ml). To the stirring solution, anhydrous diisopropylethylamine (0.124 g, 0.167 ml, 0.959 mmol, 4 eq.) was added under nitrogen. 2-Cyanoethyl-N, N-diisopropylchloro-phosphoramidite (0.063 g, 0.059 ml, 0.266 mmol, 1.1 equivalents) was added dropwise and the reaction allowed to stir for one hour. After this time, ethyl acetate (50 ml) was added to the mixture and the organic layer washed with saturated potassium chloride (20 ml), dried over anhydrous sodium sulfate and the solvent removed *in vacuo*. The resulting oil was purified by wet flash column chromatography, eluting 100% ethyl acetate, using silica pre-equilibrated with 1% triethylamine. Phosphoramidite **[31]** was obtained as a yellow oil (0.195 g, 0.232 mmol, 96.0% yield), R_f (B) 0.75, ^{31}P NMR, ($CDCl_3$), referenced to phosphoric acid, δ_P 147.79 (s).

(4,6-Dimethoxy-pyrimidin-2-yl)-ethyl-methyl-amine [32]



2-Amino-4,6-dimethoxy pyrimidine (0.250g, 1.61 mmol) was combined with acetaldehyde (0.071g, 0.090 ml, 1.61 mmol, 1.00 eq.) and titanium IV isopropoxide (0.572, 0.599 ml, 2.013 mmol, 1.25 eq.) and allowed to stir for one hour at room temperature under a drying tube. After this time 5 ml of ethanol was added followed by sodium cyanoborohydride (0.068 g, 1.079 mmol, 0.670 eq.) and the reaction allowed to proceed for 20 hours. After this time 1.50 ml of distilled water was added and the resulting inorganic precipitate removed by filtration and washed with ethanol. The filtrate was concentrated in vacuo and re-dissolved in ethyl acetate. The remaining inorganics were removed by filtration and the filtrate concentrated in vacuo. The product was isolated by flash column chromatography eluting 100 % DCM to yield the secondary amine **[32]** in 73 % (0.216 g, 1.18 mmol), R_f (G) 0.61, ^{1}H NMR (d_6-acetone) δ_H 1.10 (3H, t, *J* 7.1, C*H*$_3$); 3.26 (2H, q, *J* 7.1, C*H*$_2$); 3.77 (6H, s, O-C*H*$_3$); 5.32 (1H, s, Ar*H*); 6.38 (1H, s, Ar*H*); 7.03 (1H, brs, N*H*). M/z (EI) 183.1008 [$C_8H_{13}N_3O_2$ (M^+) < 2.9 ppm].

3.6.3 Synthesis of the Modified Oligonucleotide

A solution of phosphitylated label **[25]** or **[31]** (60 mg ml^{-1}) was placed in monomer port 5 of the instrument. The protocol was set to add the label phosphoramidite as the final monomer of the automated synthesis. Efficient coupling of the monomer was ensured by double delivery to the column, with an extended coupling cycle of 15 minutes for each monomer delivery. The instrument was programmed to remove the final monomethoxytrityl or trityl protecting group on completion of the synthesis.

Removal of the remaining protecting groups and cleavage of the oligo from the solid support was carried out using concentrated aqueous ammonia as outlined in chapter 2 section 2.6.4, as were the procedures for the reverse phase HPLC purification and subsequent de-salting of the modified oligonucleotide.

Chapter 4

Spectroscopic Analysis of SERRS Labelled Oligonucleotides

4.0 Spectroscopic Analysis of SERRS Labelled Oligonucleotides

4.1 Introduction

The main aim of this piece of work was to obtain SERRS spectra from the novel azobenzotriazole dye labelled oligonucleotides (outlined in chapters 2 and 3). This involved the initial analysis of the individual labels and labelled oligos by UV-visible spectroscopy followed by development of a SERRS sample preparation procedure for optimum signals. Secondly the performance of the labelled oligonucleotides was investigated through a series of experiments that probed the nature of the benzotriazole – colloid interaction, an essential contributing factor to effective SERRS detection. This is a preliminary assessment of the novel SERRS labelled oligonucleotides and is not a comprehensive analytical study, since this would have required a large volume of sample which was not available and is outside the scope of this thesis.

Finally, preliminary ultraviolet melting studies were carried out for an azobenzotriazole dye labelled oligonucleotide to assess how the SERRS label affected formation of a double helix.

4.2 UV-Visible Spectra of Azobenzotriazole Dyes and Labelled DNA

The analysis of the novel SERRS labels and labelled oligonucleotides by UV-visible spectroscopy was required for two reasons. Firstly the absorption maximum was required to give an indication of the optimum exciting laser frequency for SERRS and secondly it was necessary to determine the concentration of the labelled oligonucleotides in solution. Of the novel dyes discussed in this thesis, there are three parent chromophores, which are shown in Figure 53.

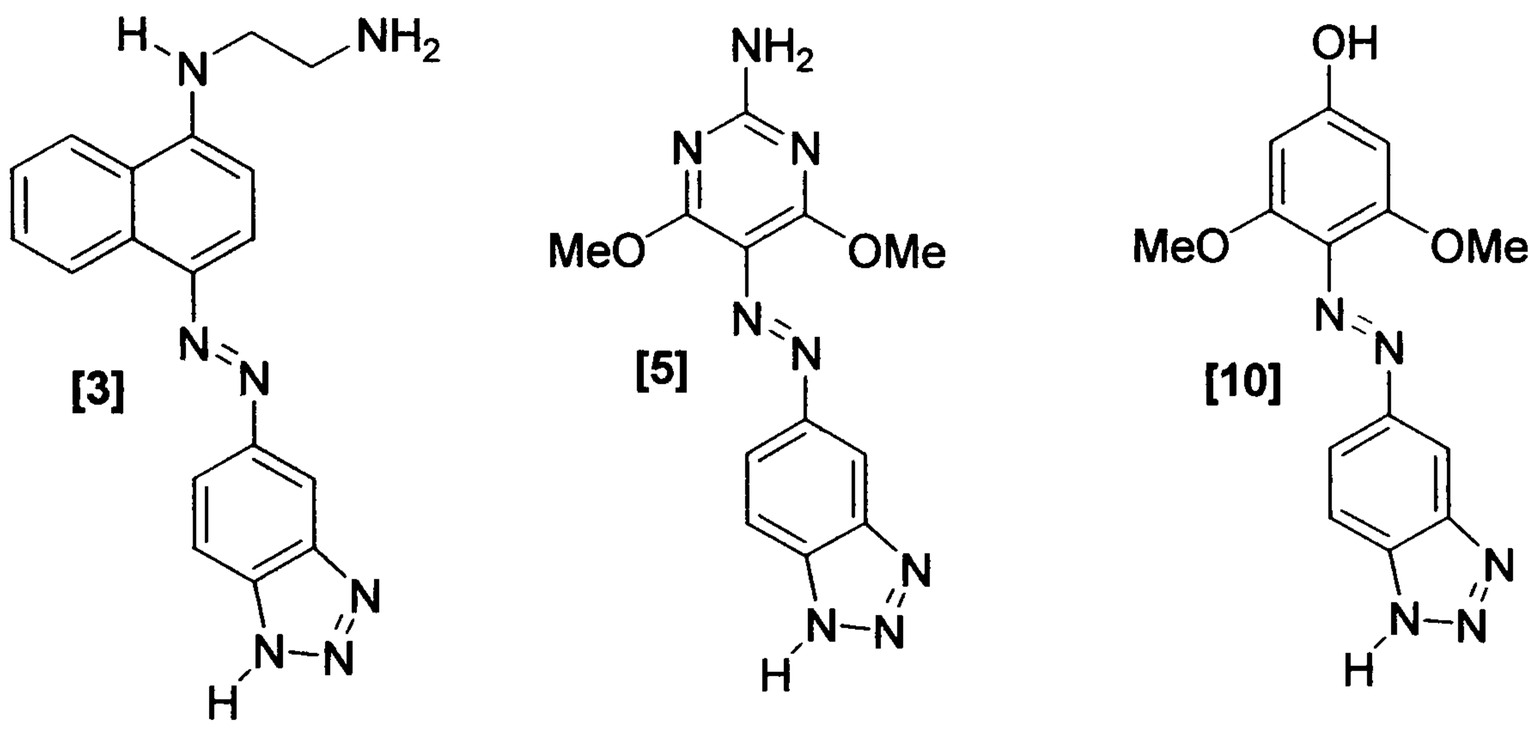


Figure 53. Parent chromophores.

N.B. The numbering system refers to that used in Chapter 3.

The UV-visible spectra were obtained for each dye at a concentration of 1 x 10^{-4} M and the λ_{max} recorded. Values of the absorption coefficients for the dyes were calculated at 260 nm to allow accurate DNA concentration determination of the SERRS labelled oligos. Table 2 shows the observed data.

Dye	λ_{Max} / nm	Absorption Coefficient ε_{260} / $mol^{-1}L^{-1}cm^{-1}$
N-[4-(5'-Azobenzotriazoyl)naphthalen-1-yl]ethylenediamine **[3]**	478.1	5690
5-(5'-Azobenzotriazoyl)-4,6-dimethoxy-pyrimidin-2-ylamine **[5]**	362.0	7840
4-(5'-Azobenzotriazoyl)-3,5-dimethoxy-phenol **[10]**	428.0	1080

Table 2. UV parameters of novel azobenzotriazole dyes.

This data suggests that of the most common visible laser lines; 457, 514 and 632 nm, the optimum exciting laser frequency for these dyes should be 457 nm, however this laser line was not available, so initial SERRS spectra were obtained using a 514 nm laser.

4.3 The SERS Spectrum of a Benzotriazole Modified Oligonucleotide

The analysis of DNA modified to contain a benzotriazole group at the 5' end, oligo 4, was carried out using SERS. The molecule does not contain a chromophore in the visible region. In order to obtain sufficient signals, the SERS sample was prepared with a large excess of modified DNA (200 μl of a 1.3 x 10^{-4} M solution in a total sample volume of 470 μl). The sample was aggregated with 20 μl of a 1 x 10^{-3} M spermine solution. The sample was then centrifuged and 90 % of the supernatant removed. The pellet containing the colloidal particles was resuspended in the remaining supernatant and analysed on a glass slide. The following SERS spectrum was obtained using a 514 nm laser source (Figure 54).

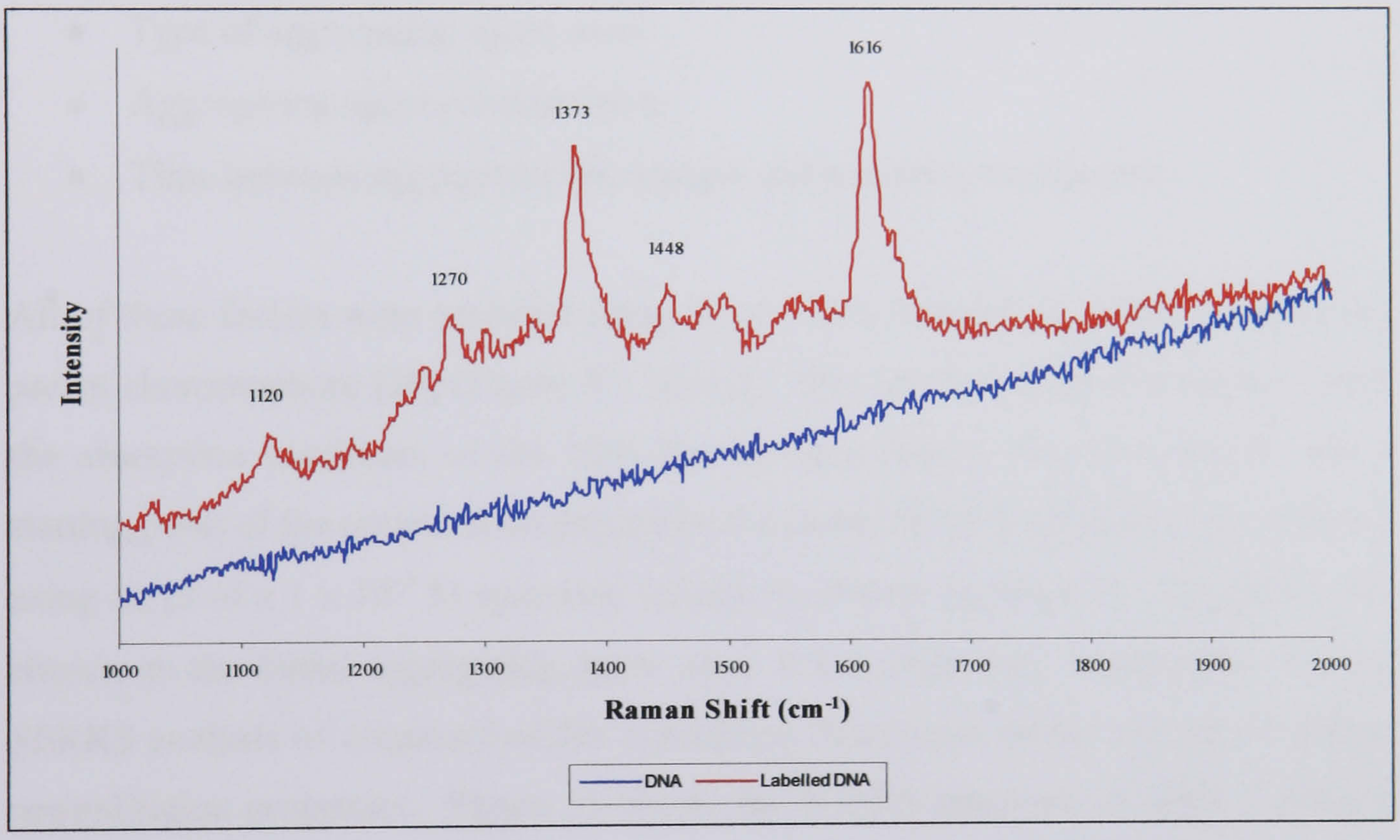


Figure 54. SERS spectrum of Oligo 4.

The spectrum shows two distinct bands at 1373 cm^{-1} and 1616 cm^{-1}, the second spectrum shows unmodified DNA treated in the same manner.

4.4 Optimisation of SERRS Conditions

In order to obtain the best possible SERRS spectra from the labelled oligonucleotides, a series of sample optimisation experiments were carried out. These were necessary since there is no precedence for SERRS of DNA labelled with an azobenzotriazole dye and therefore their properties are completely unknown.

There are several factors that must be assessed when obtaining reproducible SERRS spectra from aggregated colloids:

- Equilibration time of the sample with the colloid to allow maximum surface adsorption.
- Type of aggregating agent used.
- Aggregating agent concentration.
- Time between aggregating the sample and acquiring the spectra.

All of these factors were assessed using oligo 5 (see chapter 3), which contains the parent choromophore **[10]** (Figure 47, above). This labelled oligo was chosen since the absorption maximum of the label lies between that of the other labels. As a starting point of the optimisation procedure the initial SERRS spectrum was obtained using 40 μl of a 1 x 10^{-3} M spermine solution to ensure aggregation. Spermine was chosen as the initial aggregating agent since it has been used successfully for the SERRS analysis of oligonucleotides containing fluorescent labels, due to its charge neutralisation properties. Figure 55 shows the SERRS spectrum of oligo 5 using a 514 nm exciting laser.

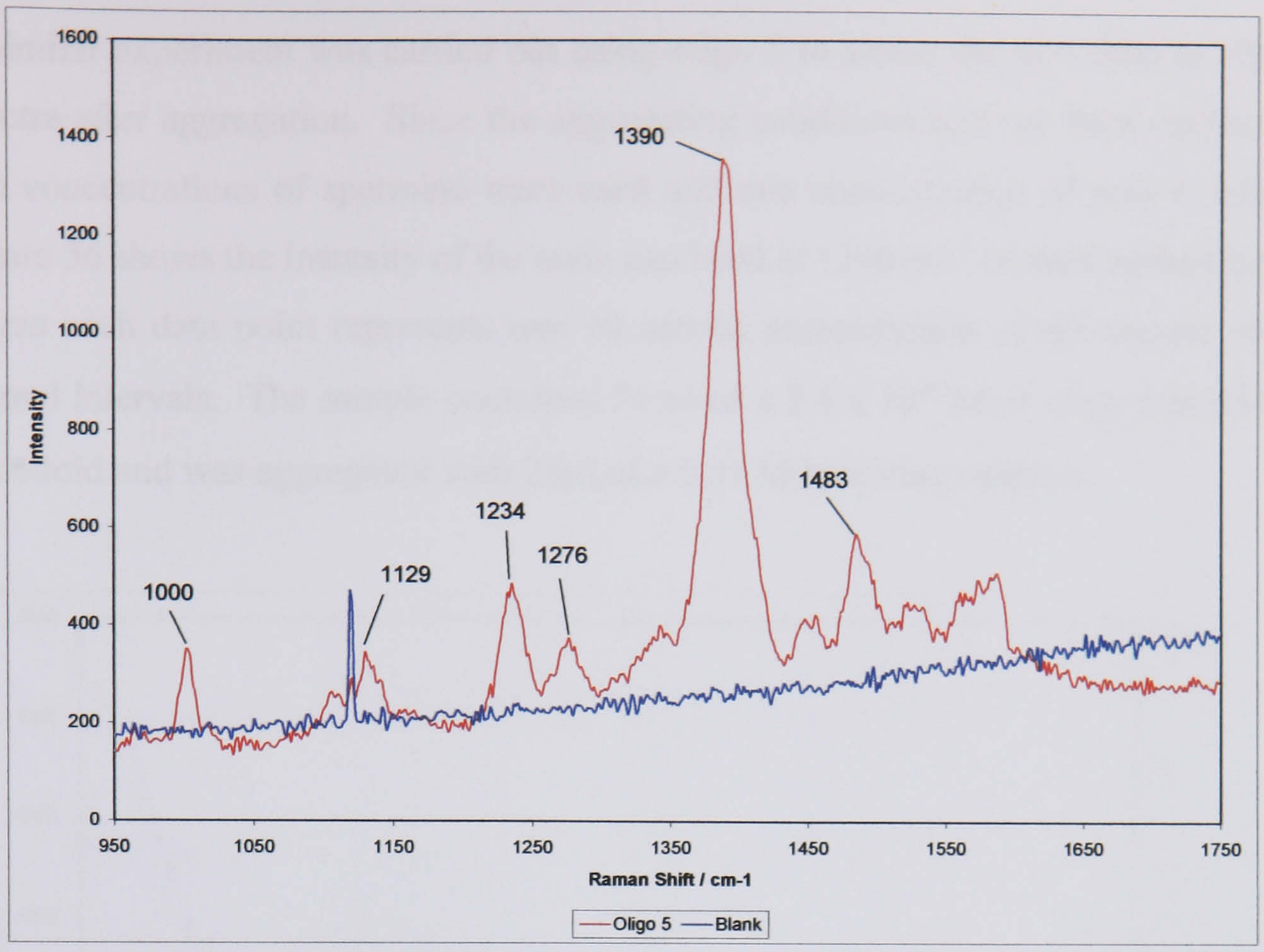


Figure 55. Initial SERRS spectrum of oligo 5.

The main feature of the SERRS spectrum of the azobenzotriazole oligo is the large azo band at 1390 cm^{-1}. The following optimisation procedures were carried out using 450 µl of colloid, 25 µl of a 4 x 10^{-6} M solution of labelled oligo with 25 µl of aggregating agent. Spectra were obtained using a 514 nm laser.

4.4.1 SERRS Signal Stability Over Time

The first of these factors to be assessed was the time dependence of the SERRS signal. This is very important since the aggregation process can take time to occur, and for the SERRS signal to stabilise, so the intensity of the signal obtained can vary depending on the time before spectral acquisition. A similar study was carried out with DNA labelled with fluorescent dyes, where samples were aggregated using spermine and spectra recorded every 30 seconds.[48] This work showed that the maximum SERRS signal occurred 60 seconds after aggregation and that the intensity stabilised after 210 seconds.

A similar experiment was carried out using oligo 5 to assess the best time to obtain spectra after aggregation. Since the aggregating conditions had not been optimised, two concentrations of spermine were used and one concentration of poly-L-lysine. Figure 56 shows the intensity of the main azo band at 1390 cm^{-1} plotted against time, where each data point represents one 10 second accumulation of the sample at 30 second intervals. The sample contained 25 µl of a 2.4 x 10^{-6} M of oligo 5 in 450 µl of colloid and was aggregated with 25µl of a 0.01 M spermine solution.

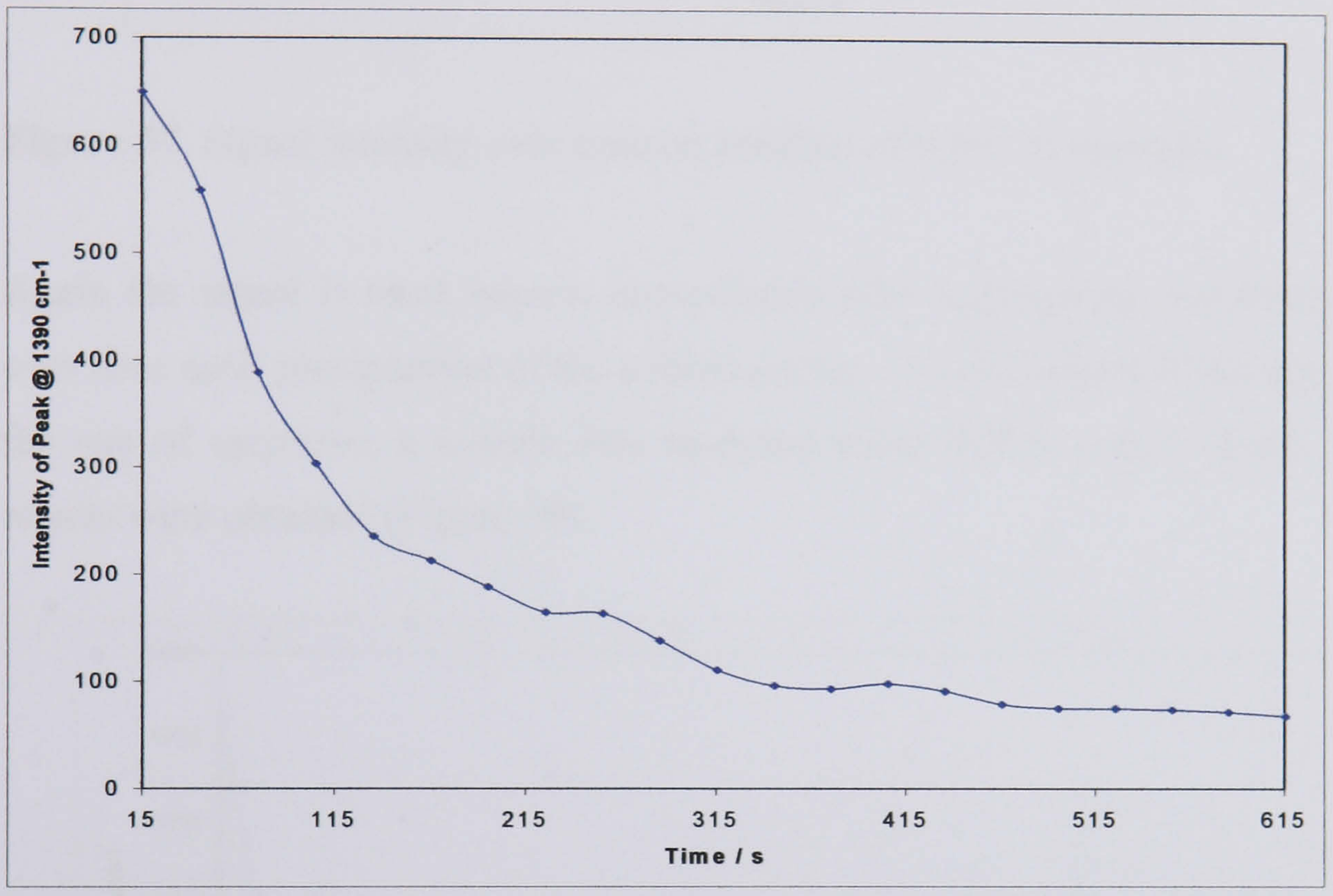


Figure 56. Signal intensity over time on addition of 0.01M spermine.

The data shows that the intensity reaches its maximum immediately after aggregation and then drops steadily stabilising around 500 seconds. At this point the spectral quality is much reduced and the colloid visibly precipitates from solution due to over aggregation. The concentration of aggregating agent was reduced by a factor of ten to see if this could provide a more stable system. Figure 57 shows the results obtained after aggregation of the same sample with 25 µl of 1 x 10^{-3} M spermine.

