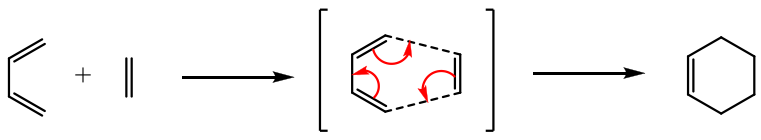
3

Conjugation of 5'-dienyl modified oligonucleotides to Tat peptide *via* Diels–Alder cycloaddition

3.1 Introduction

The Diels-Alder reaction was discovered by Otto Diels and Kurt Alder in 1929. [1] The reaction occurs between a 1, 3- diene and a dienophile to form a six-membered ring adduct. The reaction occurs *via* a pericyclic mechanism – the concerted movement of electrons *via* a cyclic transition state (**Scheme 3.1**) – resulting in the formation of two new σ C-C bonds and a new π-bond. The reaction is also known as [4 + 2] cycloaddition.



Scheme 3.1 – *Diels-Alder reaction of a diene and a dienophile to form a six-membered ring*

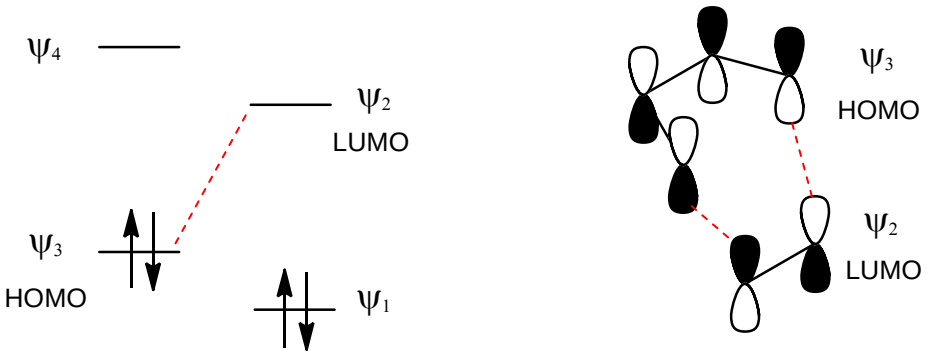
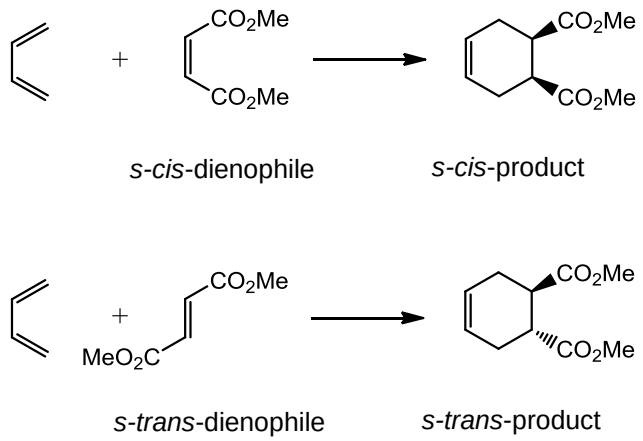


Figure 3.1 – *Interaction of the HOMO of the diene and the LUMO of the dienophile in the Diels-Alder cycloaddition*

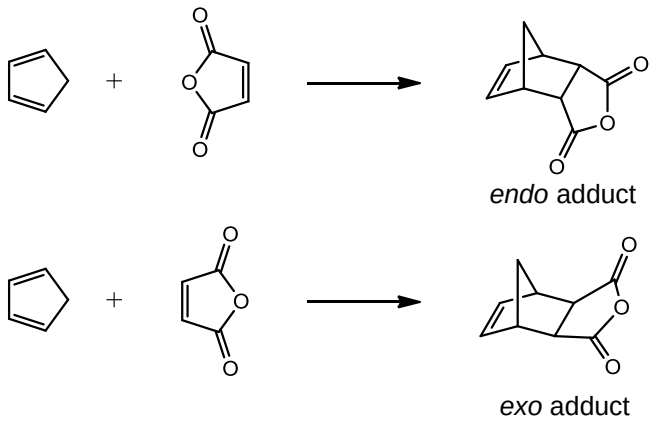
The reaction requires harsh conditions of heating to proceed; however, the dienophile can be activated by conjugation to an electron-withdrawing group. Similarly, the presence of an electron-donating group on the diene improves the reaction outcome. In these cases, the interaction between the highest occupied molecular orbital (HOMO) of the diene and the lowest unoccupied molecular orbital (LUMO) of the dienophile is energetically more favourable (**Fig. 3.1**).