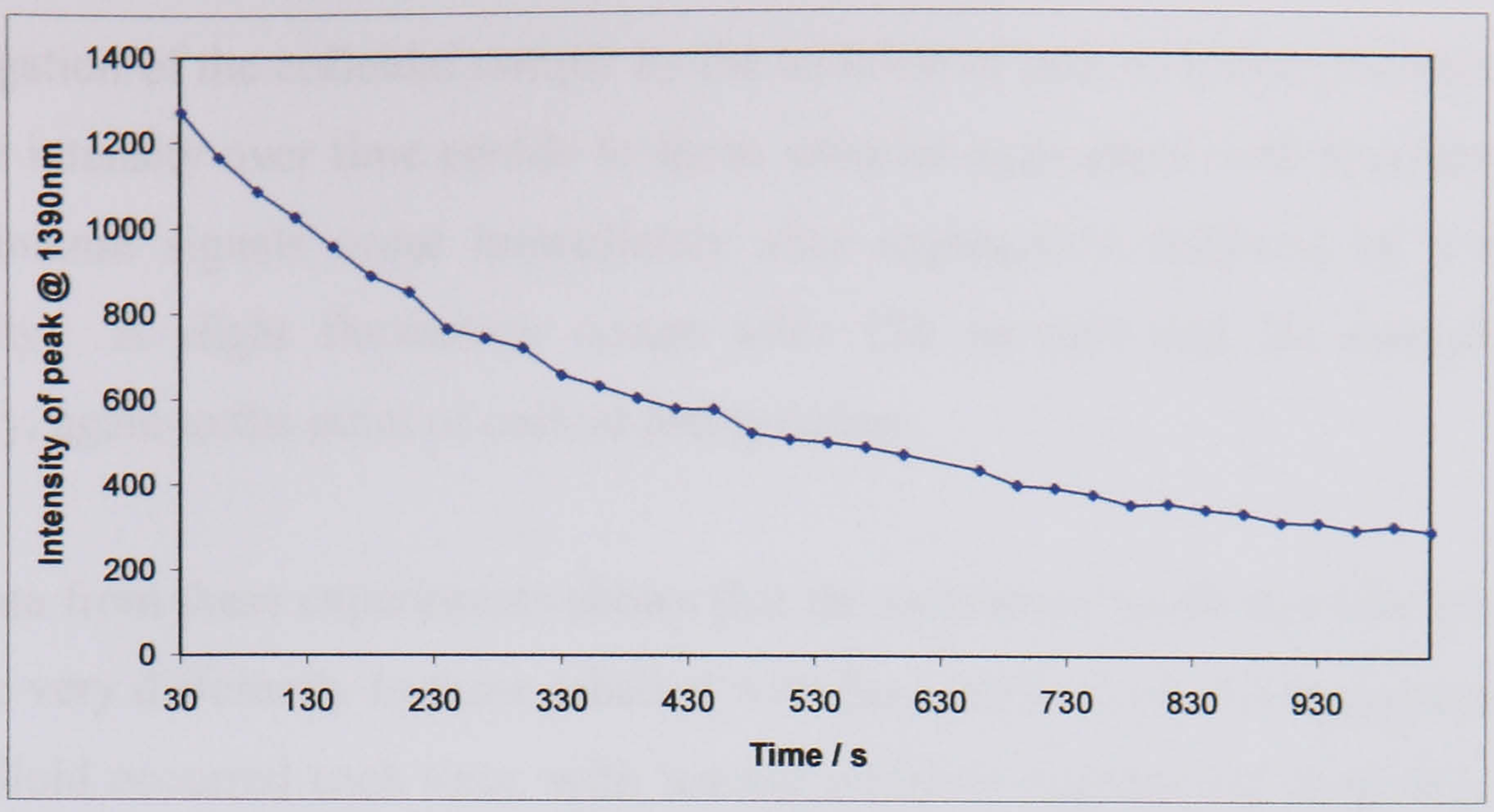


Figure 57. Signal intensity over time on addition of 0.001 M spermine.

Again the signal is most intense immediately after aggregation and drops steadily over time until precipitation of the colloid occurs. To investigate if this was a due to the use of spermine, a sample was analysed using 0.01% poly-L-lysine. Similar results were obtained (Figure 58).

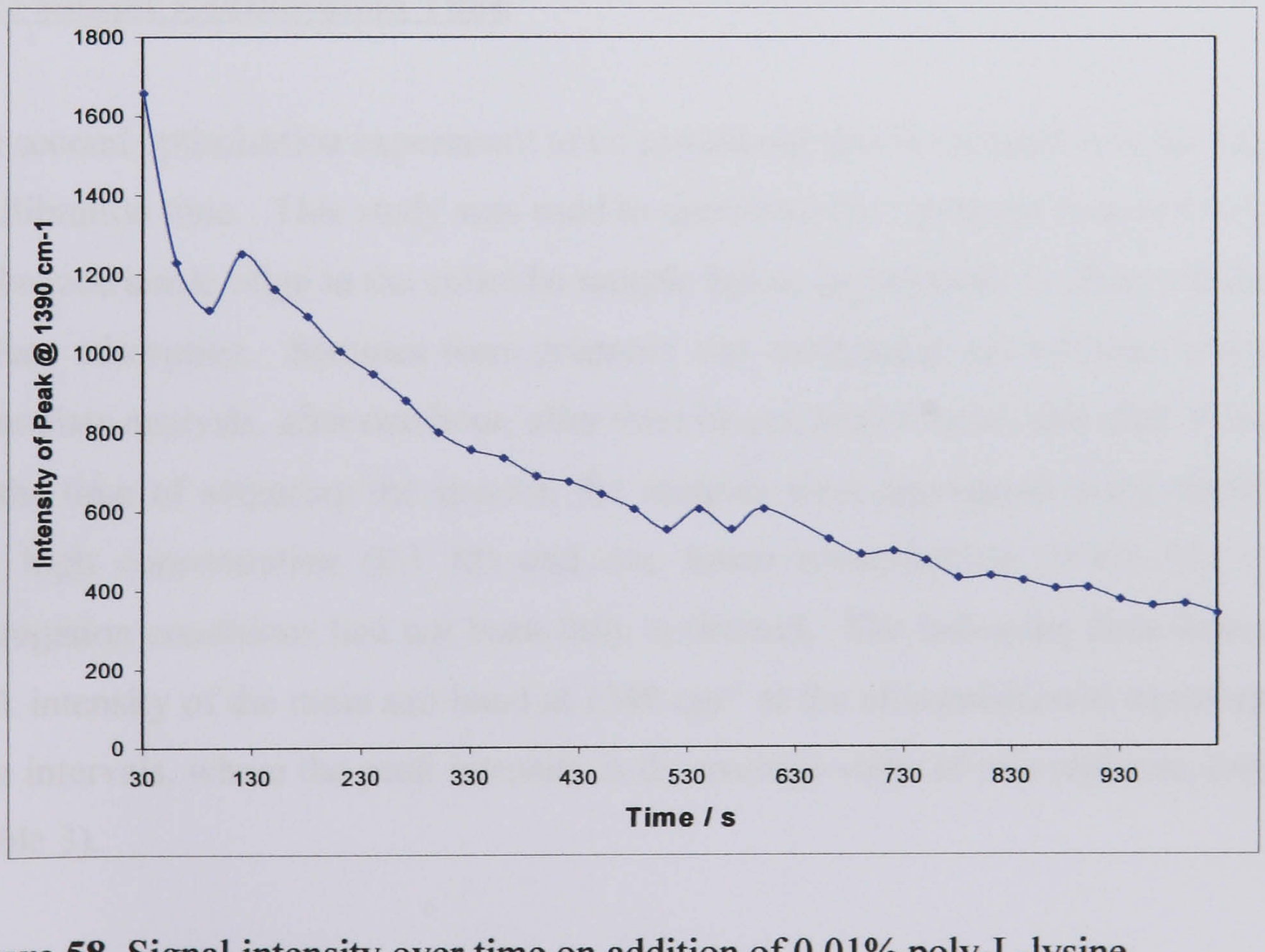


Figure 58. Signal intensity over time on addition of 0.01% poly-L-lysine.

Aggregation of the colloidal sample by the addition of poly-L-lysine provides a very similar intensity over time profile to those samples aggregated with spermine. The most intense signals occur immediately after aggregation followed by a drop in intensity. A slight fluctuation occurs after 130 seconds and the intensity falls steadily, again to the point of colloid precipitation.

The data from these experiments shows that the azobenzotriazole dye labelled oligos behave very differently to those labelled with fluorescent dyes. Over aggregation of the colloid occurred each time, with several different aggregating conditions. This suggests that the azobenzotriazole oligo contributes to the aggregation process in some way, possibly through the improved surface adsorption properties of the specifically designed labels. The interaction was investigated in more detail and is outlined later in this chapter. It can be concluded from these experiments that to obtain the most intense signals, spectral acquisition must be carried out immediately after sample aggregation.

4.4.2 Sample Equilibration Time

The second optimisation experiment to be carried out was investigation of the sample equilibration time. This study was used to determine the optimum time to leave the azobenzotriazole oligo in the colloidal sample before aggregation, to allow maximum surface adsorption. Samples were prepared and analysed at several time intervals: immediate analysis, after one hour, after three hours, after 6 hours and after 24 hours. At the time of acquiring the spectra, the samples were aggregated using spermine, one high concentration (0.1 M) and one lower concentration (0.001 M), since aggregation conditions had not been fully optimised. The following data shows the peak intensity of the main azo band at 1390 cm^{-1} at the aforementioned equilibration time intervals, where the peak intensity is the average value of two replicate samples (Table 3).

Equilibration Time	0 hrs	1 hrs	3 hrs	6 hrs	24 hrs
Peak Intensity with 0.1 M Spermine	490.33	876.54	673.95	685.96	496.73
Peak Intensity with 0.001 M Spermine	857.23	1627.25	1325.70	1436.25	1346.50

Table 3. Signal intensity with varying equilibration time.

These results show that a vast improvement in signal intensity can be achieved by equilibration of the labelled oligonucleotide and the colloid for at least one hour prior to sample aggregation and spectral acquisition. The data for the two concentrations of spermine follow the same trend. A dramatic increase in signal intensity is observed after one hour of equilibration, the intensity drops after three hours and slightly increases again after six hours, decreasing again after a period of twenty four hours.

These results indicate that optimum SERRS conditions are obtained after longer equilibration times. This could be attributed to the bulky nature of the oligonucleotide, since it could take some time to diffuse to the colloidal surface to allow the benzotriazole group to complex to the silver surface to form a thermodynamically stable configuration of clusters or polymeric forms to provide optimum position on the colloidal surface thus increasing the SERRS signal.

Preliminary experiments into the mode of surface attachment of azobenzotriazole dyes with citrate reduced silver colloid are discussed later in this chapter (section 4.5), however this aspect requires more detailed study which is outwith the scope of this work.

For sample preparation considerations, the maximum signal intensity can be achieved by equilibration of the sample for one hour prior to aggregation however, more robust results are obtained between three and six hours equilibration time, since the peak intensities fluctuate only slightly and are therefore more stable.

4.4.3 Optimisation of Aggregation Conditions

The final experiment in the optimisation procedure was the assessment of the best aggregation conditions, including type of aggregating agent and the optimum concentration. Three very different aggregating agents were assessed.

- Sodium chloride, where the sodium ions reduce the surface charge.
- Poly-L-lysine, an ionic polymer that surrounds the colloidal particles inducing controlled aggregation.
- Spermine, an effective DNA charge neutralisation agent that was used previously for the SERRS detection of DNA labelled with fluorescent dyes.

Two different concentrations were analysed for each aggregating agent. The samples were prepared using the optimised conditions outlined in sections 4.3.1 and 4.3.2 of this chapter, where the labelled oligonucleotide was equilibrated with the colloidal sample for 4.5 hours and the SERRS spectrum recorded immediately after aggregation. Again, the intensity of the main azo band at 1390 cm^{-1} was used as a marker to assess the optimum conditions. Table 4 shows the results obtained.

Aggregating Agent	Concentration added	Peak Intensity
Sodium Chloride	1 M	556.50
	0.5 M	241.49
Poly-L-lysine	0.1% w/v	153.14
	0.001 % w/v	180.53
Spermine	1×10^{-3} M	633.37
	1×10^{-4} M	1312.04

Table 4. Optimisation of aggregating agent in SERRS samples.

The most intense spectra were obtained from the samples aggregated with spermine in particular the sample aggregated with 25 µl of 1×10^{-4} M of spermine. The lowest intensity signals were obtained from the samples aggregated with poly-L-lysine. Therefore, the best aggregating agent for use with these novel probes is spermine. This is not surprising, since spermine has proven superior in the SERRS analysis of oligonucleotides labelled with fluorescent dyes.[47-49] This could be due to the four amino groups in the spermine molecule providing four positive charges, which in solution, are ideally spaced to interact with successive phosphate groups on the backbone of the labelled ologonucleotide. This effects charge neutralisation of the phosphate backbone of the oligonucleotide facilitating adsorption to the colloidal surface. This could also have an impact on the aggregation process of the SERRS sample since colloidal particles containing poly-anionic oligonucleotides would experience less repulsion, making the aggregation process more favourable and possibly more controlled.

The final part of this experiment was the optimisation of the spermine concentration added to the SERRS samples. This was evaluated in a similar way to the previous experiment. Five concentrations of spermine were included in the study, 0.1 M, 0.01 M, 1×10^{-3} M, 1×10^{-4} M and 1×10^{-5} M and 25 µl of each was used to aggregate the SERRS samples prepared as described in the previous section. Table 5 shows the

results obtained. Again each peak intensity represents the average of two replicate samples.

Concentration of Spermine / M	Peak Intensity @ 1390 cm^{-1}
0.1	255.30
0.01	234.06
1×10^{-3}	563.37
1×10^{-4}	1016.86
1×10^{-5}	69.62

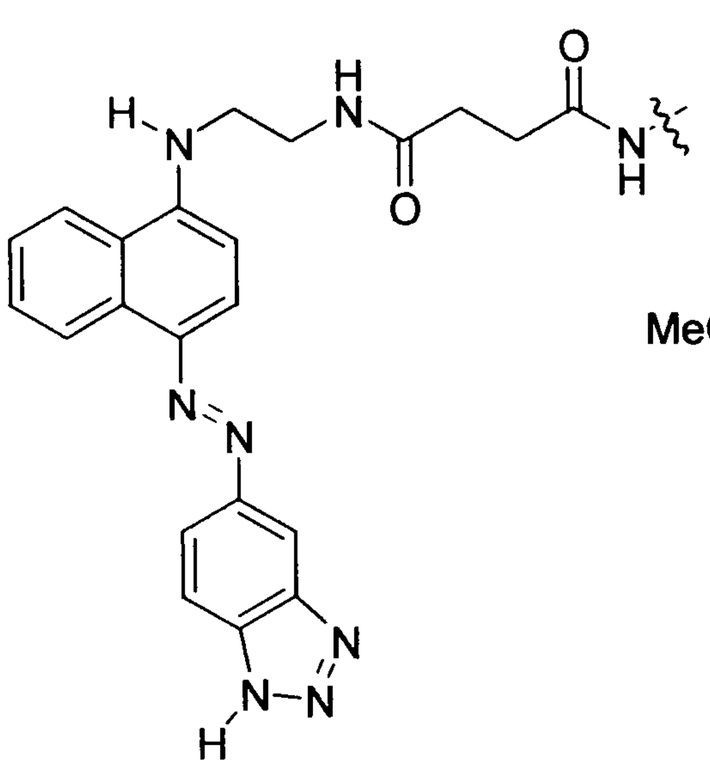
Table 5. Optimisation of spermine concentration in SERRS samples.

Very similar peak intensities were obtained after aggregation with 0.1 M and 0.01 M spermine at around 250 counts. The intensity more than doubled after decreasing the concentration to 1×10^{-3} M (563 counts) and then again to 1016 on addition of 1×10^{-4} M. The intensity dropped of suddenly to 69 counts on addition of 1×10^{-5} M spermine. These results suggest that adding 1×10^{-4} M spermine is the optimum concentration to produce the most intense spectrum.

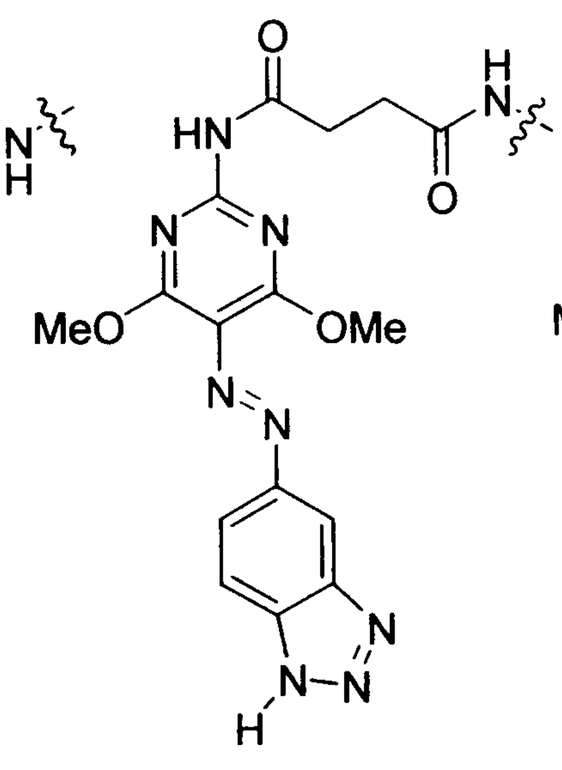
4.4.4 SERRS Spectra of Azobenzotriazole Dye Modified Oligonucleotides

Using the optimised conditions outlined earlier in this chapter, SERRS spectra were obtained of each modified oligonucleotide at an overall concentration of 1.25 x 10^{-8} M, using a 514 nm and a 785 nm laser source. Figure 59 shows the labelled oligonucleotides used for this study.

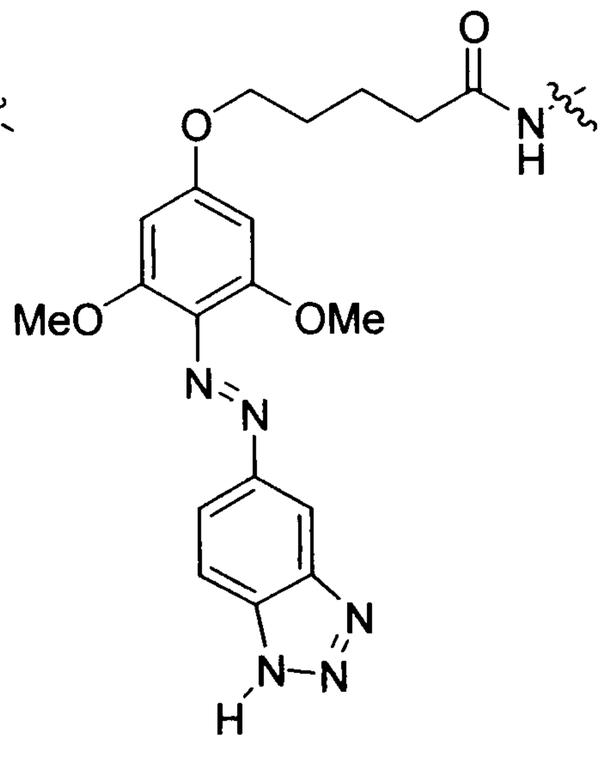
Amino-linked oligonucleotides



Oligo 1

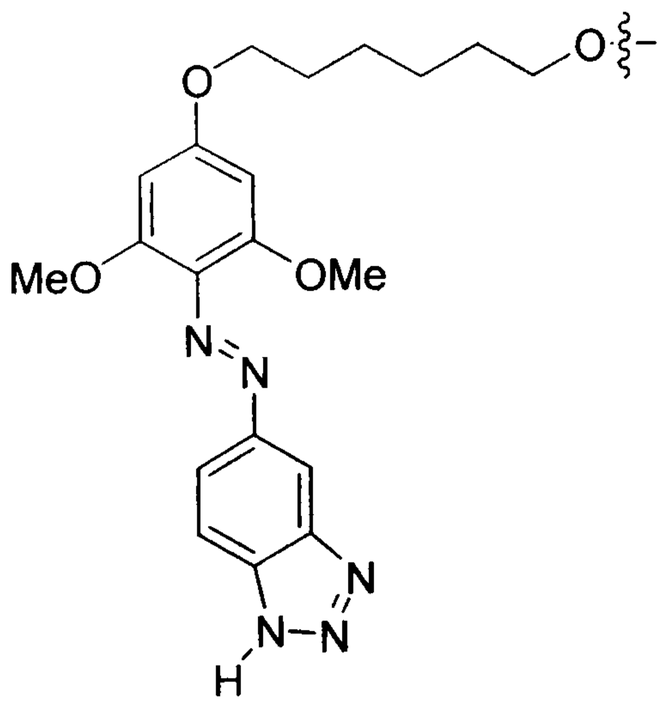


Oligo 2



Oligo 3

Phosphoramidite labelled oligonucleotide



Oligo 5

Figure 59. Labelled oligonucleotides under investigation.

The spectrum of each oligonucleotide was obtained in exactly the same way with identical sample preparation to allow direct comparison. In each case 25 µl of 2.5 x 10^{-7} M labelled oligonucleotide was allowed to equilibrate with 450 µl of colloid to allow maximum surface coverage before analysis. The samples were then aggregated using 25 µl of a 1 x 10^{-4} M spermine solution and analysed immediately after aggregation. Accumulation times were kept constant throughout the study at one accumulation of 10 s, Figure 60 shows the data obtained using a 785 nm laser source.

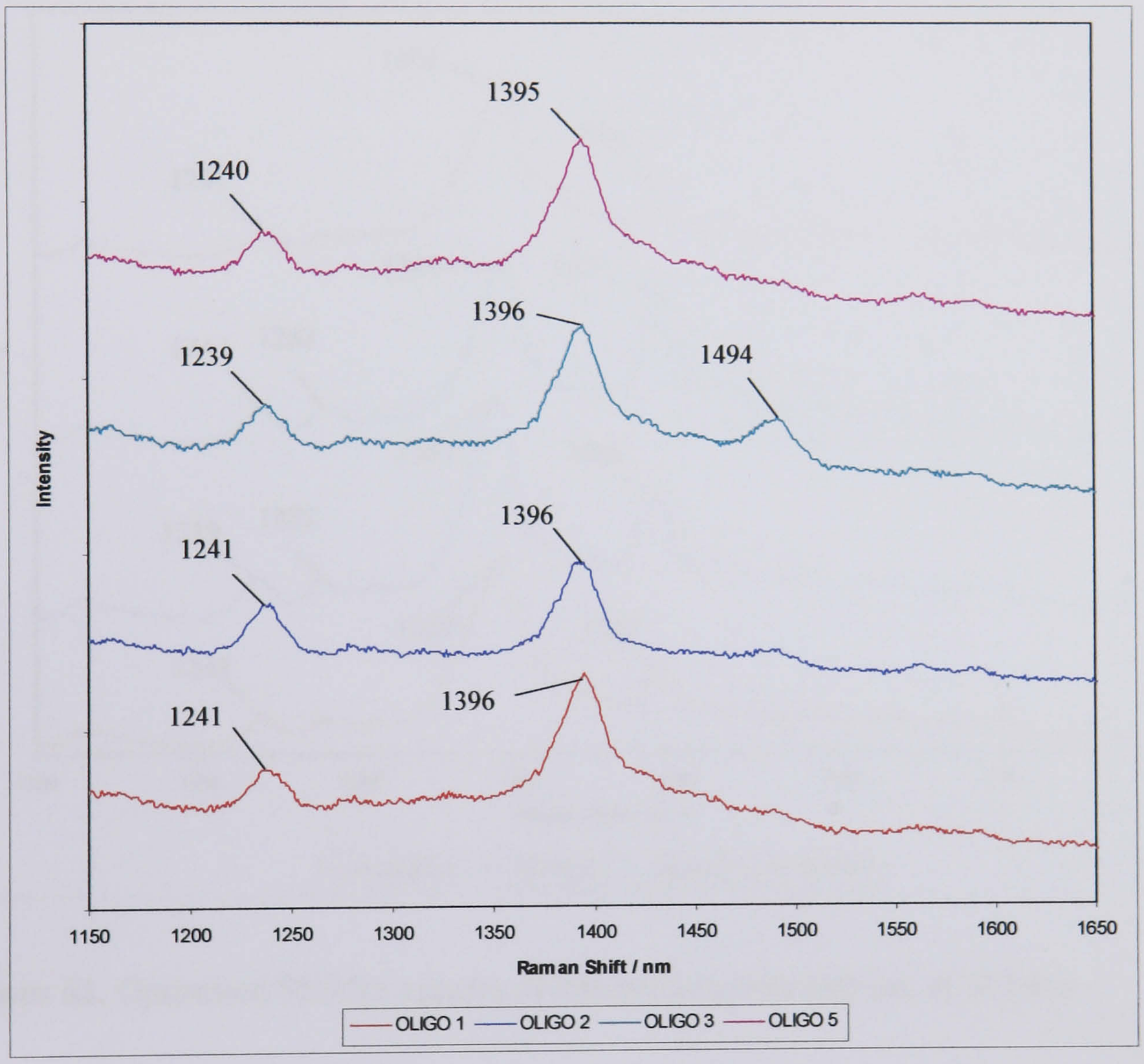


Figure 60. Optimised SERRS spectra of labelled oligonucleotides at 785 nm.

The spectra of all four oligos are very similar with the main feature being the azo stretch at around 1396 cm^{-1}. The similarity in the spectra could be attributed to the

exciting laser frequency since 785 nm is far away from the absorption maxima of the SERRS labels, ranging from 362 nm to 478 nm. This leads to less intense, less detailed signals since the resonance enhancement is lower far from the absorption maximum of the dye, so the main enhancement comes from surface adsorption.

The SERRS spectra were then obtained as described for the 785 nm study, using the 514 nm laser line and some further detail emerges from the spectra, however the main feature remains that of the azo bond at 1395 cm^{-1} (Figure 61).

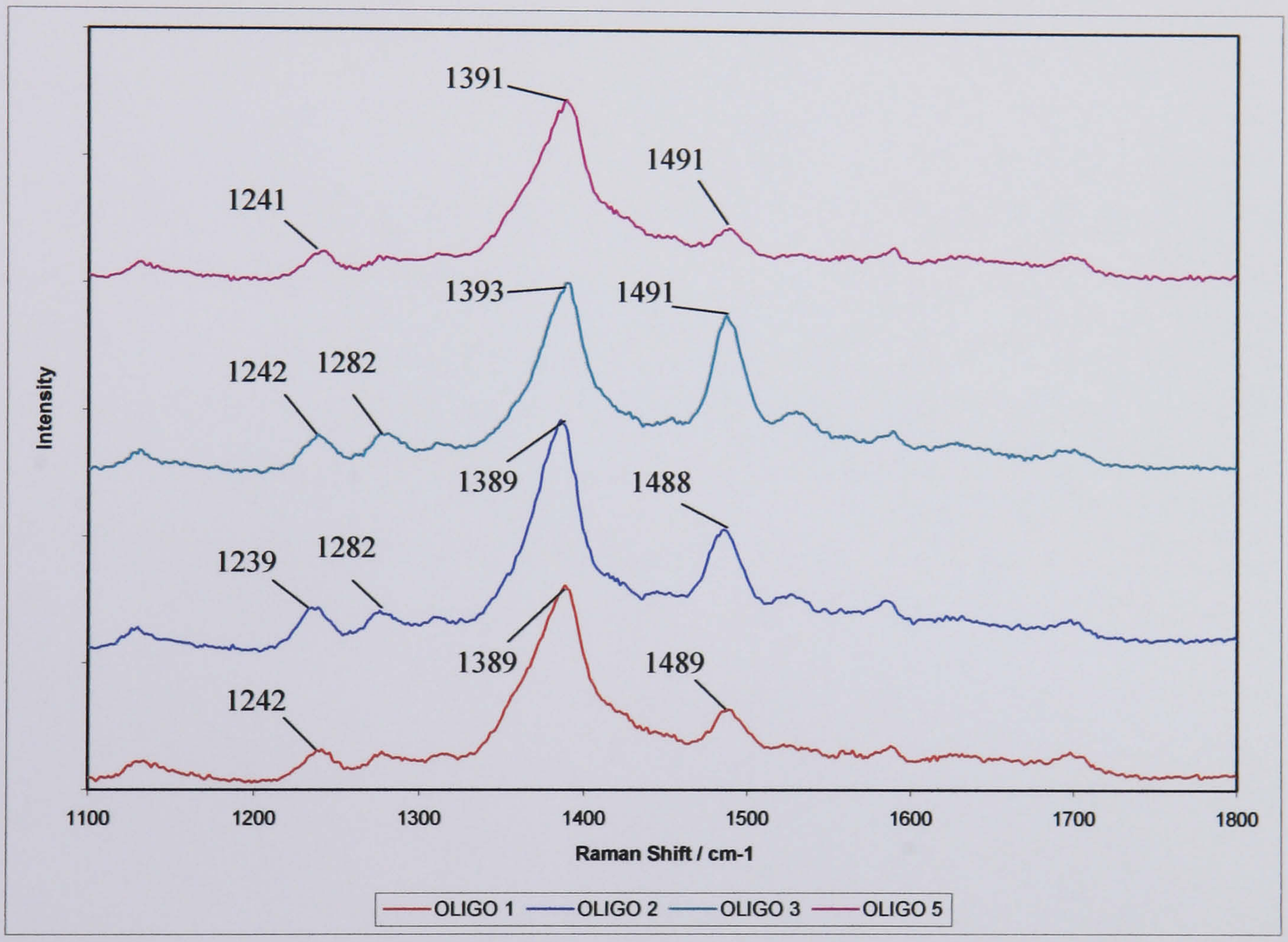


Figure 61. Optimised SERRS spectra of labelled oligonucleotides at 514 nm.

The position of the main azo band varied slightly for each oligo, between 1389 cm^{-1} and 1393 cm^{-1}. The position and relative intensity of the peak at 1489 cm-1 also differed for each labelled oligo, although all four labels gave largely the same spectrum. Further spectral resolution could be obtained by using a 457 nm laser

since this lies closer to the absorption maxima of the azobenzotriazole dyes, however this laser line was not available.

The lowest concentration at which signals could be detected was assessed for each SERRS labelled oligonucleotide to give an estimation of the detection limit. The lowest concentration at which discernible signals could be obtained in each case was at an overall sample concentration of 2.5×10^{-9} M.

4.5 Qualitative Investigation of the Colloid-Benzotriazole Interaction

The nature of the colloid-benzotriazole interaction is of vital importance. The interaction must be robust enough to keep the labelled oligo at the colloidal surface to allow surface enhancement to occur. It is thought that the benzotriazole group displaces the citrate layer at the colloidal surface and forms an irreversible bond. However this has never been proven. In order to prove this theory, an experiment was devised to probe this interaction. Since the formation of the irreversible bond between the benzotriazole group and the colloidal surface requires displacement of the citrate layer, a qualitative test to monitor the citrate levels in the colloidal sample was employed.

Luque-Perez and co-workers developed an assay to determine the levels of citric acid in soft drinks by reaction of the acid with iron III ions.[64] Under UV conditions the acid reacts with the iron III ions to produce iron II ions, these in turn can chelate diamines. One such compound is 1,10-phenanthroline. On chelation with iron II ions, 1,10-phenanthroline forms a complex with a λ_{max} of 518 nm. Therefore the UV- visible spectrum gives a qualitative indication of the citrate levels present in the sample by analysis of the resulting complex. Figure 62 shows a schematic of this process.

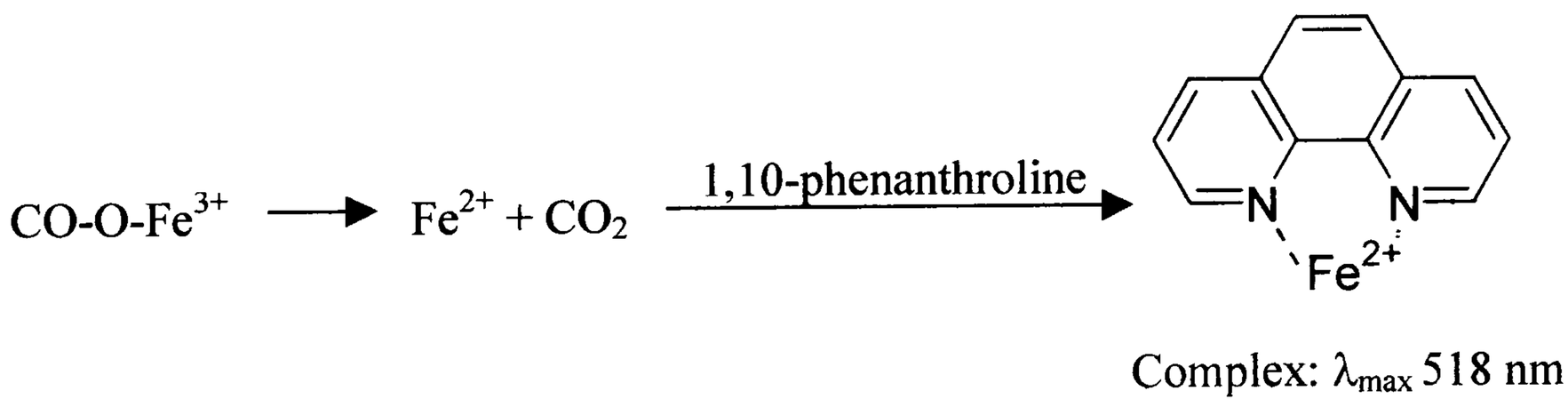


Figure 62. Method of citric acid determination

This method was adapted to determine the citrate level of the colloid. The assay conditions required to be optimised for this particular system. Since the colloidal particles have a plasmon resonance of around 400 nm, these were removed from the sample prior to treatment with the reagents to prevent interference. The standard sample volume was set as 1 ml of colloid with 100 µl of distilled water in the case of a blank or 100 µl of dye solution for the main study. Each sample was centrifuged until the silver particles formed a pellet in the sample tube and 1 ml of the supernatant was removed and treated first with 50 µl of a 0.05 M iron III nitrate solution in 0.01 M hydrochloric acid. The reaction was then carried out by exposure to UV light to form Fe II ions in solution and then 50 µl of a 0.05 M solution of 1,10-phenanthroline was added to form the desired iron complex.

Formation of the complex was apparent by eye, a change in colour from colourless to orange / red was observed on addition of the 1,10-phenanthroline solution. Further analysis of the sample by UV gave a qualitative estimate of the citrate content of the colloidal sample.

The citrate level of blank colloid was measured to give a background; since excess citrate is used for the preparation of the citrate reduced silver colloid and is therefore present in the colloid to some extent. The blank sample consisted of 1 ml of colloid and 100 µl of distilled water. The assay was carried out as described above and the background citrate level of the colloid determined. Figure 63 shows the UV spectra from the blank during activation and after addition of the 1.10-phenanthroline solution.

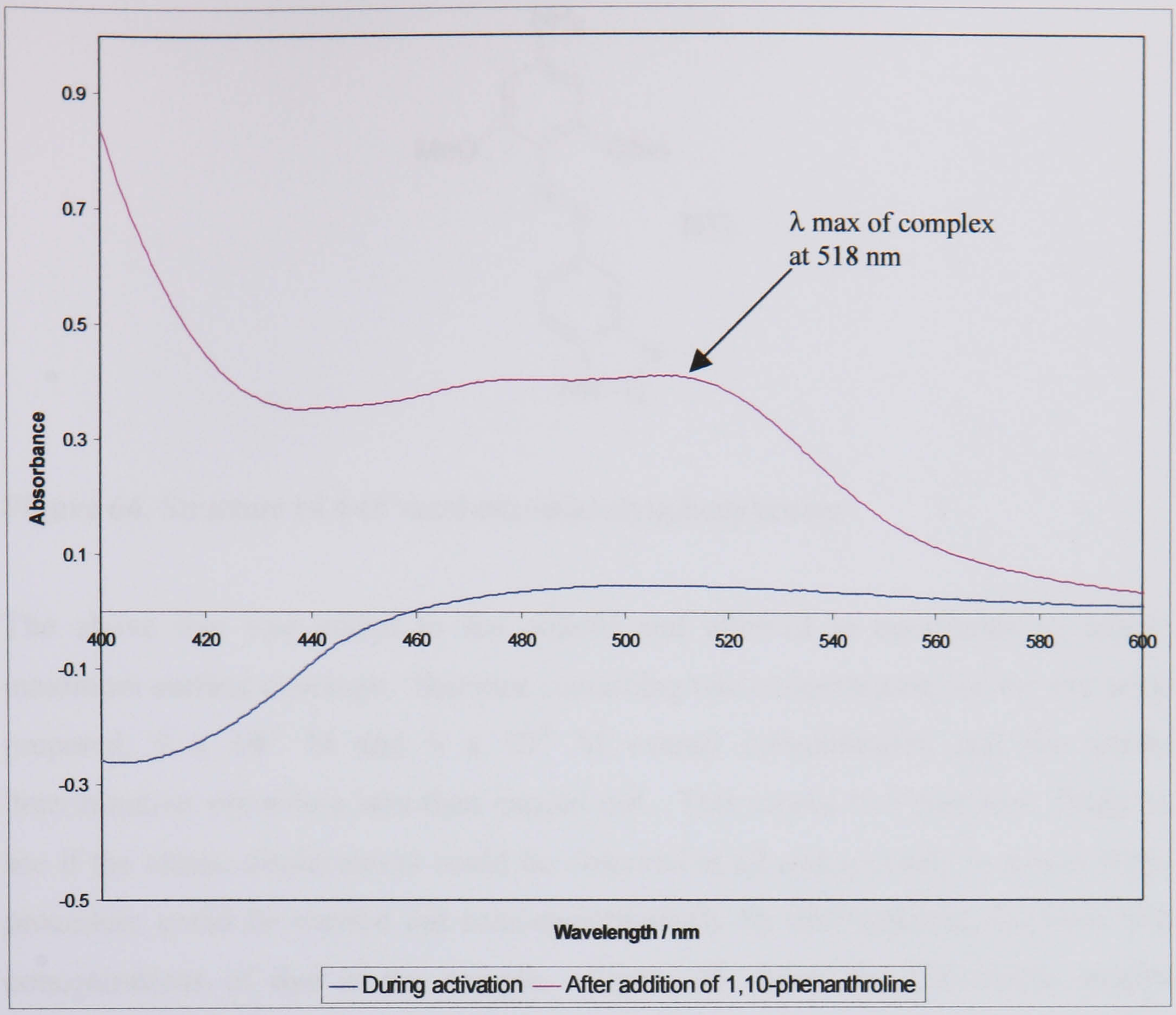
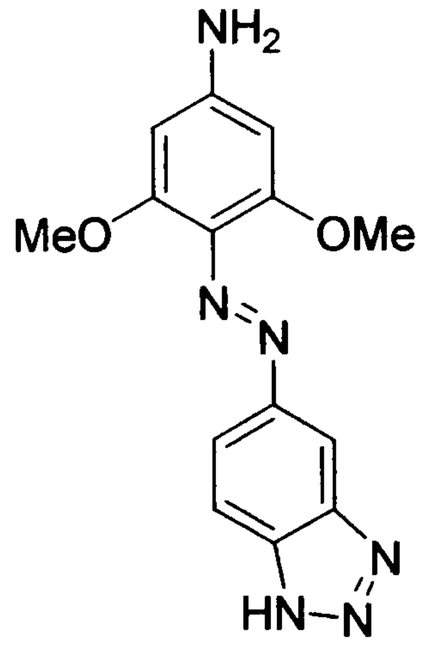


Figure 63. UV spectra of blank colloid during activation and after addition of 1,10-phenanthroline.

It should be noted that the amount of complex formed varies with each batch of colloid, due to variations in citrate present. Therefore a background citrate level must be determined for each colloid batch used to ensure a fair comparison. Similarly, all data to be compared was obtained using the same batch of colloid for continuity of results.

The assay was carried out with colloid containing the azobenzotriazole dye, 4-(5'-azobenzotriazolyl)phenylamine (Figure 64), which is known to be a very good SERRS dye.[30]



[37]

Figure 64. Structure of 4-(5'-azobenzotriazolyl)phenylamine

The above dye was added to the colloid and allowed to equilibrate to ensure maximum surface coverage. Samples containing two concentrations of the dye were prepared, 9 x 10^{-5} M and 9 x 10^{-6} M overall concentration, and the citrate determination procedure was then carried out. This served two purposes, firstly to see if the citrate displacement could be observed at all and secondly to assess if the procedure could be carried out semi-quantitatively by distinguishing between two concentrations of dye in the sample. Figure 65 shows the UV-visible spectra obtained.

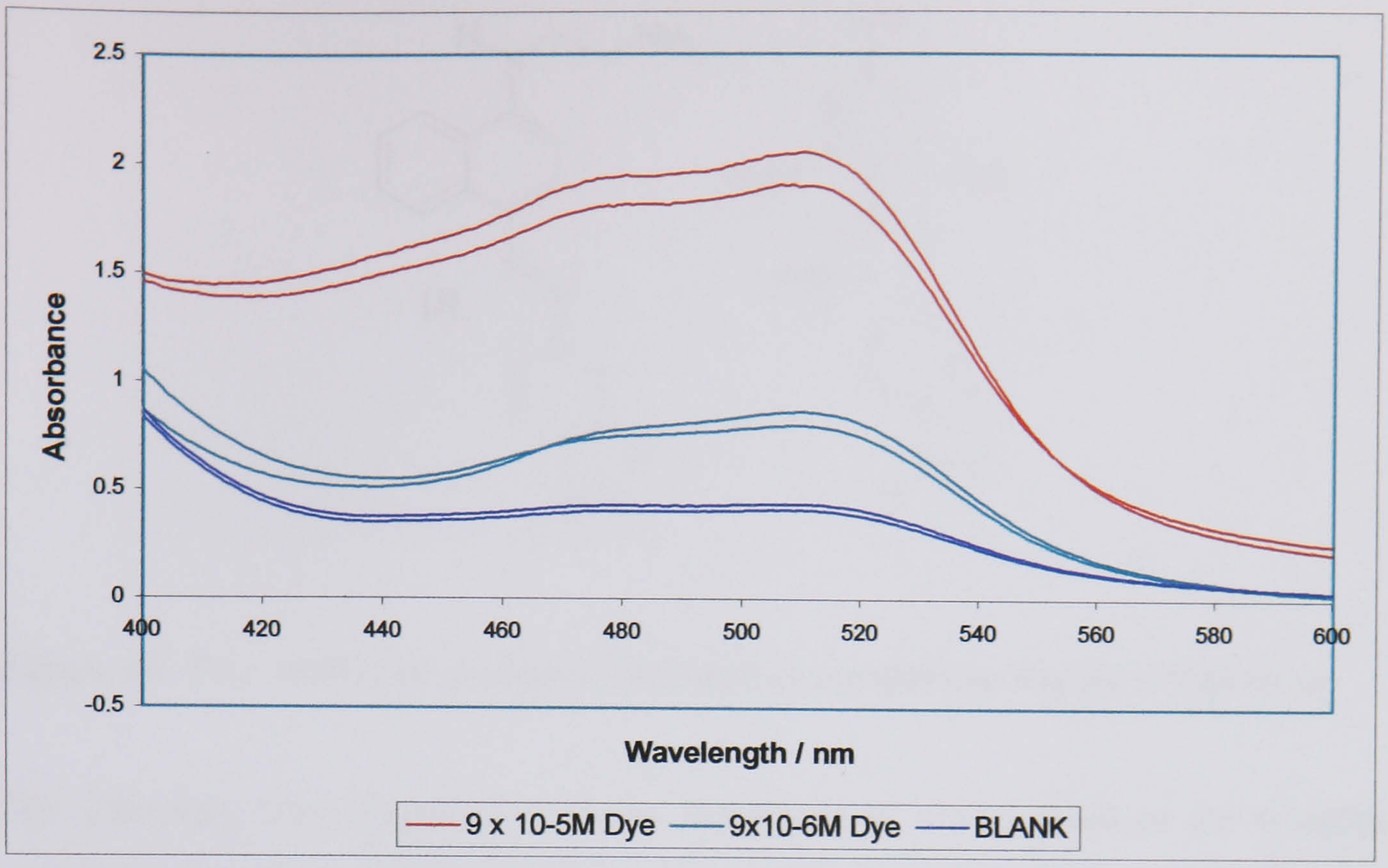


Figure 65. Results of citrate determination assay.

The above data shows that there is a substantial increase in colloidal citrate levels on addition of an azobenzotriazole dye. As described previously, there is a background citrate level for blank colloid, in this case providing enough complex for an absorbance of around 0.4. Addition of 1 x 10^{-4} M azobenzotriazole dye provides an absorbance of around 0.8 and finally, 1 x 10^{-3} M dye provides an absorbance of around 2.0. This result confirms that the citrate level of the colloid increases on addition of an azobenzotriazole dye and that the level increases with increasing concentration of dye added. This is strong evidence to suggest that the benzotriazole group does indeed displace citrate from the surface of the colloid to form a bond.

This effective assay for the displacement of citrate from the colloidal surface was used to assess the interaction of two further azobenzotriazole dyes with the colloid. This allowed the comparison of the three dyes in terms of binding efficiency with the surface. Figure 66 shows the further two dyes used in the comparison.

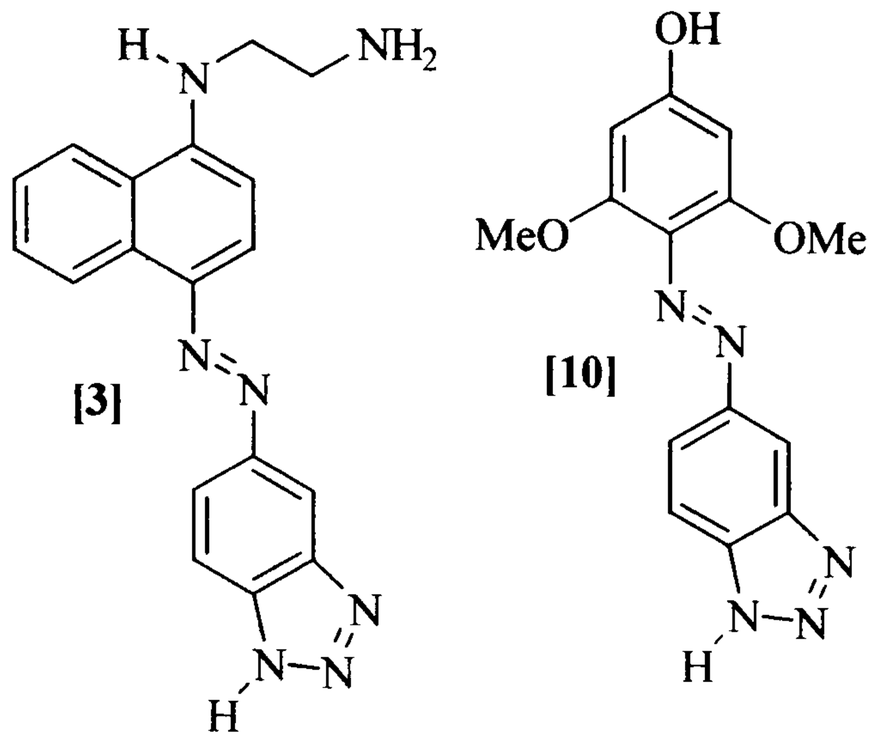


Figure 66. Two additional azobenzotriazole dyes compared using the citrate assay.

The following data (Figure 67) shows the levels of citrate detected for samples containing each dye. Each sample was prepared in the exactly the same way, 100 μl of a 1 x 10^{-3} M solution of the dye was added to 1000 μl colloid and the standard citrate assay carried out (detailed above). Each spectrum represents the average of three replicate samples.

The three azobenzotriazole dyes have different sub-structures and properties, with particular reference to the functional groups associated with the dyes. One has a primary aromatic amine, one a naphthyl group containing both a secondary aromatic and primary aliphatic amines and the final dye contains an aromatic phenol. These groups should behave differently in solution, providing differing degrees of activation towards the triazole ring and therefore conferring differing stability to the benzotriazole – colloid surface complex.

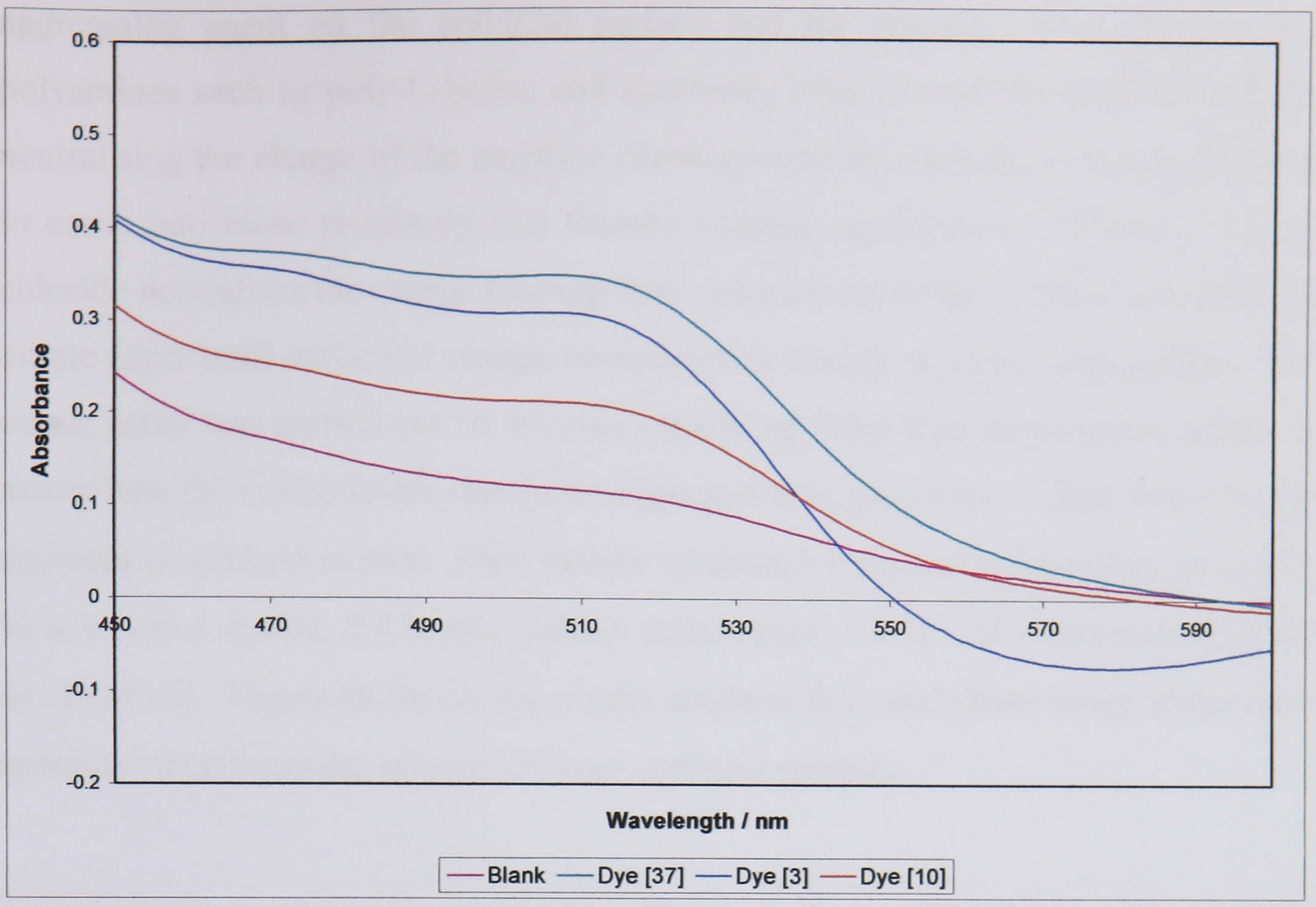


Figure 67. Comparison of azobenzotriazole dyes using a citrate assay.

The spectra clearly show a difference in the level of citrate displaced from the colloidal surface by each dye. This could be due to the properties of the dye affecting the surface adsorption process. For example by altering the rate of adsorption to the surface, differing stability of the dye – colloid complex allowing an equilibrium of adsorption and desorption, or the arrangement of molecules on the colloidal surface causing differences in surface coverage from dye to dye. The nature of these differences requires further study to provide conclusive evidence.

Finally, the citrate assay was used to investigate the interaction of some common aggregating agents with citrate reduced silver colloid. The choice of aggregating agent can have a large effect on the quality of the SERRS spectrum obtained, as demonstrated previously in this chapter, therefore it is important to understand how the aggregation process occurs in order to choose the most effective reagent. This difference in efficiency may be due to the mode of action of each particular

aggregating agent on the colloidal surface and the analyte. It is thought that polyamines such as poly-L-lysine and spermine, wrap around the colloidal surface neutralising the charge of the negative citrate groups allowing the colloidal particles to come into close proximity and thereby causing aggregation. Whereas sodium chloride neutralises the charge through ionic interactions of the sodium ions with the citrate layer until sufficient charge neutralisation occurs to allow aggregation. The citrate assay was carried out on samples containing these three aggregating agents to assess how the citrate levels change on aggregation to give some insight into how the aggregation process occurs. Each sample contained 100 µl of aggregating agent (0.1 % w/v poly-L-lysine, 0.4 % w/v sodium chloride and 1 x 10^{-3} M spermine) and 1000 µl of colloid. Figure 68 shows the results obtained from the citrate assay where each spectrum represents the average of three replicate samples.

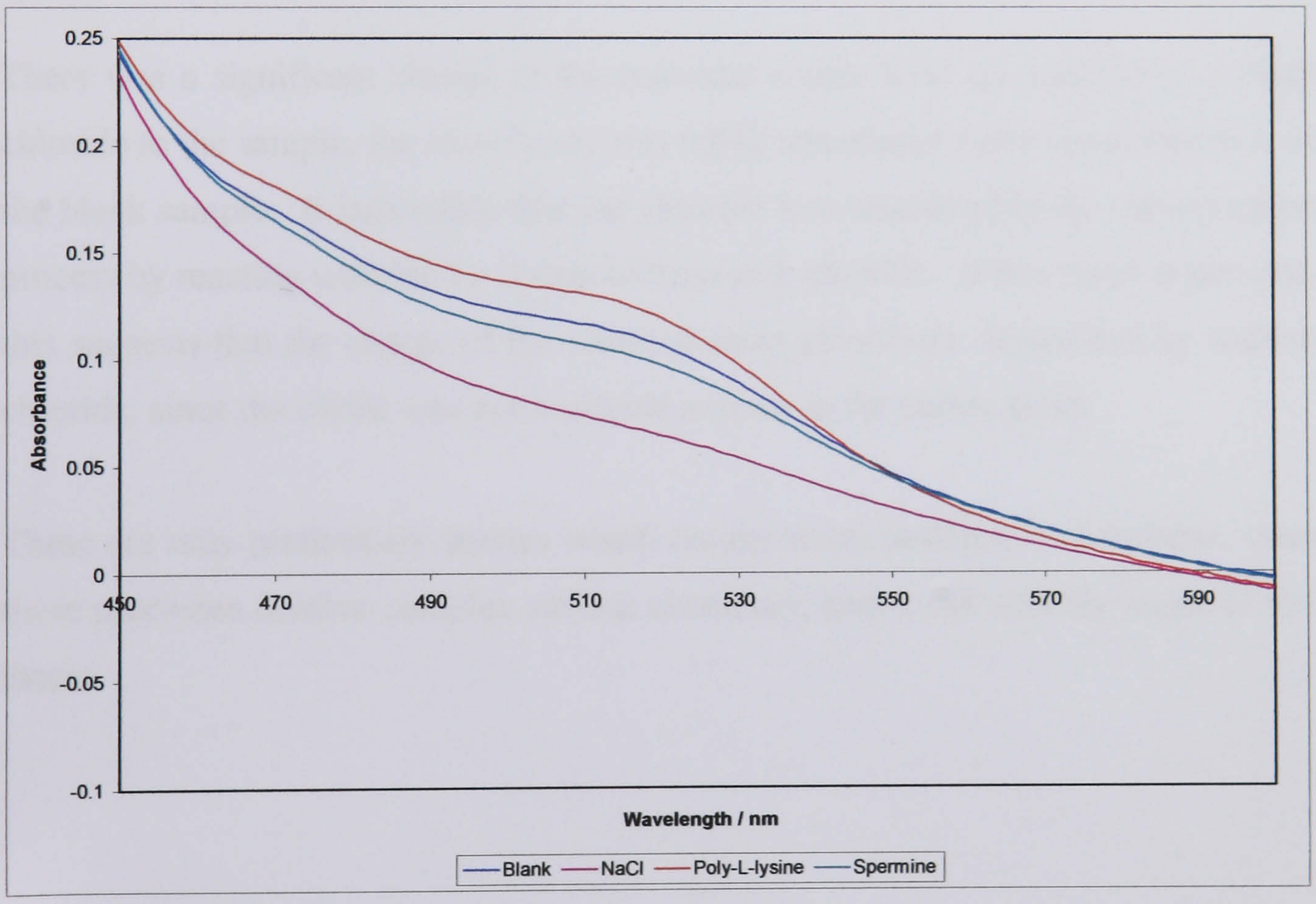


Figure 68. Comparison of aggregating agents using the citrate assay.

It can be seen from the above data that the three aggregating agents behave differently. Although the citrate levels for samples containing poly-L-lysine and spermine were similar to that of the blank sample (all gave enough complex for an absorbance of around 0.1), the poly-L-lysine sample had the highest citrate levels, slightly in excess of the level of blank colloid (a difference of 0.014 absorbance units). This suggests that the poly-L-lysine displaces some additional citrate from the colloidal surface during the aggregation process. Slightly lower levels of citrate were found in the samples containing spermine (a difference of 0.009 absorbance units), suggesting that less citrate was present in solution. Since no significant changes in citrate level were observed, it can be concluded that no dramatic alterations in the surface citrate layer occurs and indeed it is possible that the polyamines act as outlined previously by wrapping around the colloidal particles neutralising the surface charge.

There was a significant change in the colloidal citrate level on addition of sodium chloride to the sample, the absorbance was 0.042 absorbance units lower than that of the blank sample. It is possible that the chloride ions interfered in the complexation process by reacting with the Fe II ions to form iron chloride. If this result is genuine, this suggests that the charge of the citrate is most effectively neutralised by sodium chloride, since the citrate was not available to react in the citrate assay.

These are only preliminary studies which require more in-depth investigation, since these processes involve complex surface chemistry, and is out with the scope of this thesis.

A second experiment was carried out to test how robust the azobenzotriazole-colloid interaction was. A series of displacement experiments were devised to see if an azobenzotriazole dye labelled oligonucleotide could be displaced from the colloidal surface by another species.

The first experiment was the attempted displacement of an azobenzotriazole dye labelled oligonucleotide by a free azobenzotriazole dye. It was thought that if the interaction between the colloid and the labelled oligo was not robust, then the bulk of the labelled oligonucleotide could hinder the formation of the metal – ligand complex which is believed to be a polymer or a cluster, thus it could be thermodynamically favourable to displace the bulky labelled oligonucleotide with a smaller dye molecule. **Oligo 1** (page 49) was used for this purpose and 4-(5'-azobenzotriazolyl)phenylamine (figure 68) as the displacing dye, figure 58 shows the SERRS spectrum of this dye at a concentration of 1×10^{-7} M.

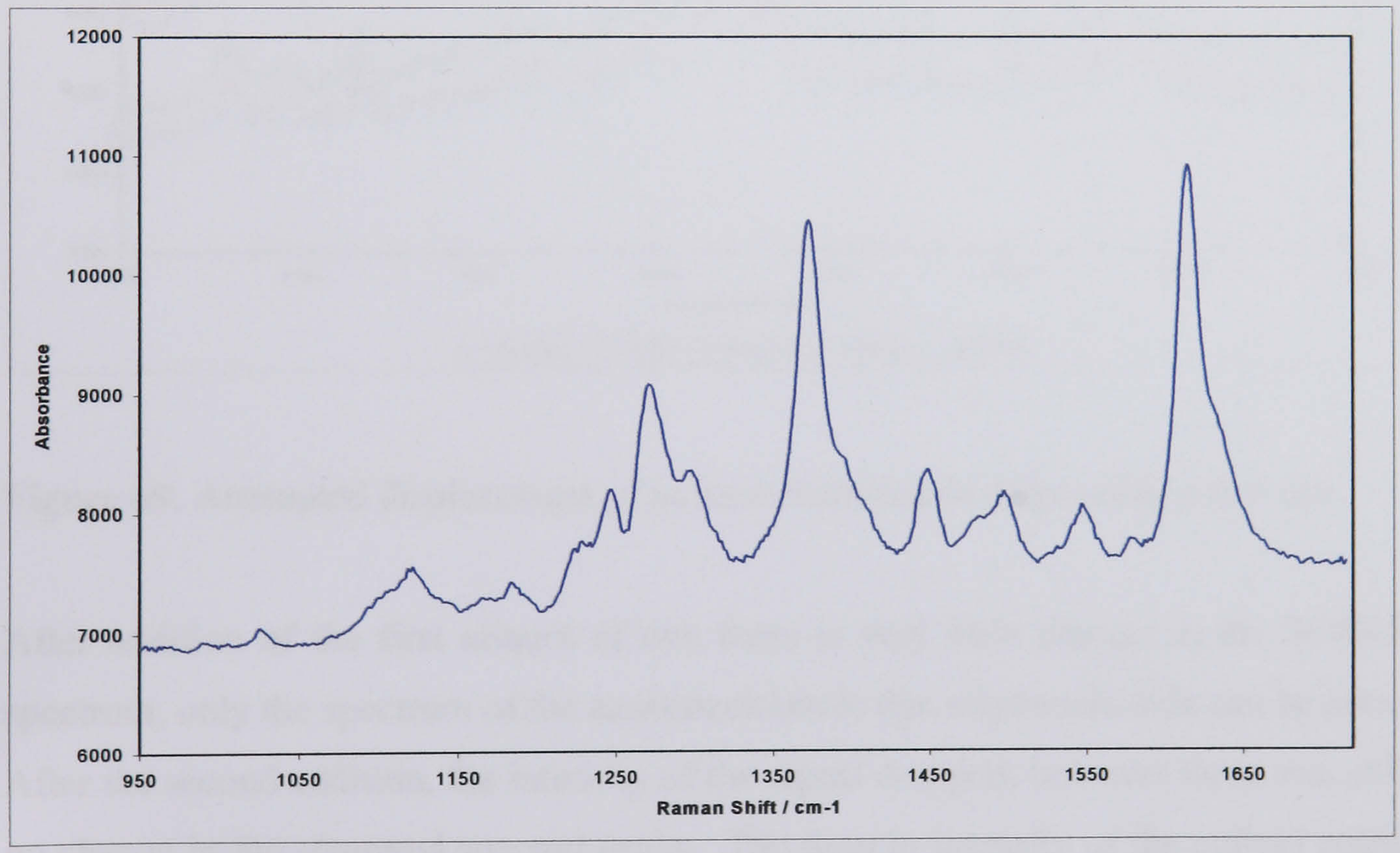


Figure 68. SERRS spectrum of 4-(5'-azobenzotriazolyl)phenylamine.

The labelled oligonucleotide was added to colloid and allowed to equilibrate for several minutes. Aggregation of the colloidal sample was ensured by the addition of 1×10^{-3} M spermine. The initial SERRS spectrum of the labelled oligo was obtained using a 514 nm laser source. Then a series of 5 μL aliquots of 4-(5'-azobenzotriazolyl)phenylamine (Figure 69) was added and the SERRS spectrum recorded five minutes after addition of the dye until the total amount of dye added was equal in concentration to the content of labelled oligo in the sample. Figure 69 shows the results obtained.

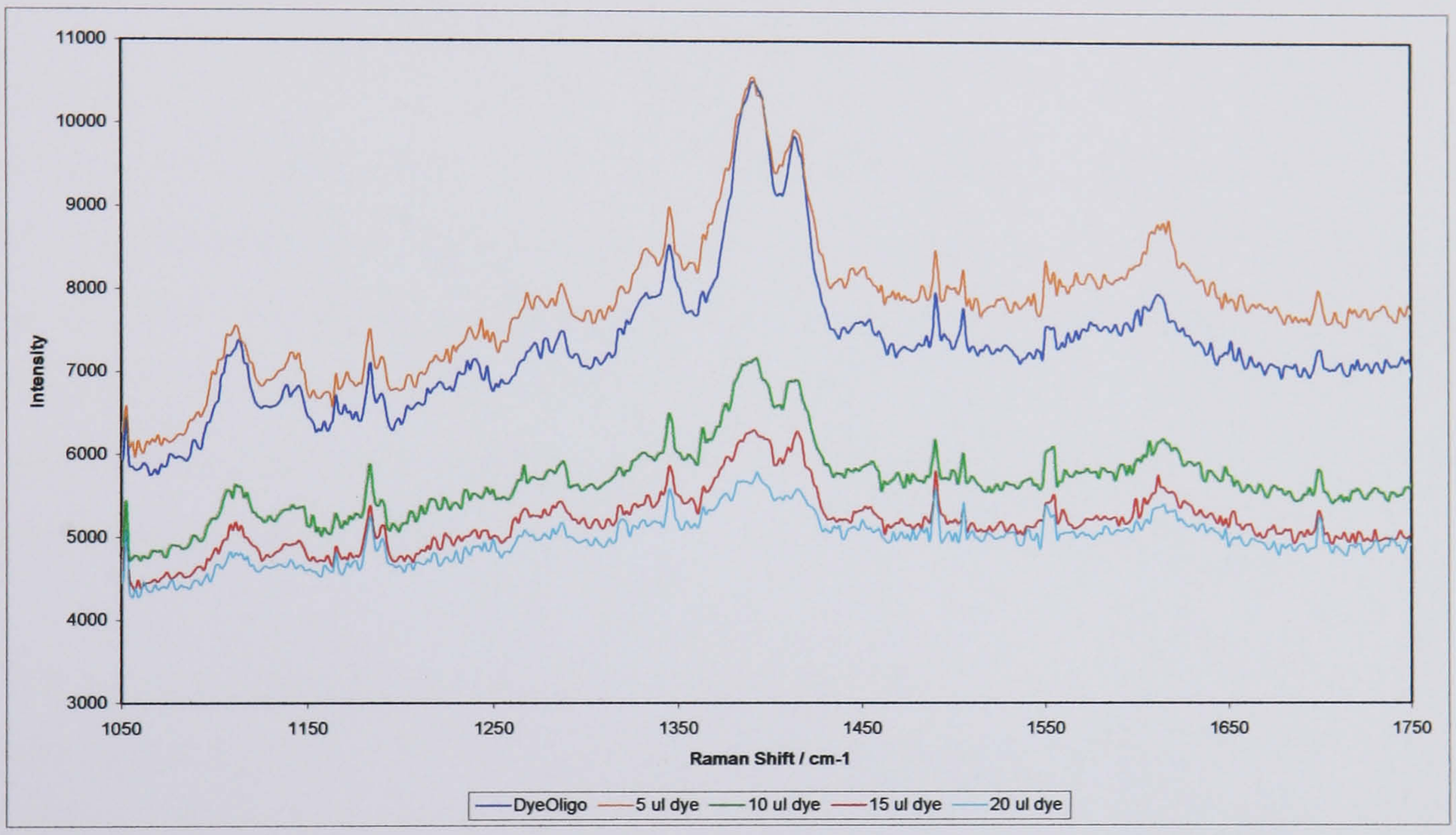


Figure 69. Attempted displacement of an azobenzotriazole oligo using a free dye.

After addition of the first aliquot of dye there is very little change in the SERRS spectrum, only the spectrum of the azobenzotriazole dye oligonucleotide can be seen. After the second addition, the intensity of the signal dropped, however there was still no change in the observed spectral peaks. The drop in intensity of the colloid could be due to precipitation of the colloidal particles caused by over aggregation. Over time the aggregated colloid sample becomes unstable and the colloid begins to precipitate. Further additions of dye resulted in the same effect however no traces of the characteristic 4-(5'-azobenzotriazolyl)phenylamine signal was observed.

This result indicates that either the bond between the azobenzotriazole dye labelled oligo and the colloidal surface is very robust, or that the DNA surrounding the particle prevents other species from reaching the colloidal surface or a combination of these two factors. In either instance, this provides the basis for reliable analysis.

4.6 Ultraviolet Melting Studies of SERRS Labelled Oligonucleotides

The aim of this section was to carry out initial Ultraviolet melting studies to assess how addition of the SERRS label to a single strand oligonucleotide affected the formation of a double helix. This is an important factor that has to be assessed, since large differences in the melting temperature (T_m), i.e. a decrease in stability of a double helix containing the SERRS label in comparison to the corresponding unmodified sequence, can have implications for the eventual intended application of any probe produced.

A large decrease in T_m over unmodified DNA indicates that the modification de-stabilises the double helix which means that hybridisation would be unfavourable, therefore the use of the modified oligonucleotide as a hybridisation probe would not be effective. Similarly, a large increase in T_m indicates that the label stabilises the formation of a double helix, again if the probe was used as a hybridisation probe, then the stabilising effect could increase the possibility of hybridisation to the wrong sequence.

The affect on melting temperature by modification by an azobenzotriazole dye was assessed using **Oligo 5**. The complementary sequence to this oligonucleotide was synthesised in addition to the unmodified sequence, shown in figure 70.

Oligo 5	5' DYE **[31]** GAA TCA CGA ATA TCA ATT TGT AGC
Unmodified	5' GAA TCA CGA ATA TCA ATT TGT AGC
Complement	5' GCT ACA AAT TGA TAT TCG TGA TTC

Figure 70. Oligonucleotides used in ultraviolet melting studies.

The oligonucleotides to be compared were dissolved in hybridisation buffer consisting of 0.1 M sodium chloride, 0.01 M sodium dihydrogenorthophosphate and 0.001 M EDTA, pH controlled at 7.0. The samples were cooled to 5 °C to allow formation of the double helix then warmed gradually by 1 °C per minute until a final temperature of 80 °C in the first instance to find the range of the melting curve for the particular sample. The UV absorbance was monitored throughout this process. Figure 71 Shows the UV melting curve obtained for both samples, one containing the unmodified oligonucleotide and the complement and the other containing the SERRS labelled oligonucleotide and the complement. In each sample the ratio of complement to oligonucleotide under investigation was kept equivalent at a concentration of 1.61×10^{-6} M.

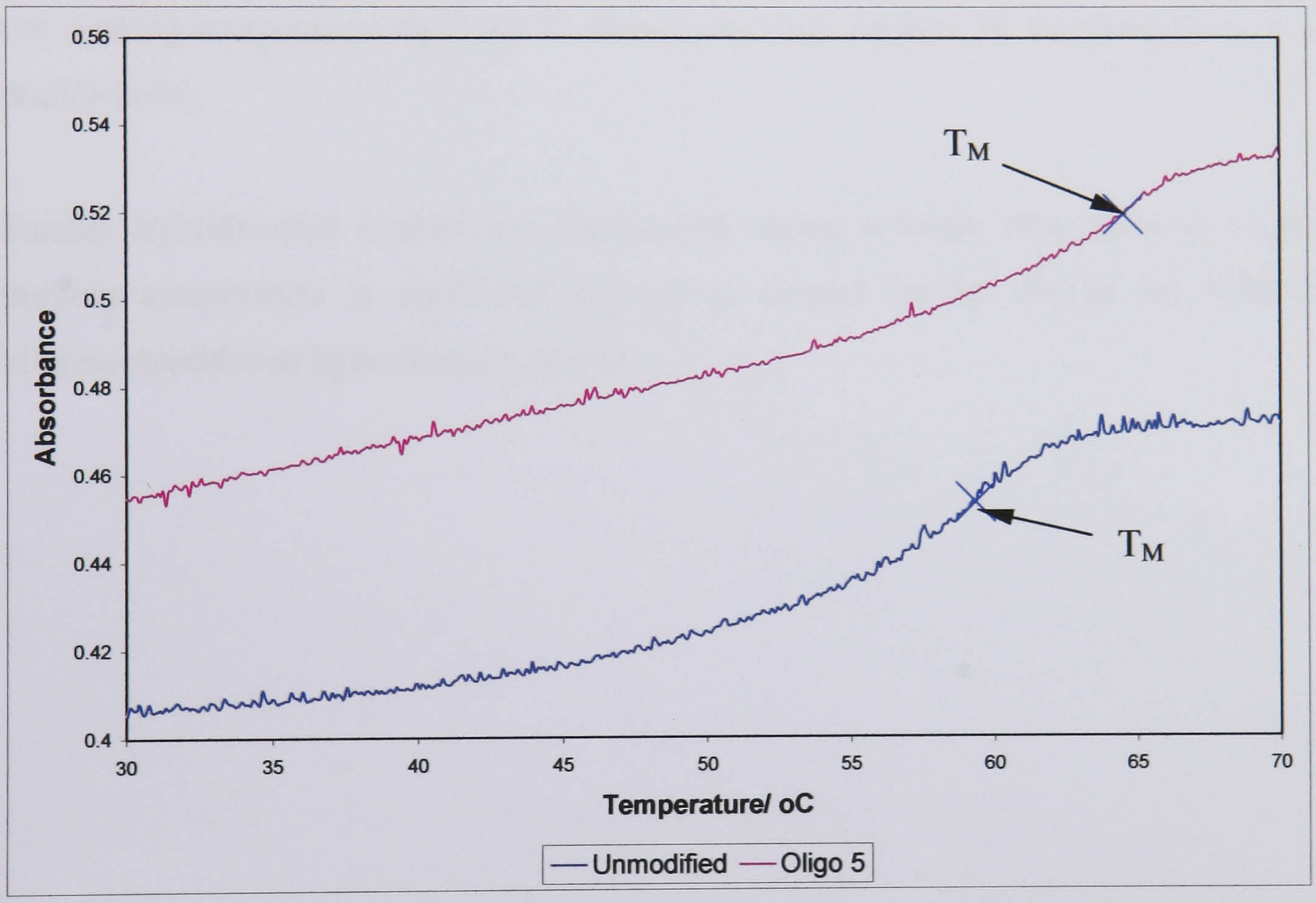


Figure 71. UV melting curves.

The melting temperature was determined by producing the 1st derivative spectrum of the melting curve which showed the main point of inflection.

The process was carried out three times for each oligonucleotide and the average value determined, following results were obtained (Table 6).

	Average T_m / °C
Oligo 5	64.49
Unmodified	59.07

Table 6. Melting temperature data.

It can be seen from the above table that modification of the 5' hydroxyl of a sequence of DNA by the addition of this particular azobenzotriazole dye increases the melting temperature by 5.42 °C thus conferring stability to the formation of a double helix.

Further hybridisation studies are required to assess whether this increase in the melting temperature is significant enough to impact on the use of the SERRS oligonucleotides as hybridisation probes.

4.7 Conclusions

A series of optimisation experiments were carried out to determine the optimum conditions for the SERRS analysis of the novel azobenzotriazole dye oligonucleotides. The optimum conditions were 3-6 hours incubation period to allow the labelled oligonucleotide to effectively adsorb to the colloidal surface, aggregation with 1 x 10^{-4} M of spermine and immediate analysis of the aggregated sample.

The SERRS spectra were obtained for each labelled oligonucleotide using a 514 nm and a 785 nm laser source. The spectra in each case were similar to each other with the azo band at 1390 cm^{-}1 as the main spectral feature. The lowest concentration at which SERRS of the labelled oligonucleotides could be detected was 2.5 x 10^{-9} M.

The SERS spectrum of an oligonucleotide containing benzotriazole at the 5' end was obtained.

Studies were carried out to elucidate the nature of the azobenzotriazole interaction with the colloidal surface. A qualitative citrate assay was developed to prove that adsorption of the benzotriazole group to the colloidal surface involved the displacement of the citrate layer surrounding the colloid particle. Further studies showed that the azobenzotriazole dye labelled oligonucleotides were not displaced from the colloidal surface by another azobenzotriazole dye, suggesting robust surface attachment.

Initial UV melting analysis of an oligonucleotide labelled with an azobenzotriazole dye shows that the modification increases the T_m by 5.42 ^{o}C over the unmodified sequence.

4.8 Experimental

4.8.1 General

Solvents were of HPLC grade and distilled water was used throughout. All chemicals were supplied by Aldrich except sodium citrate, which was supplied by Fisons. UV-visible spectra were recorded using a Perkin Elmer Lambda 35 Spectrometer. SERRS and SERS spectra were obtained using a Renishaw Ramascope 2000 instrument. An Omnichrome argon-ion laser was used to provide the 514.5 nm source. All UV melting data was recorded using a Cary 300 Bio Spectrometer.

4.8.2 Concentration Determination of Modified Oligonucleotides

The concentrations of the DNA stock solutions were measured using the UV absorption coefficient and measurements from a dilution of the oligonucleotide. The samples measured contained 20μl of the DNA stock solution, diluted to 2ml in distilled water. The absorbance value of the sample was noted at 260nm (the absorption maximum of the DNA bases). From the Beer Lambert Law (equation 1) the absorbance value is converted to the concentration of the sample.

$$A = \varepsilon_{260} c l \qquad \textbf{Equation 1}$$

The absorption coefficient (ε_{260}) must be determined for each oligonucleotide as it is a cumulative sum of the absorption coefficients of the individual DNA bases. The absorption coefficient values for each DNA base are outlined below:

$G = 11{,}700\ mol^{-1}\ cm^{-1}$

$A = 15{,}400\ l\ mol^{-1}\ cm^{-1}$

$C = 7{,}300\ l\ mol^{-1}\ cm^{-1}$

$T = 8{,}800\ l\ mol^{-1}\ cm^{-1}$

The following general equation (2) gives the total absorption coefficient of a labelled oligonucleotide, where L is the absorption coefficient of the free label at 260 nm. Note a hypochromicity factor of 0.9 is included. The absorption coefficient of any added label (L) is added as this contributes to the total value for the oligo.

$$a = \{(dG \times n) + (dA \times n) + (dC \times n) + (dT \times n)\}\ 0.9 + L \qquad \textbf{Equation 2}$$

4.8.3 Colloid Preparation

Silver colloid was prepared by a Lee and Miesel procedure, modified as outlined below.

All glassware was soaked in aqua regia (HNO_3: HCl 1:3, v/v) overnight before being washed with a soap solution and rinsed thoroughly with distilled water. Silver nitrate was suspended in degassed, distilled water (500ml). This solution was heated with constant stirring, and an aqueous solution of tri-sodium citrate (10ml, 1% w/v) was added just before the solution began to boil. The heat was reduced and the solution was kept boiling gently for a further 90 minutes with constant stirring.

The quality of the colloid was assessed by UV-visible spectroscopy. A sample of the colloid was diluted with distilled water and the UV-vis spectrum obtained (using distilled water as the background). The absorption maximum (λ_{max}) of the colloid was 401 nm, with a half height peak width of 65nm. The ideal colloid has a λ_{max} of 400-403 nm and a half height peak width of 60-65nm, these criteria ensure a highly monodispersed colloid.

4.8.4 SERRS Signal Variation Over Time Study

Before the experiment was carried out, a blank sample was analysed to ensure no contamination was present. The blank preparation procedure was as follows, 50 µl of distilled water was added to 400 µl of colloid and then aggregated with 25 µl of a 1×10^{-3} M solution of spermine. An aliquot of the sample was transferred to a microtitre plate for analysis.

After obtaining a satisfactory blank spectrum, 25 µl of oligo 5 and 25 µl of distilled water was added to 400 µl of colloid. The sample was aggregated by the addition of

25 μl of spermine as before for the blank. The first SERRS spectrum was obtained 15 s after aggregation and subsequent spectra obtained at 30 s intervals for a total time of 10 to 20 minutes depending on the quality of the signal. The spectrometer grating was fixed at 1400 cm^{-1} throughout the experiment, using a x10 objective. Each spectrum consisted of one accumulation of 10 s in length.

4.8.5 Optimisation of Sample Equilibration Times

A series of identical samples were prepared containing 25 μl of 4 x 10^{-6} M labelled oligo 5 and 400 μl of colloid. The samples were left to equilibrate for 0 hrs, 1 hr, 3 hrs, 6 hrs and 24 hrs. After the equilibration time was complete, each sample was aggregated using spermine. Half of the samples were aggregated with 25 μl of 0.1 M spermine and the other half with 1 x 10^{-3} M. The spectra were obtained using a x10 objective with one accumulation of 10 s for each sample.

4.8.6 Optimisation of Aggregation Conditions

All samples contained 450 μl of colloid, 25 μl of 4 x 10^{-6} M oligo 5 and were allowed to equilibrate for 4.5 hours prior to analysis. Samples were then aggregated with 25 μl of the chosen aggregating agent and then analysed using a x 10 objective with one accumulation of 10s.

4.8.7 Optimised SERRS Spectra of Azobenzotriazole Dyes

Each labelled oligo was analysed in the same way, 25 μl of the oligo was pre-equilibrated with 450 μl of colloid for 4.5 hours. After this time, the samples were aggregated by the addition of 25 μl of 1 x 10^{-4} M spermine. Spectra were obtained using a x10 objective with one accumulation of 10 s for each sample.

4.8.8 SERS Spectrum of a Benzotriazole Modified Oligo

Oligo 4, containing a benzotriazole group, (200 μl of a 1.5 x 10^{-5} M solution) was added to 200 μl of colloid and the sample aggregated with 20 μl of a 1 x 10^{-3} M spermine solution. The sample was centrifuged at 6000 rpm for 15 minutes and 90 % of the resulting supernatant removed. The silver pellet was resuspended and placed onto a glass slide for SERS analysis. The spectrum was obtained using a x20 objective and acquiring three accumulations of 10 s.

4.8.9 Qualitative Assessment of the Benzotriazole-Colloid Interaction by a Citrate Assay

Each sample was prepared by addition of 100 μl of distilled water for a blank, or 100 μl of dye solution to 1 ml of colloid. The samples were allowed to equilibrate for 15 minutes and then centrifuged for 15 minutes at 6000 rpm. After this time 1.0 ml of the supernatant was removed and treated with 50 μl of iron III solution (0.05 M $Fe(III)(NO_3)_3.9H_2O$ in 0.01 M HCl). The samples were then activated by exposure to UV light (a UV spectrum was recorded in the region 200 nm to 800 nm) and developed by the addition of 50 μl of 1,10-phenanthroline (0.05 M solution in distilled water). The UV-visible spectrum of the sample was then recorded.

4.8.10 Displacement Studies

A sample containing 20 μl of a 1 x 10^{-7} M solution of oligo 1, 460 μl of colloid and 20 μl of 1 x 10^{-3} M spermine was prepared in a quartz cuvette. The initial SERRS spectrum was obtained, then sequential aliquots of 5 μl of azobenzotriazole dye were added and the SERRS spectrum recorded 15 minutes after addition of each aliquot.

Chapter 5

Use of SERRS Labelled Oligonucleotides as Nanoparticle Probes

5.0 Use of SERRS Labelled Oligonucleotides as Nanoparticle Probes

5.1 Introduction

The final aim of this research was to apply the SERRS labelled oligonucleotides to a potential application that would best demonstrate their unique properties. One such area is in the field of specific DNA sequence detection using 'nanoparticle probes'. Mirkin and co-workers have carried out research utilising gold colloidal particles derivatised with thiol modified oligonucleotide probes as a colorimetric means of detecting the presence of specific sequences of DNA.[65-68] In this approach, the modified oligonucleotide of chosen sequence was bound to the colloidal surface through a thiol group at either the 3' or 5' end to form the nanoparticle probe. Two such nanoparticle probes can hybridise with a target DNA strand of complementary base sequence to form an extended network of nanoparticles linked together by duplex DNA. When this process occurs the optical properties of the gold colloid changes.

As prepared, the gold colloid is reddish in colour, however on hybridisation the colloidal particles are drawn closer together which alters the plasmon resonance and shifts the colour to blue. Only addition of the correct target sequence allows hybridisation to induce the aggregation process and colour change.

A similar procedure was attempted using SERRS labelled oligonucleotides to modify citrate reduced silver colloid, with the benzotriazole group as the means of surface attachment in place of the thiol group. The SERRS labelled oligonucleotides are well suited to such an application since the azobenzotriazole moiety is designed to bind irreversibly to a metal surface in order to give SERRS. This provides a robust means of attaching the oligonucleotide to the colloidal surface. Further, the presence of the azo chromophore allows SERRS detection and provides a molecular fingerprint of the nanoparticle. Since aggregation of the colloid is required before

SERRS is observed, this provides a sensitive method of detecting specific DNA sequences, as hybridisation and therefore colloidal aggregation only occurs when the target sequence is present and complementary to the probe sequences. However, the full development of a detection system is outwith the scope of this thesis. As an assessment of the feasibility of such an application, the initial stage of preparation of a SERRS nanoparticle probe was attempted.

5.2 Preparation of SERRS Nanoparticles

A procedure for the modification of the colloidal surface was developed using a method devised by Mirkin *et al.* for the modification of gold colloid as a template.[66] Figure 72 shows a schematic of Mirkin's nanoparticle probe preparation procedure.

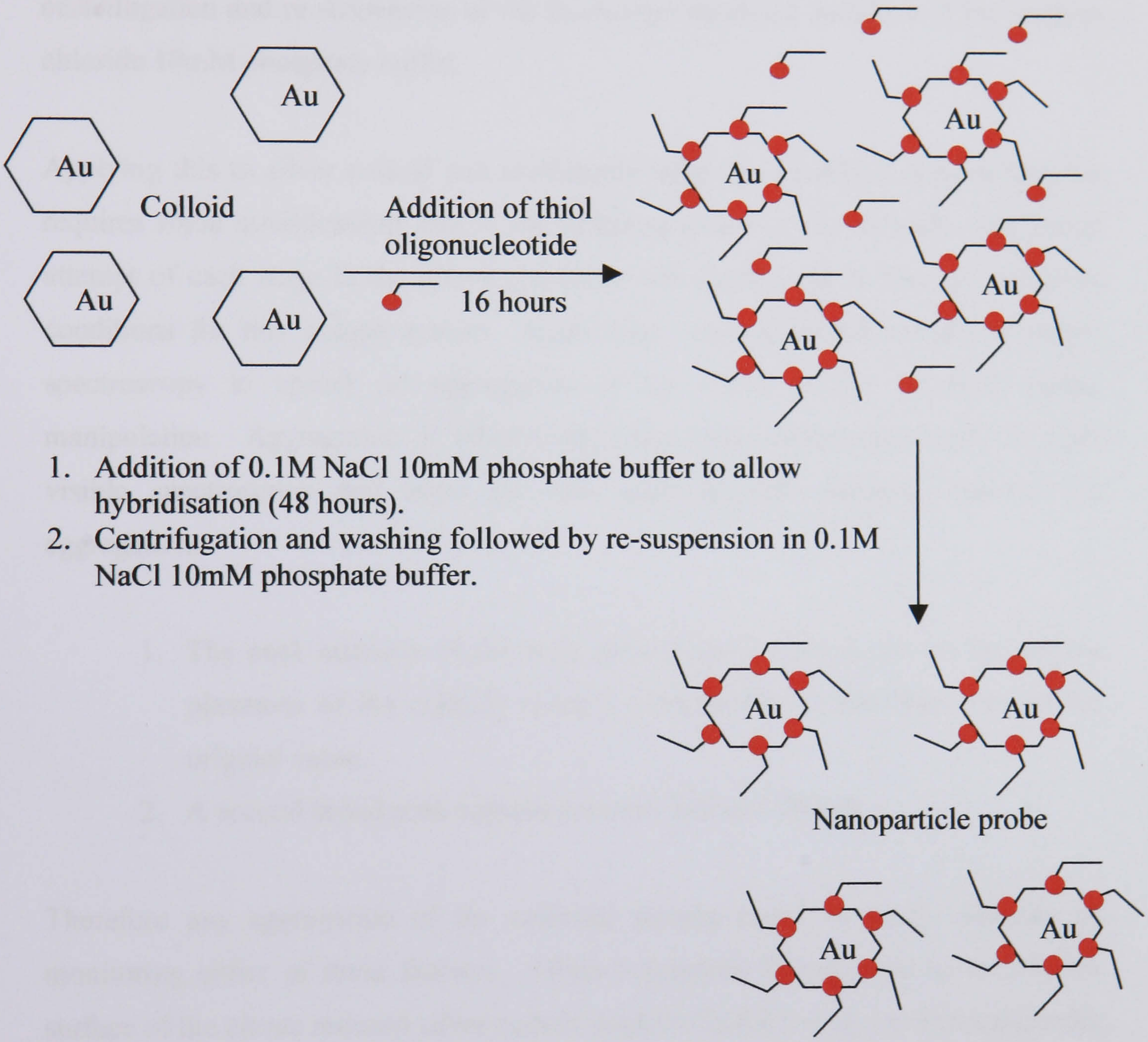


Figure 72. Schematic of the nanoparticle probe preparation procedure.

The first step of this procedure was incubation of the thiol oligonucleotide with the gold colloid for 16 hours at room temperature to allow surface adsorption to occur and give maximum surface coverage. The second step involved the introduction of

the salt buffer required for hybridisation of the oligonucleotides to form duplex DNA, this was carried out by the addition of an aliquot of a 10mM phosphate buffer. After 10 minutes equilibration, sodium chloride solution was added to a final concentration of 0.1M to aid hybridisation, and the thiol-oligo colloidal suspension left to equilibrate over a 40 hour period. Finally a washing step was carried out several times to remove any unbound thiol-oligo, this was carried out by centrifugation and re-suspension of the thiol-oligo modified colloid in 0.1M sodium chloride 10mM phosphate buffer.

Applying this to silver colloid and azobenzotriazole dye modified oligonucleotides requires some modifications due to the differing nature of the colloids. An initial attempt of each stage in the above procedure was carried out to find the optimum conditions for this unique system. Each stage was monitored using UV-visible spectroscopy to ensure no aggregation of the silver colloid occurred during manipulation. Aggregation of silver colloid has been studied previously by UV-visible spectroscopy and there are two main spectral changes observed on aggregation.

1. The peak intensity of the main absorption at 400nm (due to the surface plasmons of the colloid) reduces dramatically to less than 50% of the original value.
2. A second broad peak appears between 550 and 700nm.

Therefore any aggregation of the colloidal sample could be easily detected by monitoring either of these features. Oligo 5 (chapter 3) was used to modify the surface of the citrate reduced silver colloid used for SERRS analysis. Throughout the following experiments a control set of samples were prepared by addition of distilled water to the colloid and treated in exactly the same way as those containing the azobenzotriazole dye oligonucleotide for comparison.

The azobenzotriazole dye oligo was mixed with the silver colloid and allowed to equilibrate for 16 hours at room temperature. Figure 73 shows the UV-vis spectra of the initial colloid and following addition of the labelled oligonucleotide.

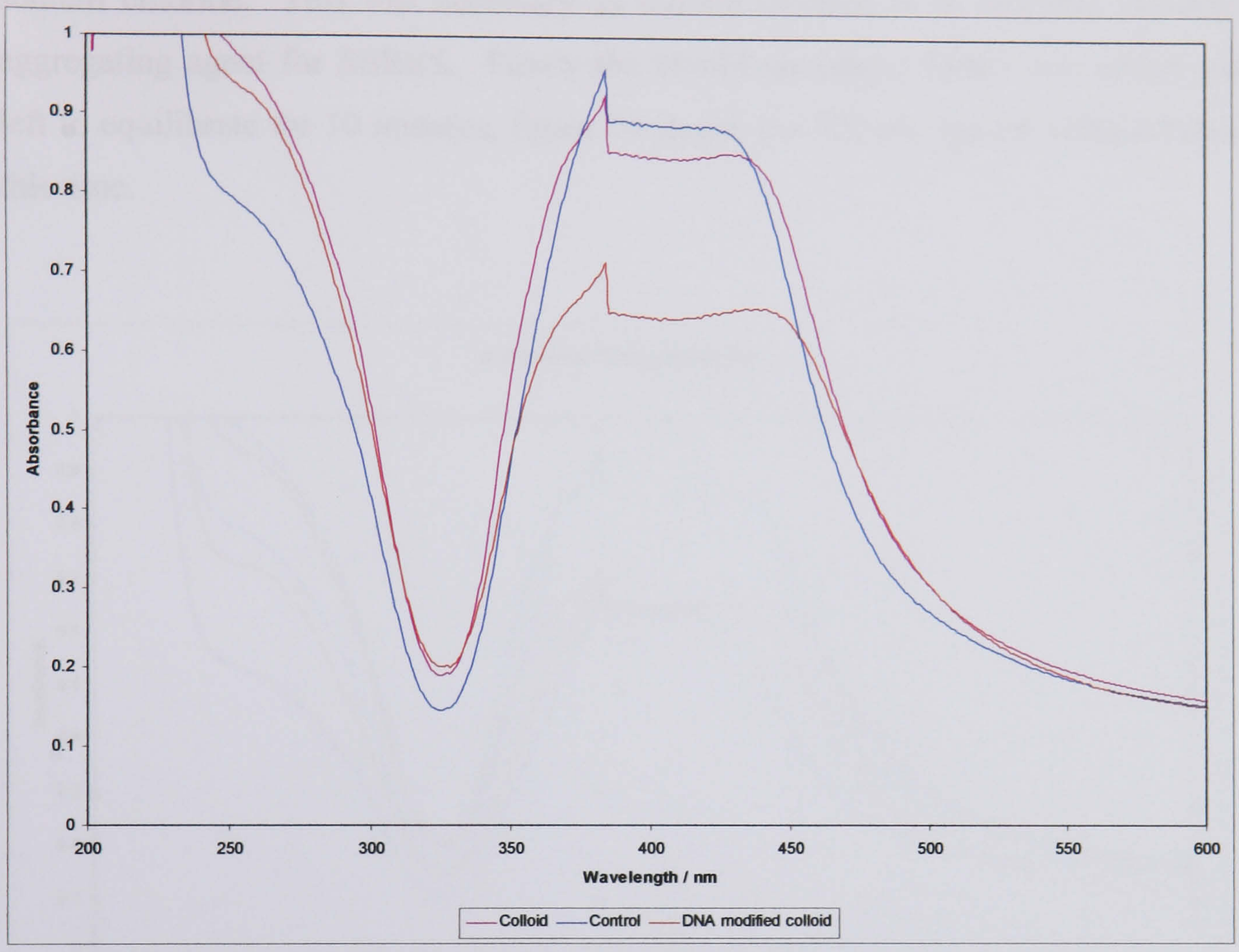


Figure 73. UV-vis spectra of colloid on addition of the azobenzotriazole oligo.

From the spectra it can be seen that there is little difference between the colloid spectrum and the control, as expected. There is a spike in the spectrum at around 384 nm which is due to a filter wheel misalignment. There is a slight reduction and flattening of the main colloid peak at 400 nm on addition of the azobenzotriazole oligo, however there is no indication of aggregation from the region around 550nm. This suggests there is no aggregation on addition of the oligo and indeed the colloid remains in solution after several days, whereas aggregated colloid normally precipitates from solution within minutes.

The second stage in the preparation of a SERRS nanoparticle is addition of the 0.1M sodium chloride 10mM phosphate buffer required for DNA hybridisation. In order to ensure no aggregation occurred the addition of the necessary buffer was separated into two steps; addition of the phosphate buffer followed by careful addition of the sodium chloride. This was necessary as sodium chloride is an effective colloidal aggregating agent for SERRS. Firstly the 10mM-phosphate buffer was added and left to equilibrate for 10 minutes, figure 74 shows the UV-vis spectra obtained after this time.

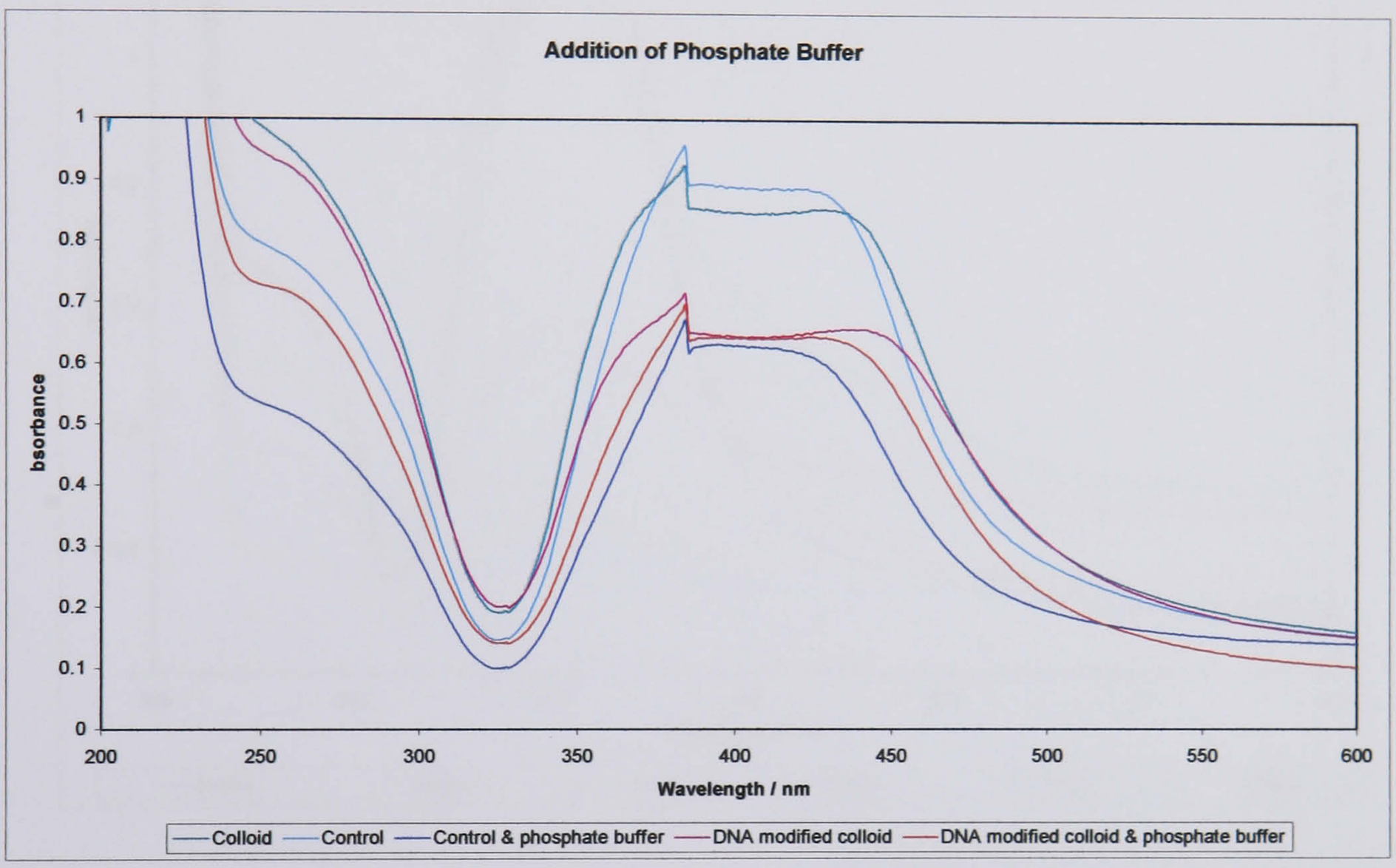


Figure 74. Effect of addition of phosphate buffer on colloidal samples

Addition of phosphate buffer to the control sample caused a decrease in the peak at 400nm but again there was no indication of aggregation in the region above 550nm, this reduction in absorbance could be simply a dilution effect on increasing sample volume. Addition of phosphate buffer to the DNA modified colloid produced no appreciable effect in the spectrum compared to the spectrum of the colloid containing DNA only.

The second step of the buffer addition was addition of sodium chloride to give an overall sample concentration of 0.1M. In order to find out the maximum amount of sodium chloride tolerated by the silver colloid, 5µl aliquots of 0.4M sodium chloride solution were added until the colloid aggregated and precipitated from solution. Figure 75 shows the UV-vis spectra obtained from addition to the control sample containing water colloid and 10mM phosphate buffer.

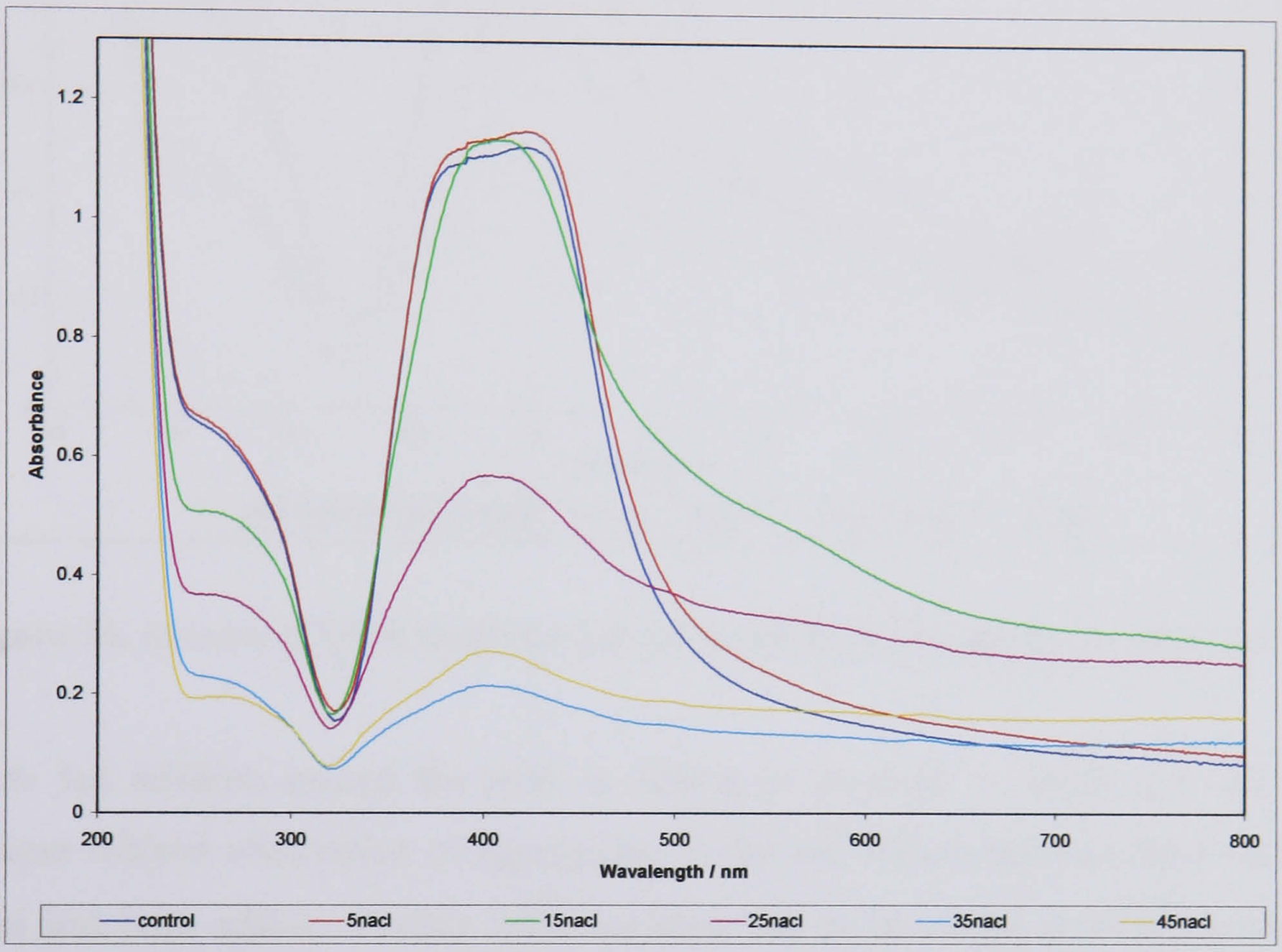


Figure 75. Spectra of the control sample on addition of aliquots of 0.4M NaCl.

From figure 4, it can be seen that the intensity of the 400nm peak reduces slightly on each addition of sodium chloride and then a sharp decrease after the addition of 25µl. An increase in absorbance is seen in the region 550-700nm after the addition of 15µl and remains until precipitation of the colloid occurs after the addition of 45µl of 0.4M sodium chloride. This follows an expected pattern due to the gradual reduction of the colloidal surface charge by sodium ions until aggregation occurs.

The sequential addition of sodium chloride to the DNA modified colloid with phosphate buffer, caused an unexpected change in UV-vis spectrum (figure 76).

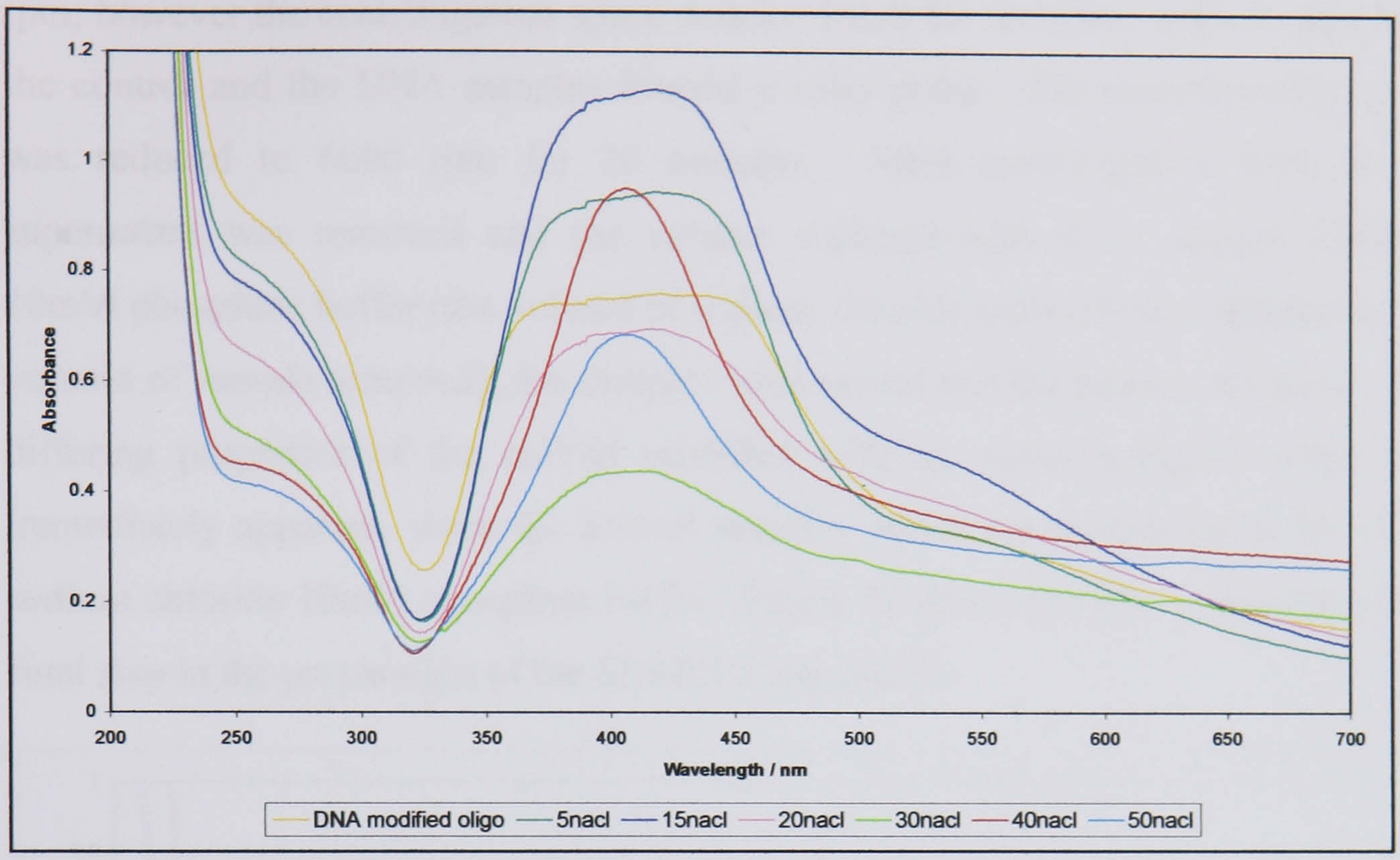


Figure 76. Spectra of DNA modified colloid on addition of aliquots of 0.4M NaCl.

Each 5µl addition caused the peak at 400nm to increase in height and become sharper without observation of aggregation in the 550-700nm region, until a total of 15µl had been added. Further additions after this point caused a reduction in the 400nm peak followed by an increase in absorption in the 550-700nm region, resulting in precipitation of the colloid. This divergence in behaviour from the control sample suggests a modification in the colloidal surface due to the surface adsorption of the azobenzotriazole oligo and hence a modification in properties occurs. It is possible that the surface is completely covered with benzotriazole groups which link together in a network providing a protective layer. At the same time the attached DNA sequence extending from the surface provides a polyanionic boundary to the colloidal particle, preventing aggregation unless this ionic character is neutralised by a polycationic species, such as spermine.

The final step in the preparation of the SERRS nanoparticle probe was the washing step to remove any free azobenzotriazole dye oligonucleotide left in solution. Following Mirkin's procedure, the samples were centrifuged for 25 minutes at 14000 rpm, however the centrifugation speed was too harsh for the silver colloid since both the control and the DNA samples formed a solid pellet. The centrifugation speed was reduced to 6000 rpm for 20 minutes. After centrifugation 50% of the supernatant was removed and the volume replaced with 0.1M sodium chloride 10mM phosphate buffer (the volume of sodium chloride replaced was identical to the volume of sample removed), the samples were mixed and the process repeated. The differing properties of the colloid modified with an azobenzotriazole oligo was immediately apparent, since the control samples would not re-suspend in the 0.1M sodium chloride 10mM phosphate buffer. Figure 77 shows the UV-vis spectra of the final step in the preparation of the SERRS nanoparticle.

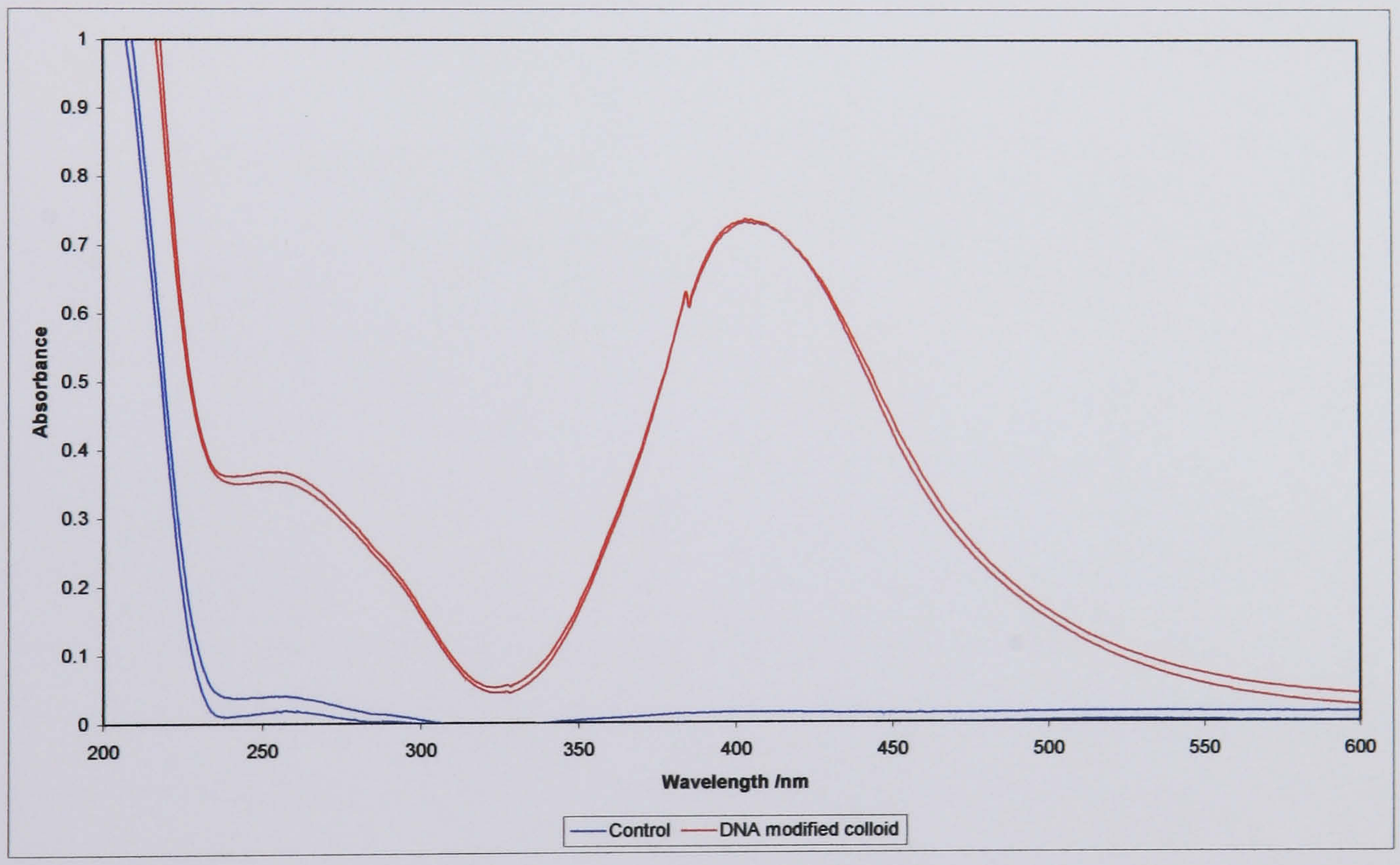


Figure 77. UV-vis spectra of samples on centrifugation and re-suspension in buffer.

The spectra of the samples after centrifugation and re-suspension in buffer show a striking difference, the control samples show complete precipitation of the colloidal particles, however the colloid modified with an azobenzotriazole oligonucleotide remains in solution without any sign of aggregation. This suggests that the azobenzotriazole-modified oligonucleotide provides robust surface attachment and in effect protects the colloidal surface.

5.3 SERRS analysis of Nanoparticles Coated with an Azobenzotriazole labelled Oligonucleotide

Finally, the SERRS activity of the SERRS nanoparticles was assessed to decide whether the azobenzotriazole dye labelled oligonucleotides would be successful for this application. To allow specific sequence detection using SERRS nanoparticles, the probes must only "switch on" i.e. a SERRS spectrum must only be detected after hybridisation of the target sequence to the nanoparticle probes. Successful hybridisation of the correct target sequence brings the nanoparticles into close proximity, effectively inducing aggregation which is necessary to detect SERRS from the labelled probes. Figure 78 shows a schematic of this process.

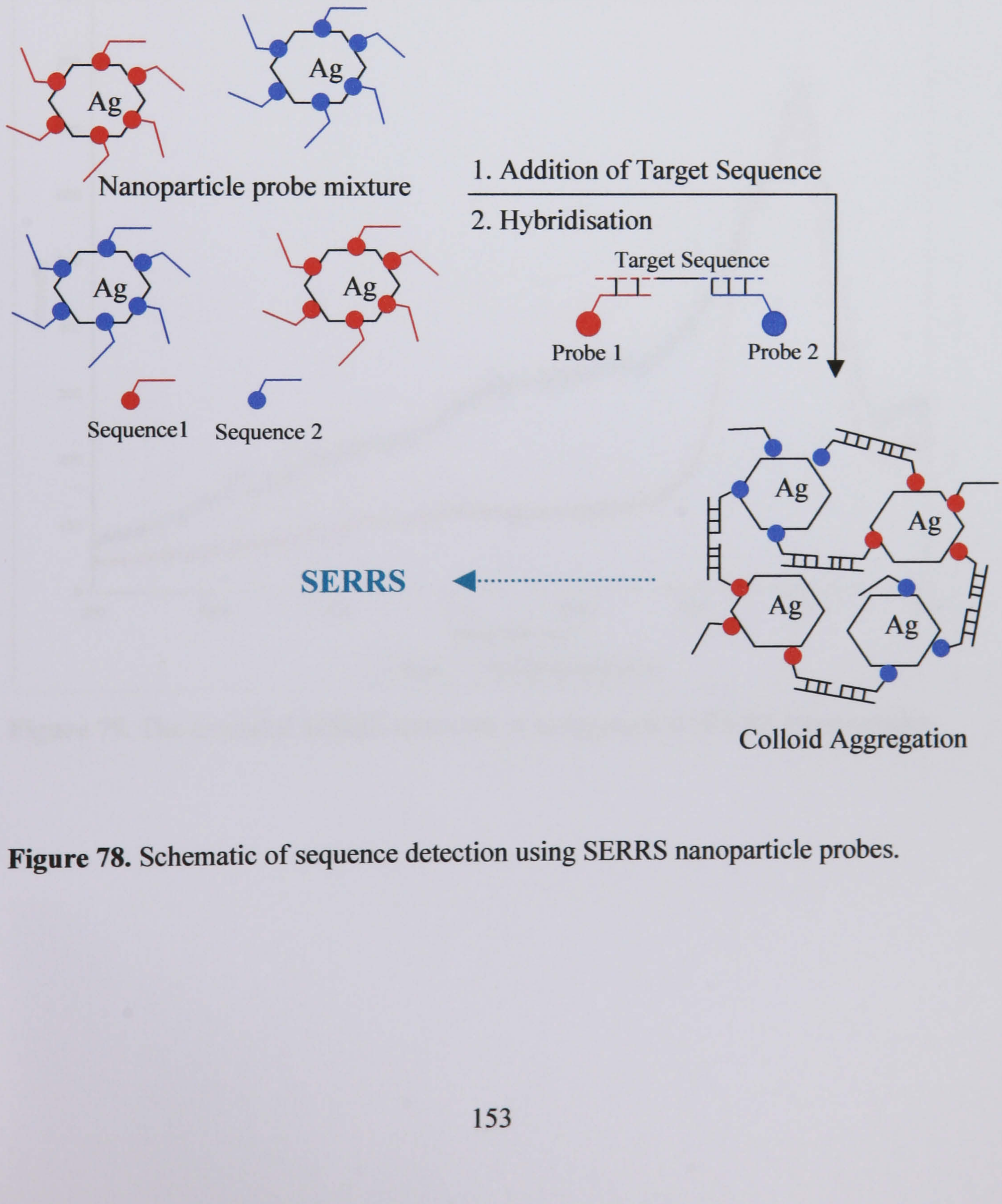


Figure 78. Schematic of sequence detection using SERRS nanoparticle probes.

Since the integrity of the technique relies upon production of a SERRS signal on aggregation of the sample and at no other time, it was crucial to confirm that the SERRS nanoparticles produced no SERRS individually but that the effect could be switched on by addition of an aggregating agent. Figure 79 shows the spectra obtained from a SERRS nanoparticle probe functionalised with **oligo 5** (prepared as outlined in section 5.2 of this chapter) in comparison to blank colloid. The blank sample consisted of 450 μl colloid, 25 μl of distilled water and 25 μl of phosphate buffer. An extended spectrum was recorded from 200 cm^{-1} to 4000 cm^{-1} to ensure that no signals were obtained in any region for the unaggregated SERRS nanoparticles.

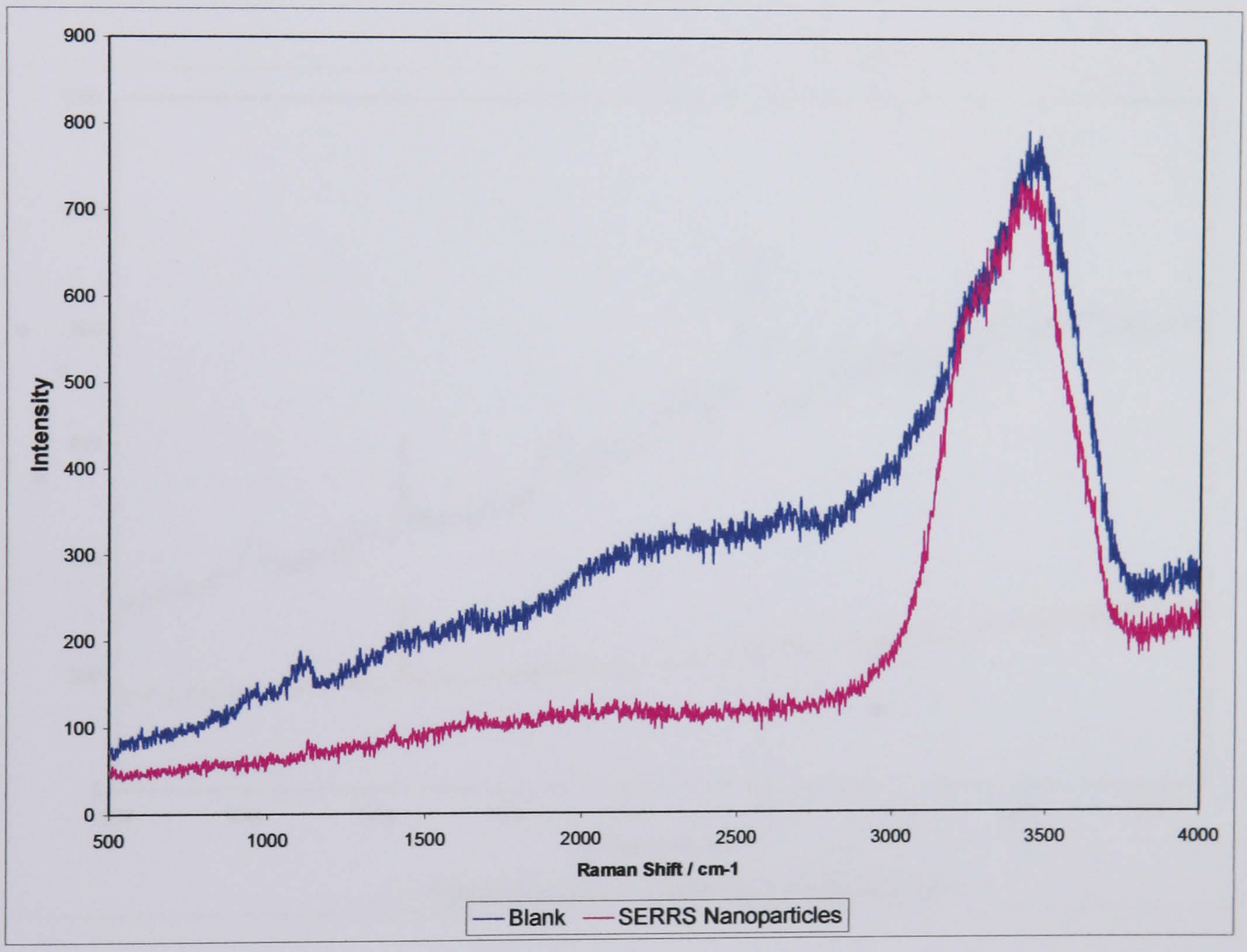


Figure 79. The extended SERRS spectrum of unaggregated SERRS nanoparticles.

It is clear from the above spectrum that no SERRS signals were obtained from the SERRS nanoparticles. In order to test the SERRS signal of the **oligo 5** labelled nanoparticles on aggregation, spermine was added. The use of spermine to effect aggregation instead of hybridisation of a target sequence, was due to the large amount of development work required to optimise the hybridisation process for such a unique system. This was beyond the scope of this project. Aggregation of the sample could be carried out easily by the addition of spermine, to assess whether the nanoparticles did in fact give SERRS.

Figure 80 shows the SERRS spectra of the above sample upon addition of 5 µl of 1 x 10^{-3} M spermine.

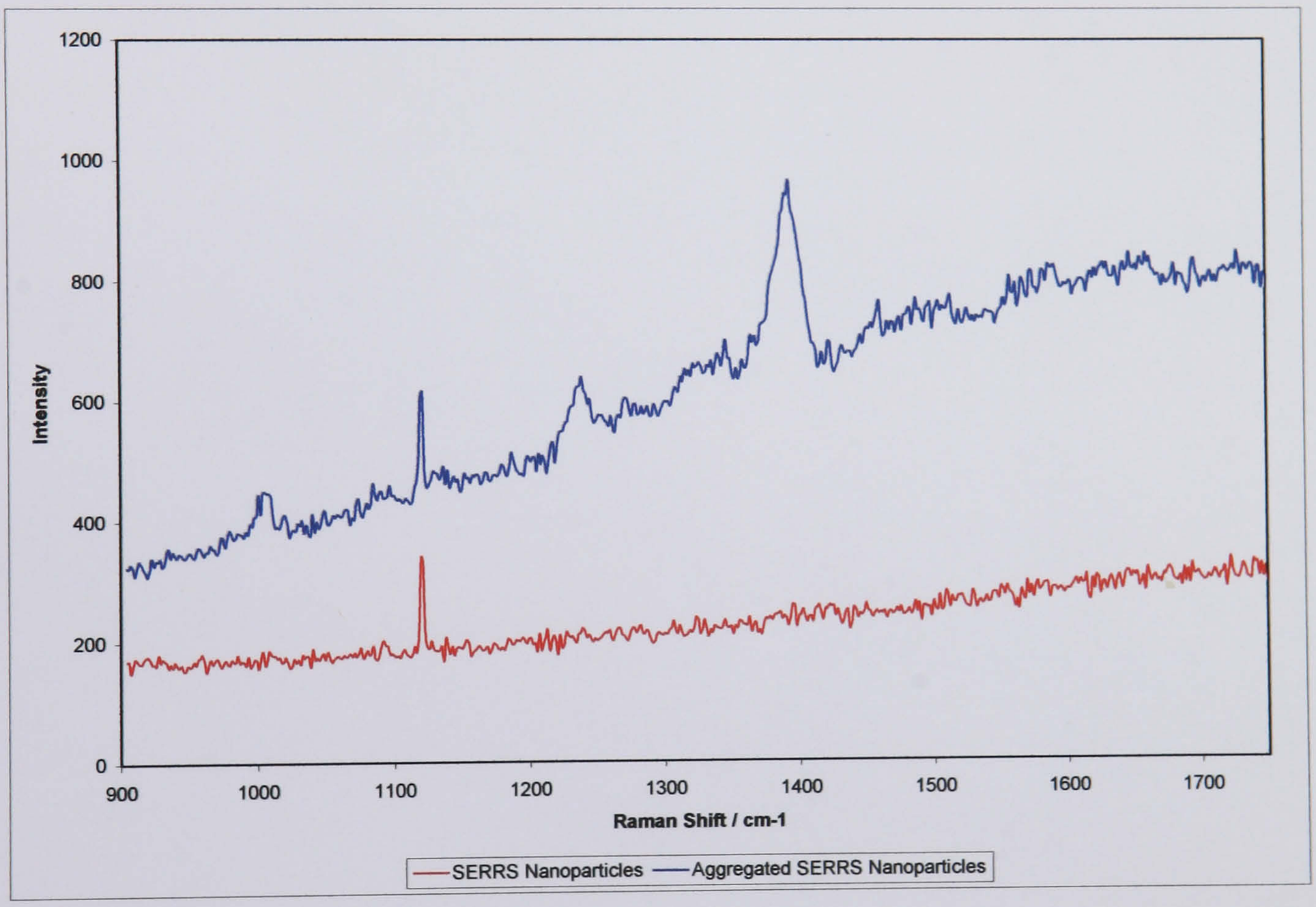


Figure 80. SERRS spectra of SERRS nanoparticles aggregated with spermine.

It can be seen from the above spectra that the main azo band at 1390 cm^{-1} is clearly present, thus confirming that the azobenzotriazole dye labelled oligonucleotide is

present in the sample. These results show that SERRS spectra can be obtained from the SERRS nanoparticles and that the effect can indeed be switched on by aggregation. This breakthrough confirms that the SERRS nanoparticles have the required properties for use in the assay describe in figure 78.

The success of this work provides the foundation for further development of the sequence specific detection method using the SERRS technique. In particular completion of the target hybridisation procedure requires to be carried out, since time did not permit for this body of work. However the aims of this chapter have been fulfilled, since a possible application of theses novel labelled oligonucleotides has been highlighted and proven in essence.

5.4 Conclusion

A procedure for the preparation of a SERRS nanoparticle was successfully developed. The surface of citrate reduced silver colloid was modified to contain an azobenzotriazole oligonucleotide, which dramatically altered the properties of the colloid. This allowed the successful preparation of the first specifically designed SERRS nanoparticle.

SERRS spectra of the nanoparticles were only obtained on aggregation, thus providing proof of principle that the SERRS nanoparticles could be prepared and have the desired properties to fulfil the ultimate gaols of the proposed assay. The success of this work provides the basis of a more indepth investigation into hybridisation of target sequences. This result constitutes an important milestone in the development of a specific sequence detection technique based on the SERRS effect.

5.5 Experimental

5.5.1 General

Solvents were of HPLC grade and distilled water was used throughout. All chemicals were supplied by Aldrich. All UV-visible spectra were recorded using a Perkin Elmer Lambda 35 Spectrometer. SERRS spectra were obtained using a Renishaw Ramascope 2000 instrument. An Omnichrome argon-ion laser was used to provide the 514.5 nm source.

5.5.2 Nanoparticle Probe Preparation Procedure

The labelled oligonucleotide was added to citrate reduced silver colloid to give an overall DNA concentration of 1.0×10^{-6} M and allowed to equilibrated for a period of 16 hours at room temperature. After this time, half of the total sample volume of 100 mM phosphate buffer was added (a mixture of 100mM $Na_2HPO_4.7H_2O$ and 100 mM of $NaH_2PO_4.2H_2O$). The samples were allowed to stand for 10 minutes before adding 15 μl of 0.4 M sodium chloride and then being left to equilibrate over 40 hours. After the equilibration period, the samples were centrifuged at 6000 rpm for 20 minutes and 50% of the supernatant volume removed. The sample volume removed was replaced by the addition of 0.1 M sodium chloride 10 mM phosphate buffer. The centrifugation and washing steps were repeated to yield the SERRS nanoparticle probe.

UV-visible monitoring of each stage of the procedure ensured no aggregation occurred.

5.5.2 SERRS Sample Preparation for Nanoparticle Probe

The blank sample was prepared by addition of 450 µl colloid to 25 µl of distilled water and 25 µl of phosphate buffer. The SERRS nanoparticle probe was analysed as prepared. An extended scan from 200 cm^{-1} and 4000 cm^{-1} was recorded by one accumulation of 10 seconds.

The SERRS nanoparticle probe sample was aggregated by the addition of 5µl of a 1×10^{-3} M solution of spermine. The sample was analysed immediately after aggregation, 5 accumulations of 10 seconds were recorded in each case with the spectrometer grating centred at 1350 cm^{-1}.

Chapter 6

Conclusions

6.0 Conclusions

A series of SERRS labels have been synthesized and attached to the 5' end of deoxyribonucleic acid (DNA), to allow analysis by surface enhanced resonance Raman scattering (SERRS) spectroscopy. This was accomplished by two successful strategies.

Three novel azobenzotriazole dyes have been synthesised. These SERRS active labels were each attached to the 5' end of a sequence of synthetic DNA *via* an amino linker. This was achieved by reaction of the activated carboxylic acid derivative of the SERRS label with DNA containing a free amine at the 5'end.

A successful methodology for the synthesis of a benzotriazole phosphoramidite has been devised. The protecting group strategy proved to be of great importance to the success of the synthetic route. Protection of the triazole ring with the monomethoxytrityl group allowed efficient conversion to the phosphoramidite. This method was extended to the synthesis of an azobenzotriazole dye phosphoramidite, where the trityl protecting group was employed for the protection of the triazole ring. This result constitutes the first example of a benzotriazole phosphoramidite.

Preliminary spectroscopic data has been obtained for the labelled oligonucleotides. Ultraviolet melting studies of a DNA sequence labelled with an azobenzotriazole dye show an increase in melting temperature (T_M) of 5 ^{o}C over the same sequence without modification, thus suggesting the label confers stability to the double helix. Initial SERRS optimisation experiments allowed the optimum sample conditions to be determined for these novel oligonucleotides. SERRS spectra have been obtained for each labelled oligonucleotide with a detection limit determined at 5×10^{-8} M.

A potential application of the labelled oligonucleotides was investigated resulting in the first ever preparation of a SERRS labelled nanoparticle probe. Initial proof of principle confirmed that the SERRS nanoparticles contained the required propties to

provide the basis for a specific sequence detection technique based on the SERRS effect.

Chapter 7

References

7.0 References

1. C. Rodger, W. E. Smith, G. Dent, M. Edmunson, *J. Chem. Soc., Dalton Trans.*, 1996, 791.
2. D. Voet, J. G. Voet, *Biochemistry,* 1995, Wiley & Sons.
3. J. D. Watson, F. H. C. Crick, *Nature,* 1953, **171**, 737.
4. J. D. Watson, F. H. C. Crick, *Nature,* 1953, **171**, 964.
5. E. Chargaff, *Experienta,* 1950, **6**, 201.
6. D. Voet, J. G. Voet, *Biochemistry,* 1995, Wiley & Sons.
7. G. M. Blackburn, M. J. Gait, *Nucleic Acids in Chemistry and Biology*, 1996, Oxford University Press.
8. M. H. Caruthers, *Science,* 1985, **230**, 281.
9. E. T. Kool, J. C. Morales, K. M. Guckian, *Angew. Chem. Int. Ed.*, 2000, **39**, 990.
10. A. Castro, R. T. Okinaka, *Analyst*, 2000, **125**, 9.
11. C. H. Munro, W. E. Smith, P. C. White, *Analyst,* 1995, **120**, 993.
12. M. Prochazka, P. Mojzes, B. Vlckova, P. Y. Turpin, *J. Phys. Chem. B,* 1997, **101**, 3161.
13. C. H. Munro, W. E. Smith, D. R. Armstrong, P. C. White, *J. Phys. Chem.,* 1995, **99**, 879.
14. T. M. Cotton, J. Kim, G. D. Chumanov, *J. Raman Spectrosc.,* 1991, **22**, 729.
15. B. N. Rospendowski, J. M. Campbell, J. Reglinski, W. E. Smith, *Eur. Biophys.J.,* 1992, **21**, 257.
16. C. Heywang, M. S. P. Chazalet, M. Masson, A. Garnier-Suillerot, J. Bolard, *Langmuir,* 1996, **12**, 6459.
17. K. Kneipp, W. Pohle, H. Fabian, *J. Mol. Struct.,* 1991, **244**, 183.
18. R. A. Alberty, R. J. Silbey, *Physical Chemistry*, 1992, Wiley.
19. M. Diem, *Introduction to Modern Vibrational Spectroscopy,* 1993, Wiley-Interscience.
20. M. Fleishman, P. J. Hendra, A. J. Mcquillan, *J. Chem. Phys. Lett.,* 1974, **26**, 163.
21. M. G. Albrecht, J. A. Creighton, *J. Am. Chem. Soc.,* 1977, **99**, 5215.

22. J. A. Creighton, C. G.Blatchford, M. G. Albrecht, *J. Chem. Soc. Far.Trans. 2,* 1978, **75**, 790.
23. P. C. Lee, D. J. Meisel, *J. Phys. Chem.,* 1982, **86**, 3391.
24. R. S. Sheng, L. Zhu, M. D. Morris, *Anal. Chem.,* 1986, **58**, 1116.
25. C. H. Munro, W. E. Smith, M. Garner, J. Clarckson, P. C. White, *Langmuir,* 1995, **11**, 3712.
26. S. Schneider, H. Grau, P. Halbig, U. Nickel, *Anlalyst,* 1993, **118**, 689.
27. C. H. Munro, W. E. Smith, P. C. White, *Analyst,* 1993, **118**, 731.
28. S. Nie, S. R. Emory, *Science*, 1997, **275**, 1102.
29. A. M. Stacy, R. P. Van Dyne, *Chem. Phys. Lett.,* 1983, **103**, 365.
30. D. Graham, C. McLaughlin, G. McAnally, J. C. Jones, P. C. White, W. E. Smith, *Chem. Comm.,* 1998, 1187.
31. K. Xi, S. K. Sharma, G. T. Taylor, D. W. Muenow, *App. Spectrosc.,* 1992, **46**, 5, 819.
32. X. Dou, T. Takama, Y. Yamaguchi, H. Yamamoto, *Anal. Chem.,* 1997, **69**, 1492.
33. I. R. Nabiev, K. V. Sokolov, O. N. Voloshin, *J. Raman Spectrosc.,* 1990, **21**, 333.
34. I. Nabiev, I. Chourpa, M. Manfait, *J. Raman Spectrosc.,* 1994, **25**, 13.
35. C. Otto, T. J. J. Van den Tweel, F. F. M. de Mul, J. Greve, *J. Raman Spectrosc.,*1986, **17,** 289.
36. S. K. Kim, T. H. Joo, S. W. Suh, M. S. Kim, *J. Raman Spectrosc.,* 1978, **17**, 381.
37. E. Koglin, J. M. Sequaris, P. Valenta, *J. Mol. Struct.,* 1982, **79**, 185.
38. J. Thornton, R. K. Force, *App. Spectrosc.,* 1991, **45**, 9.
39. A. Helmenstine, M. Uziel, T. Vo-Dinh, *Journal of Toxicology and Environmental Health,* 1993, **40**, 195.
40. T. Vo-Dinh, K. Houck, D. L. Stokes, *Anal. Chem.,* 1994, **66**, 3379.
41. N. R. Isola, D. L. Stokes, T. Vo-Dinh, *Anal. Chem.,* 1998, **70**, 1352.
42. T. Vo-Dinh, D. L. Stokes, G. D. Griffin, M. Volkan, U. J. Kim and M. I. Simon, *J. Raman Spectrosc.,* 1999, **30**, 785.
43. F. Zimmermann, B. Hossenfelder, J. C. Panitz, A. Wokaun, *J. Phys. Chem.,* 1994, **98**, 12796.

44. F. Zimmermann, B. Zimmermann, J. C. Panitz, A. Wokaun, *J. Raman Spectrosc.,* 1995, **26**, 435.
45. A. Beljebbar, G. D. Sockalingum, J. F. Angiboust, M. Manfait, *Spectrochimica Acta Part A,* 1995, **51**, 2083.
46. S. Sanchez-Cortes, P. Miskovsky, D. Jancura, A. Bertoluzza, *J. Phys. Chem.,* 1996, **100**, 1938.
47. D. Graham, W. E. Smith, A. M. T. Linacre, C. H. Munro, N. D. Watson, P. C. White, *Analytical Chemistry,* 1997, **69**, 22, 4703.
48. D. Graham, B. J. Mallinder, W. E. Smith, *Biopolymers (Biospectroscopy),*2000, **57**, 85.
49. D. Graham, B. J. Mallinder, W. E. Smith, *Angew. Chem. Int. Ed.*, 2000, **39**, 6, 1061.
50. K. T. Carron, Y. Guan, K. N. Hau, *Corrosion Sci.,* 1995, **51**, 367.
51. D. W. Will, G. Breipohl, D. Langer, J. Knolle, E. Uhlmann, *Tetrahedron,* 1995, **51**, 44, 12069.
52. N. D. Sinha, J. Biernat, H. Koster, *Tetrahedron Lett.,* 1983, **24**, 52, 5843.
53. D. Graham, G. McAnally, *Unpublished Results*.
54. Shine, *Aromatic Rearrangements*; Elsevier, 1967.
55. C. Scala-Valero, D. Doizi, G. Guillaumet, *Tetrahedron Lett.,* 1999, **40**, 4803.
56. Fries, Gueterbock, Kuehn, *Justus Liebigs Ann. Chem.*; 1934, 511, 213.
57. A. R. Katritzky, F. B, Ji, W. Q. Fan, P. Beretta, M. Bertoldi, *J. Heterocyclic Chem.,* 1992, **29**, 1519.
58. Y. Hirokawa, H. Yamazaki, N. Yoshida, S. Kato, *Bioorg. Med. Chem. Lett.*, 1998, **8**, 1973.
59. D. Graham, R. Brown, W. E. Smith, *Chem. Commun.*, 2001, 1002.
60. M. L. Bode, P. T. Kaye, R. George, *J. Chem. Soc. Perkin Trans. 1,* 1994, 3023.
61. K. C. Nicolaou, R.E. Dolle, D.P. Papahatjis, J.L. Randall, *J. Am. Chem. Soc.*, 1984, **104**, 15, 4189.
62. R. Brown, W. E. Smith, D. Graham, *Tetrahedron Lett.*, 2001, **42**, 2197.
63. R. J. Matteson, K. M. Pham, D. J. Leuck, K. A. Cowen, *J. Org. Chem.*, 1990, **55**, 8, 2552.

64. E. Luque-Perez, *Analytica Chimica Acta*, 1998, **366**, 231.

65. R. Elghanian, J. J. Storhoff, R. C. Mucic, R. L. Letsinger, C. A. Mirkin, *Science*, 1997, **277**, 1078.

66. J. J. Storhoff, R. Elghanian, R. C. Mucic, C. A. Mirkin, R. L. Letsinger, *J. Am. Chem. Soc.*, 1998, **120**, 1959.

67. J. J. Storhoff, A. A. Lazarides, R. C. Mucic, C. A. Mirkin, R. L. Letsinger, G. C. Schatz, *J. Am. Chem. Soc.*, 1998, **120**, 4640.

Chapter 8

Appendices

Pergamon

Tetrahedron Letters 42 (2001) 2197–2200

TETRAHEDRON LETTERS

Synthesis of a benzotriazole phosphoramidite for attachment of oligonucleotides to metal surfaces

Rachel Brown, W. Ewen Smith and Duncan Graham*

Department of Pure and Applied Chemistry, University of Strathclyde, 295 Cathedral Street, Glasgow G1 1XL, UK

Received 7 April 2000; revised 10 January 2001; accepted 17 January 2001

Abstract—A method for the addition of a benzotriazole moiety to the 5′-terminus of an oligonucleotide via phosphoramidite chemistry has been developed. Use of a monomethoxytrityl protecting group on the benzotriazole allowed fast on-column detritylation purification by reverse-phase HPLC. Surface enhanced Raman scattering (SERS) of the modified oligonucleotides was obtained from silver colloid.

Oligonucleotides containing a metal complexing group are of interest for the attachment of oligonucleotides to metal surfaces. Traditionally oligonucleotides have been attached to gold surfaces by a thiol linker via the 5′-terminus of the oligonucleotides.[1,2] Recently Mirkin and co-workers have reported extensive use of gold colloid coated in oligonucleotides for DNA sequence analysis and also fabrication of nano-structures.[3–6] In this letter we report an alternative linker that allows attachment of oligonucleotides to other metal surfaces such as silver and copper. This allows oligonucleotide modified metal surfaces to be constructed, but is also of particular interest for surface enhanced Raman scattering (SERS). For SERS to occur the target molecule must be adsorbed onto a suitable metal surface.[7,8] The metal surface used in our spectroscopic studies is silver colloid and as such requires a suitable complexing agent to form an irreversible complex between the complexing agent and the metal. A suitable complexing agent for this purpose is benzotriazole.[9] Benzotriazole is known to form a polymeric layer with silver and copper metals and is commonly used as an anti-corrosion agent to prevent tarnishing.[10,11] As part of our research we have developed a convenient route for the synthesis of a benzotriazole phosphoramidite and used the monomer to prepare 5′-benzotriazole modified oligonucleotides. This allowed oligonucleotides to complex to metal surfaces via the 5′-terminus unlike unmodified oligonucleotides which do not complex and hence do not produce SERS.

Keywords: benzotriazole; HPLC; nucleic acids; phosphoramidites; solid-phase synthesis; SERS.

* Corresponding author. Tel.: (+44) 141 548 4701; fax: (+44) 141 552 0876; e-mail: duncan.graham@strath.ac.uk

Previously we have added a benzotriazole moiety to an amino linker at the 5′-end of an oligonucleotide but this takes time, requires a manual-coupling step and is not as high yielding. In our preferred approach we synthesised a benzotriazole phosphoramidite that contained an alkyl spacer between the benzotriazole and the phosphorus. The spacer ensured that the action of the benzotriazole was not affected by the presence of the larger oligonucleotide.

The starting material for the synthesis of the phosphoramidite was benzotriazole-5-carboxylic acid, which is commercially available (Fig. 1). In a separate reaction 6-aminohexanol was protected with a *tert*-butyldiphenylsilyl group to yield 6-*tert*-diphenyl-silanyloxyhexylamine (**1**). The amine was then reacted with the carboxylic acid to form an amide linkage (**2**) using carbonyldiimidazole as the activating agent. Carbonyldiimidazole was chosen as it has been used before with benzotriazole carboxylic acid and allows amide formation at the acid function without the need for protection of the triazole ring system.[12] However, the benzotriazole ring proton still required protection prior to the formation of the phosphoramidite. Thus, the monomethoxytrityl group was chosen for this purpose as it is compatible with solid-phase synthesis of oligonucleotides and allows trityl on purification. The benzotriazole linker was protected using monomethoxytrityl chloride in pyridine with a catalytic amount of dimethylaminopyridine. This yielded the fully protected compound *N*-[4-methoxytrityl)-benzotriazoyl-5-carboxylic acid-(6-tertbutyl-diphenyl-silanyloxy-hexyl)-amide (**3**) in 54% yield. A benzoyl protecting group was also tested but subsequent phosphitylation resulted in a poor yield hence the use of the more favoured

0040-4039/01/$ - see front matter
PII: S0040-4039(01)00108-3

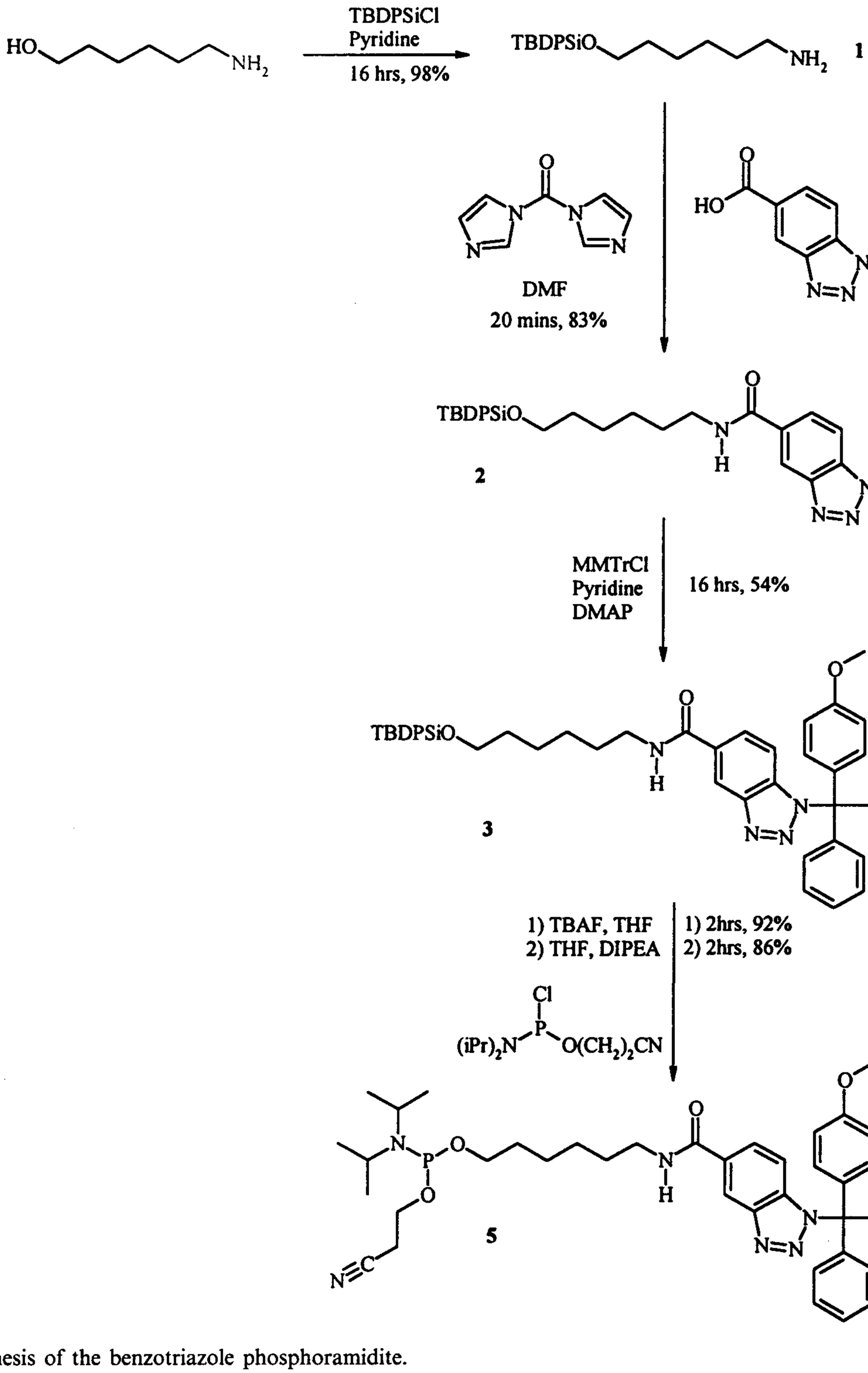


Figure 1. Synthesis of the benzotriazole phosphoramidite.

monomethoxytrityl group. *N*-Acylated benzotriazole can act as an acylating agent itself as the benzotriazole is comparable to a weak halide in leaving group nature in this case.

Removal of the silyl-protecting group was accomplished by tetrabutylammonium fluoride in THF followed by treatment with DOWEX H^+ in 92% yield to produce the primary alcohol for phosphitylation (**4**). A standard phosphitylation was performed in THF using 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite and diisopropylethylamine to yield the product (**5**) in 86% after 2 hours. The purity of the phosphoramidite was confirmed by ^{31}P NMR (148.78 ppm) prior to dissolution in anhydrous THF and used in routine solid-phase oligonucleotide synthesis.

Two 12 mer sequences and one 30 mer were synthesised using fast deprotection monomers[13] and the benzotriazole monomer added at the 5′-terminus via an extended coupling cycle using a double delivery and 15 minute coupling time. Deprotection by ammonia at room tem-

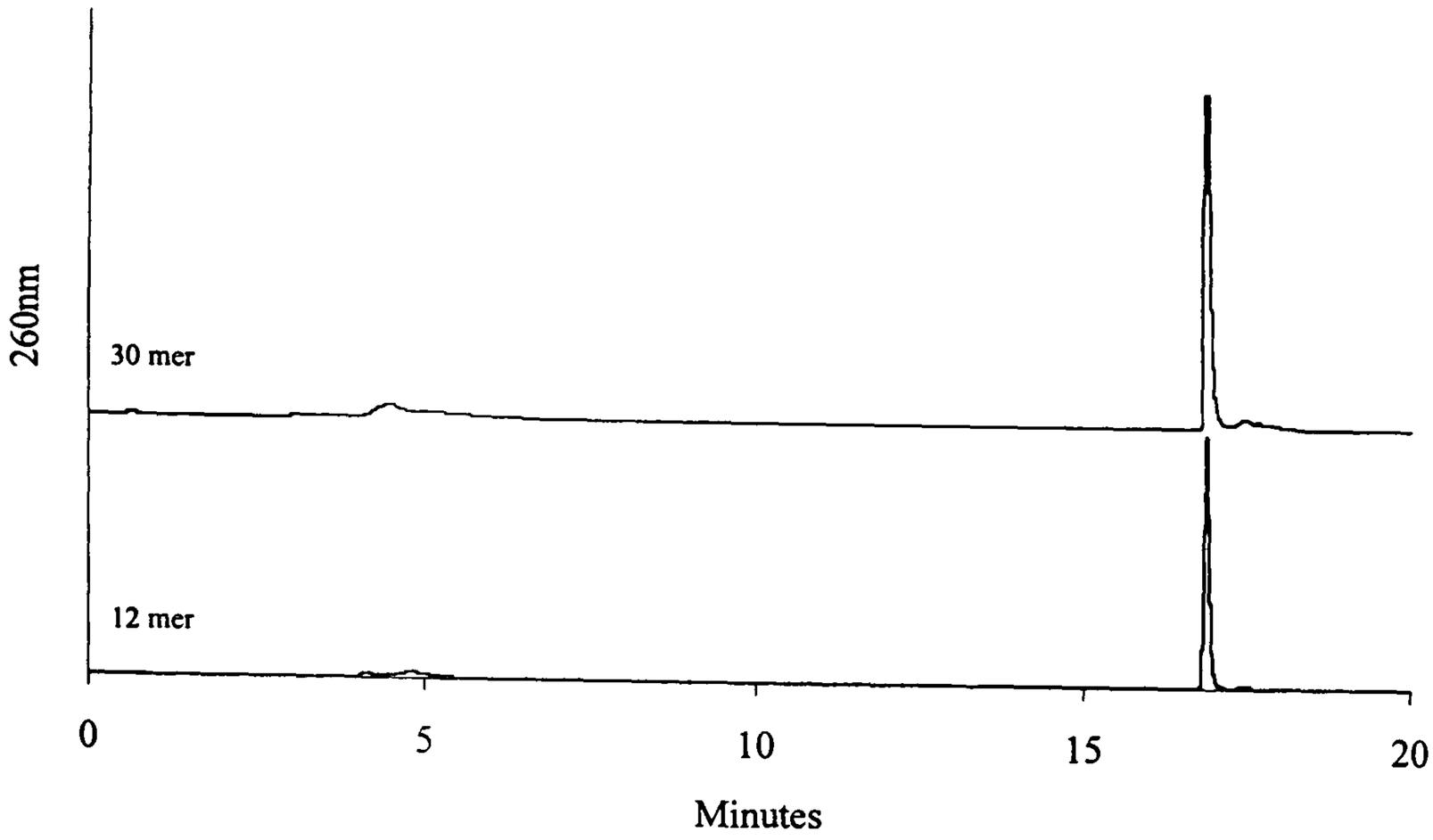


Figure 2. Analytical HPLC trace of on-column detritylation and purification for the two sequences at 260 nm. 12 mer 5′-BT TCT ATA TTC ATC and 30 mer 5′-BT GTA TCT ATA TTC ATC ATA GGA AAC ACC ATT.

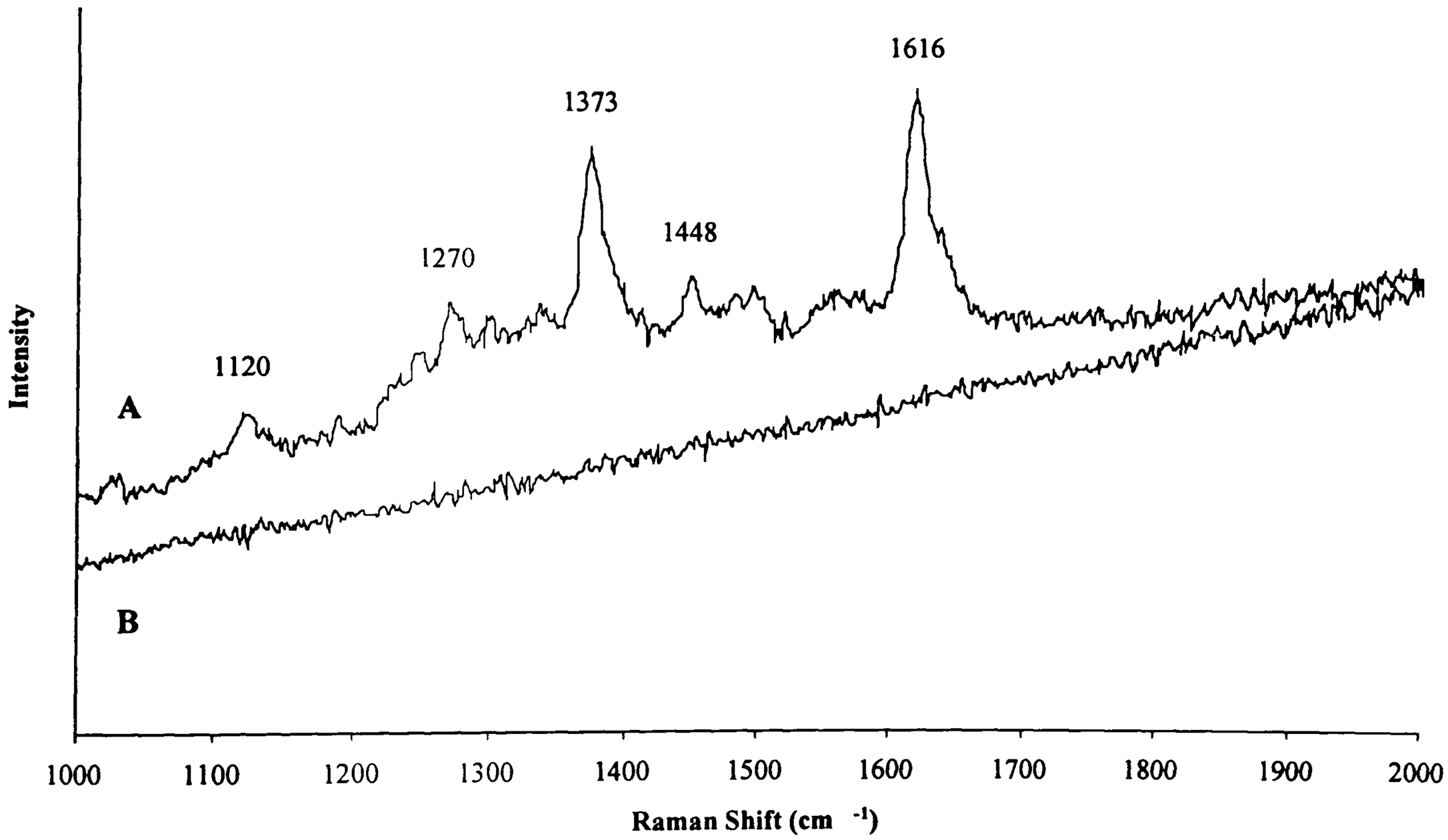


Figure 3. SERS spectra of benzotriazole modified oligonucleotide (A) and the control spectra of colloid with an unmodified oligonucleotide (B).

perature for 2 hours was followed by purification by reverse phase HPLC using a Poros oligo R3™ column and on column detritylation[14] (Fig. 2). The coupling efficiency was estimated as being between 80 and 90% by HPLC integration. The identity of both oligonucleotides was confirmed by electrospray MS.[15]

The benzotriazole oligonucleotides were then investigated for their ability to complex to a silver surface by examination of their SERS activity. SERS can only be seen for molecules on the surface of the silver colloid hence the oligonucleotide will only produce SERS if attached to the metal surface. The metal surface used was that of citrate reduced silver colloid, which has previously produced excellent SERS from benzotriazole azo dyes.[9] In this case the oligonucleotides were premixed with spermine (to neutralise the phosphate backbone[16]) and added to the silver colloid then left for 15 minutes to allow surface complexation. The colloidal suspension was concentrated by centrifugation and the supernatant removed before examination of the residue by SERS.[17] SERS signals were obtained from the benzotriazole oligonucleotides at a level of 5×10^{-8} mol, however, nothing could be seen from the control sample of colloid with DNA and spermine (Fig. 3). The bands arising at 1373 and 1616 cm^{-1} were assigned to the triazole ring and the phenyl ring systems of the benzotriazole, respectively, with the band at 1270 cm^{-1}

arising from the phenyl-triazole ring system. The signals were similar to those obtained for the benzotriazole carboxylic acid starting material as expected.

In conclusion, we have developed a simple route for the synthesis of a benzotriazole phosphoramidite and used it to incorporate benzotriazole into oligonucleotides via routine solid-phase synthesis. This has produced oligonucleotides with specific metal complexing properties. The benzotriazole modified oligonucleotides were shown to produce SERS from silver colloid thus providing evidence of surface attachment. This method of oligonucleotide modification will be of use in anchoring DNA to a number of metal surfaces for use in both structural and analytical studies.

Acknowledgements

The authors wish to thank the BBSRC for the award of a David Phillips fellowship to D.G. and Astra Zeneca for funding R.B.

References

1. Herne, T. M.; Tarlov, M. J. *J. Am. Chem. Soc.* **1997**, *119*, 8916–8920.
2. Steel, A. B.; Levicky, R. L.; Herne, T. M.; Tarlov, M. J. *Biophys. J.* **2000**, *79*, 975–981.
3. Mirkin, C. A.; Letsinger, R. L.; Mucic, R. C.; Storhoff, J. J. *Nature* **1996**, *382*, 607–609.
4. Storhoff, J. J.; Elghanian, R.; Mucic, R. C.; Mirkin, C. A.; Letsinger, R. L. *J. Am. Chem. Soc.* **1998**, *120*, 1959–1964.
5. Mitchell, G. P.; Mirkin, C. A.; Letsinger, C. A. *J. Am. Chem. Soc.* **1999**, *121*, 8122–8123.
6. Taton, T. A.; Mirkin, C. A.; Letsinger, R. L. *Science* **2000**, *289*, 1757–1760.
7. Fleischmann, M.; Hendra, P. J.; McQuillan, A. J. *J. Chem. Phys. Lett.* **1974**, *26*, 163–166.
8. Hildebrandt, P.; Stockburger, M. *J. Phys. Chem.* **1984**, *88*, 5935–5944.
9. Graham, D.; McLaughlin, C.; McAnally, G.; Jones, J. C.; White, P. C.; Smith, W. E. *Chem. Commun.* **1998**, *11*, 1187–1188.
10. Altura, D.; Nobe, K. *Corrosion* **1972**, *28*, 345.
11. Ling, Y.; Guan, Y.; Han, K. N. *Corrosion* **1995**, *51*, 367–375.
12. Hirokawa, Y.; Yamazaki, H.; Yoshida, N.; Kato, S. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 1973–1978.
13. Sinha, N. D.; Davis, P.; Usman, N.; Perez, J.; Hodge, R.; Kremsky, J.; Casale, R. *Biochimie* **1993**, *75*, 13–23.
14. Buffer A—50 mM NH_4OAc pH 10; Buffer B—50 mM NH_4OAc pH 10+5% MeCN; Buffer C—0.2% TFA; Buffer D—65% MeOH 35% H_2O. Method run on Biocad Sprint HPLC load, 3 min A, 3 min B, 3 min A, 4 min C, 3 min A, 4 min D at 5 ml/min.
15. Electrospray MS found 3971.0, calculated 3970.8.
16. Graham, D.; Smith, W. E.; Linacre, A. M. T.; Munro, C. H.; Watson, N. D.; White, P. C. *Anal. Chem.* **1997**, *69*, 4703–4707.
17. SERS signals were accumulated using a Renishaw 2000 Raman Microprobe instrument with 514.5 nm excitation. Spectra were accumulated three times for 30 seconds.

CHEMCOMM Communication
www.rsc.org/chemcomm

SERRS detection of PNA and DNA labelled with a specifically designed benzotriazole azo dye†

Duncan Graham,* Rachel Brown and W. Ewen Smith

Department of Pure and Applied Chemistry, University of Strathclyde, 295 Cathedral Street, Glasgow, UK G1 1XL. E-mail: duncan.graham@strath.ac.uk; Fax: (+44) 141 552 0876; Tel: (+44) 141 548 4701

Received (in Cambridge, UK) 8th March 2001, Accepted 27th April 2001
First published as an Advance Article on the web 15th May 2001

PNA and DNA have been detected for the first time with a specifically designed non-fluorescent SERRS active label; this is also the first use of SERRS to detect PNA.

In the post human genome project era a significant effort will centre on the detection of known nucleic acid sequences and variations within those sequences. Currently most methods of achieving this detection rely on the use of specific molecular biological assays and a physical detection technique which selectively identifies a label in a sensitive and discriminatory manner. Fluorescence and radio labelling are the most widely used physical techniques.[1,2] Recently we have reported the detection of labelled oligonucleotides using surface enhanced resonance Raman scattering, SERRS.[3–5] The main benefits of using SERRS rather than fluorescence are the ability to discriminate between labels in a mixture, without separation, at femtomole levels or below and the more extensive, simpler labelling chemistry which can be employed.[4]

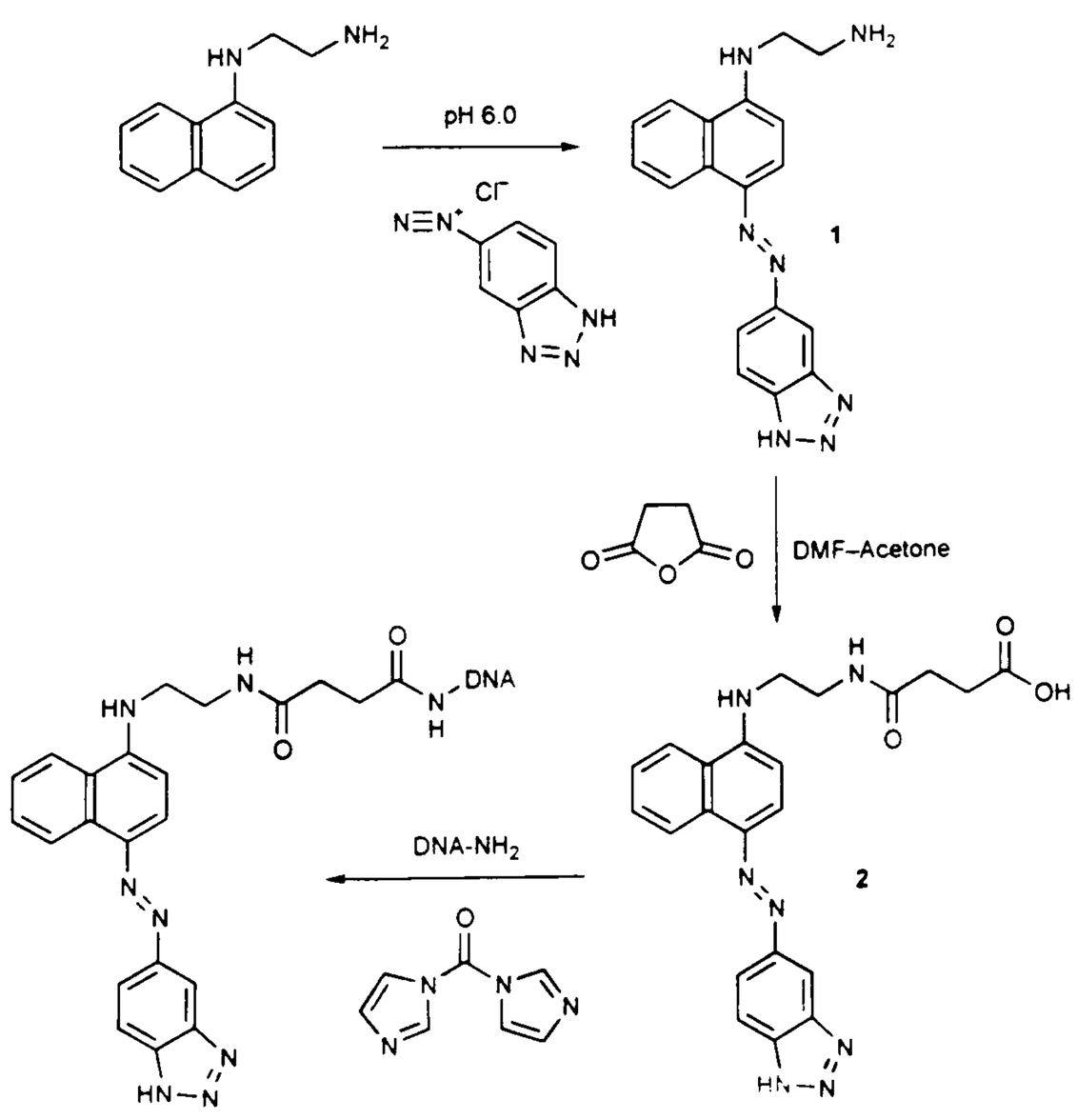
To obtain SERRS a dye requires to be adsorbed onto a roughened surface of certain metals of which the most widely used are silver and gold. The dye is required to obtain molecular resonance enhancement and the surface attachment provides enhancement by interaction with the plasmons on the metal surface. The combined enhancement processes provide a Raman signal of equivalent sensitivity to that of fluorescence. The sharp vibrational signals provide much better selectivity and since many dyes which are not fluorophores are effective, the chemistry can be much simpler and more extensive. A very effective metal surface used in this laboratory is that of citrate reduced silver colloid.[6] Aggregating agents such as sodium chloride or acid provide the most effective roughened surface and tune the surface plasmon to match the frequency of the excitation used.[7] In all previous SERRS studies involving DNA we have used commercially available fluorophores as the labels, as fluorescence is quenched in SERRS thus allowing the enhanced light scattering to be observed. Surface adsorption has been by electrostatic attraction of positively charged ammonium groups on the label to negatively charged citrate groups on the surface of the metal colloid used in our studies. In this approach the adsorption is not robust and can be affected by the presence of other agents such as metal chelators and no use is made of the extensive additional chemistry available for use with SERRS methodologies.

Previously we reported the synthesis of benzotriazole monoazo dyes specifically designed to provide SERRS by complexing directly to the silver surface, through the benzotriazole moiety which displaced the citrate surface layer.[8] In this communication we report the synthesis of a derivative of one of the previous dyes which is capable of undergoing solid phase addition to both DNA and PNA to make the oligomers SERRS active. The benefits of using these labels are that they are easy to produce, they give distinctive SERRS signals which are different from the previously used fluorophores, and they bind *via* complexation to the silver surface in a way which produces virtually irreversible binding.

The route followed for synthesis of the dye is shown in Scheme 1. *N*-(1-Naphthyl)ethylenediamine was chosen due to the presence of the primary aliphatic amine. Once an azo linkage has been produced the electron density on the aromatic amine is pulled into the ring system[9] dramatically reducing the reactivity of the amine as a nucleophile. Thus the aliphatic amine provides a reactive functionality to further derivatise the dye once synthesised. Dye synthesis was achieved by a diazonium coupling using 5-aminobenzotriazole to produce the orange dye, *N*-[4-(5′-azobenzotriazolyl)naphthalen-1-yl]ethylenediamine [**1**]. This dye was further functionalised to produce *N*-[2-(4′-(5″-azobenzotriazolyl)naphthalen-1-yl)aminoethyl]-succinamic acid [**2**]. The acidic dye was then capable of addition to amino linked DNA and PNA *via* amide formation on the solid phase.

A DNA oligomer was synthesised by routine solid phase phosphoramidite chemistry[10] and a monomethoxytrityl protected amino link added to the 5′-terminus.[11] Removal of the monomethoxytrityl group and addition of the activated dye produced the 5′-dye labelled DNA on the solid phase. The dye was activated by addition of carbonyl diimidazole to form the active ester. Cleavage and deprotection produced the crude labelled oligonucleotide which was purified by ion exchange HPLC. The coupling efficiency was estimated as >83% by integration of the peaks from the HPLC.

PNA has a N-terminus which can be used to react in the same way as the amino link in DNA, however two Fmoc protected amino-3,6-dioxaoctanoic acid (AEEA-OH) spacers[12] were



Scheme 1 Synthesis of the benzotriazole label and addition to amino linked DNA.

† Electronic supplementary information (ESI) available: full experimental details. See http://www.rsc.org/suppdata/cc/b1/b102241p/

DOI: 10.1039/b102241p

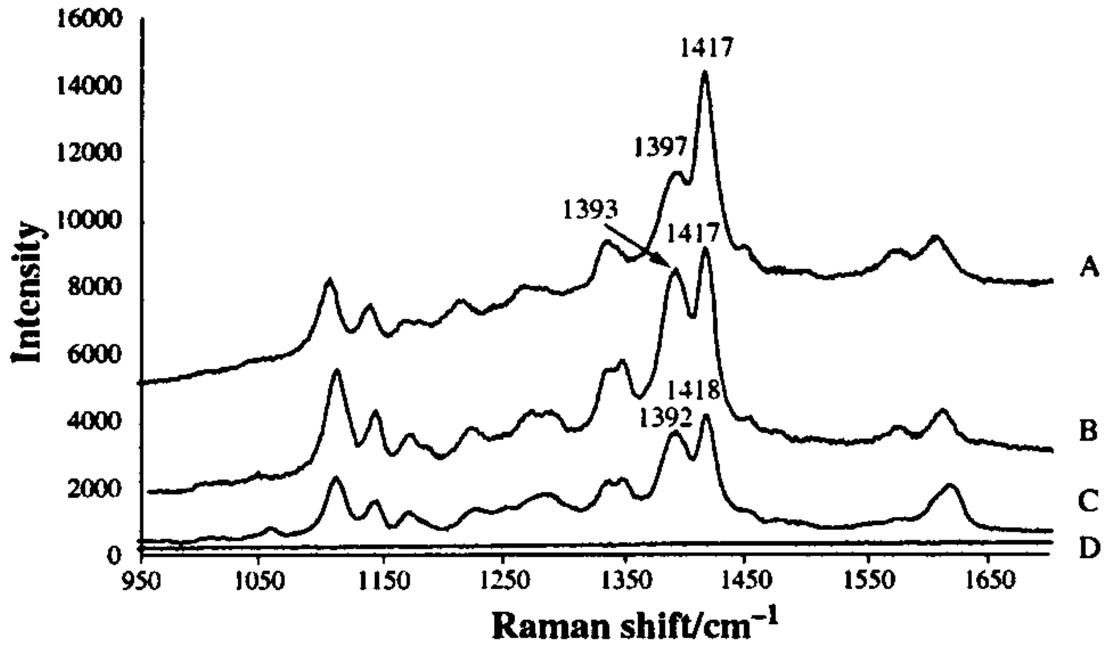


Fig. 1 The SERRS spectra obtained for free dye (A), dye labelled DNA (B), dye labelled PNA (C) and aggregated colloid with no dye (D). The spectra were obtained under different conditions and are scaled to allow comparison.

added to distance the dye from the PNA sequence. An 8mer was synthesised using Fmoc/Bhoc chemistry[12] and the two spacers added to the N-terminus. Removal of the Fmoc protecting group from the terminal spacer allowed addition of the benzotriazole carboxylic acid dye in the same way as for the DNA. Use of the dye as a monomer in the PNA synthesis cycle resulted in a mixture of products and poor coupling due to the unprotected triazole system, hence the use of the carbonyl diimidazole. Deprotection yielded the crude oligomer as a purple solid due to protonation of the azo linkage. Purification by reverse phase HPLC gave the pure dye labelled PNA with an estimated coupling efficiency of ~55% for the dye. The low coupling could be from a number of sources, such as cross-reaction of the unprotected triazole group. However, as only one monomer was being added this yield was acceptable.

The oligomers were then investigated for their ability to produce SERRS using citrate reduced silver colloid. A Renishaw microprobe system was used with 1 mW of 514.5 nm excitation at the sample. The optimum conditions for obtaining SERRS of both benzotriazole labelled oligomers vary. For the DNA oligomer the highest signal intensity was obtained when the phosphate backbone was neutralised by the addition of spermine prior to addition to the silver colloid. Use of an excess of spermine also provides aggregation of the colloid to produce the desired SERRS signals.[4] The spectrum is shown in Fig. 1B. The spectrum is consistent with that of the uncoupled dye (Fig. 1A) and displays the stretches at ~1393 cm^{-1} and 1417 cm^{-1} that are indicative of an azo tautomer.

A citrate assay was used to monitor the release of citrate from the surface after the addition of the benzotriazole dye, to the colloid. This proved that citrate was released from the surface after addition of the benzotriazole dye, but if spermine was added alone to induce aggregation then citrate was not released. This indicates that the benzotriazole is indeed complexing directly with the metal and not by a charge–charge interaction which appears to occur during spermine aggregation. Additionally, SERRS signals can be obtained from the dye labelled oligonucleotide without the use of spermine by using alternative aggregating agents such as phosphate buffer or nitric acid. This is in direct contrast to the oligonucleotides labelled with the fluorophores which in previous studies only produced intense SERRS with spermine. Thus we can conclude that the benzotriazole dye acts as a surface complexing agent and displaces citrate to provide attachment of modified oligonucleotides to a silver surface.

The conditions for obtaining SERRS of the PNA oligomer differ in that the oligomer is dissolved in 0.1% TFA and has a neutral backbone. Thus there is no need for the use of spermine as a charge neutralising agent. Also the TFA acts as an aggregating agent, again negating the need for the addition of spermine as an aggregating agent. The addition of the labelled PNA oligomer to a colloidal suspension produced a strong and distinct SERRS spectrum identical to that obtained for the labelled DNA (Figure 1C). In order to investigate the SERRS of the PNA labelled oligomer, a set of standard aggregating agents was tested with the labelled 8mer and the resulting SERRS recorded (Table 1). The values show that the intensity of the signals for the labelled PNA can be improved by the addition of an aggregating agent. However, if the solution is made basic by the addition of triethylamine then the intensity of the signals decreases, indicating that the PNA provides optimal signal to noise ratios in acidic conditions which are compatible with the conditions used for the standard synthesis of PNA. Spermine still appears to enhance the intensity of the spectrum, as it is an efficient aggregating agent.

Table 1 The intensity of the major peak in the PNA spectrum with different aggregating agents. All spectra were recorded in one scan of ten seconds and at 2×10^{-11} mole equivalents

Species studied	Intensity of peak at 1418 cm^{-1}
Colloid + TFA	No peaks
Colloid + BtDYE PNA + triethylamine	866
Colloid + BtDYE PNA	1074
Colloid + BtDYE PNA + spermine	1269
Colloid + BtDYE PNA + NaCl	2341
Colloid + BtDYE PNA + TFA	4148
Colloid + triethylamine	No peaks

In conclusion, we have synthesised benzotriazole azo dye labelled oligomers specifically designed to give SERRS. This dye is not an effective fluorophore and gives an indication of the additional labelling chemistry available for use with SERRS. The dye has been added to both DNA and PNA and SERRS obtained from both species. This type of label has additional benefits since the covalently attached benzotriazole group gives strong bonding, particularly to silver and copper surfaces, and provides a new and effective DNA–metal bonding chemistry. This is the first time that SERRS has been obtained from PNA and it is clear that it is easier to obtain SERRS from PNA than DNA.‡§

Notes and references

‡ The authors wish to thank the BBSRC for the award of a David Phillips Fellowship to D. G. and Astra Zeneca for funding to R. B.

§ Oligomers synthesised were: DNA 5′-BtDye X GTG CTG CAG GTG TAA ACT TGT ACC AG 3′ (X = amino link) and PNA (N)-BtDye OO ACA TTT GA (C) (O = AEEA spacer).

1 L. J. McBride and M. D. Oneill, *Am. Laboratory*, 1991, **23**, 52 *et seq.*
2 A. Castro and J. G. K. Williams, *Anal. Chem.*, 1997, **69**, 3915.
3 D. Graham, W. E. Smith, A. M. T. Linacre, C. H. Munro, N. D. Watson and P. C. White, *Anal. Chem.*, 1997, **69**, 4703.
4 D. Graham, B. J. Mallinder and W. E. Smith, *Biopolymers (Biospectroscopy)*, 2000, **57**, 85.
5 D. Graham, B. J. Mallinder and W. E. Smith, *Angew. Chem., Int. Ed.*, 2000, **39**, 1061.
6 C. H. Munro, W. E. Smith, M. Garner, J. Clarkson and P. C. White, *Langmuir*, 1995, **11**, 3712.
7 C. Rodger, W. E. Smith, G. Dent and M. Edmondson, *J. Chem. Soc., Dalton Trans.*, 1996, 791.
8 D. Graham, C. McLaughlin, G. McAnally, J. C. Jones, P. C. White and W. E. Smith, *Chem. Comm.*, 1998, 1187.
9 D. Graham, A. R. Kennedy and S. J. Teat, *J. Heterocycl. Chem.*, 2000, **37**, 1555.
10 M. H. Caruthers, *Science*, 1985, **230**, 281.
11 S. L. Beaucage and R. P. Iyer, *Tetrahedron*, 1993, **49**, 1925.
12 R. Castle, I. S. Jensen and M. Egholm, in *Peptide Nucleic Acids: Protocols and Applications*, ed. P. E. Nielsen and M. Egholm, 1999.