For the Diels-Alder cycloaddition to proceed, the diene must lie in the *s-cis*-conformation; in the *s-trans*-conformation the terminal carbons are too far apart to react effectively with the dienophile. The cycloaddition is stereoselective; since the reaction occurs in one step *via* a transition state neither the diene nor the dienophile has time to rotate. As such, the stereochemistry of each is maintained in the product (**Scheme 3.2**); *s-cis*-dienophiles give *s-cis*-products and *s-trans*-dienophiles give *s-trans*-products.



Scheme 3.2 – *The Diels-Alder cycloaddition is such that the stereochemistry of the reactants is retained in the product*

The Diels-Alder reaction of cyclic dienophiles affords stereoisomeric products, dependent on the positioning of the dienophile relative to the diene in the transition state (**Scheme 3.3**). If the diene and the dienophile are aligned such that the dienophile substituents point towards the π-system of the conjugated diene, the result is the *endo* adduct; if they are aligned such that the dienophile substituents point away from the π-system of the conjugated diene, the result is the *exo* adduct. Due to kinetic control, the major product in the reaction is the *endo* adduct.

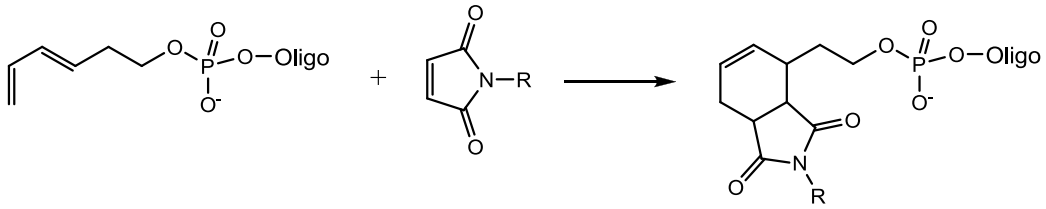


Scheme 3.3 – *The Diels-Alder cycloaddition produces both the endo and the exo adducts*

In 1980, Breslow and Rideout reported that the rates of Diels-Alder cycloadditions are greatly enhanced in aqueous systems. [2] It was observed that using water as the reaction solvent increased the rate up to 700-fold. For this reason, the Diels -Alder reaction has been utilized for conjugation of water-soluble biomolecules such as oligonucleotides.

3. 1. 1 Diels-Alder cycloaddition for oligonucleotide bioconjugation

The first report of the Diels-Alder cycloaddition for oligonucleotide bioconjugation was by Hill *et al*. [3] in 2001. Phosphoramidites of 1, 3-hexadiene and 1, 3-cyclohexadiene were synthesised and incorporated into oligonucleotide sequences at the 5'-terminus, using standard phosphoramidite methodology. 5'- 1, 3-hexadienyl modified oligonucleotides were then conjugated successfully to seven different maleimide derivatives (**Scheme 3.4**). Optimum conditions were found to be a two-fold excess of the maleimide derivative at a temperature of 25 – 55 ^{o}C, over a time of between two and twenty hours. The authors also found that pH was influential on the rate of the cycloadditions, with conjugation of both linear and cyclic dienyl modified oligonucleotides to biotin maleimide at lower pH (5.5) reaching completion more quickly than those performed at higher pH (6.3).

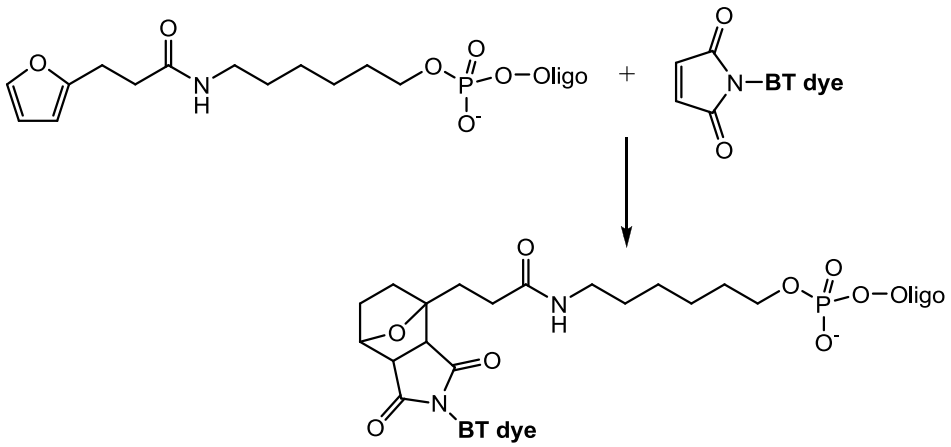


Scheme 3.4 – *Diels-Alder cycloaddition of 1, 3-hexadienyl modified oligonucleotides with maleimides, as reported by Hill et al.* [3]

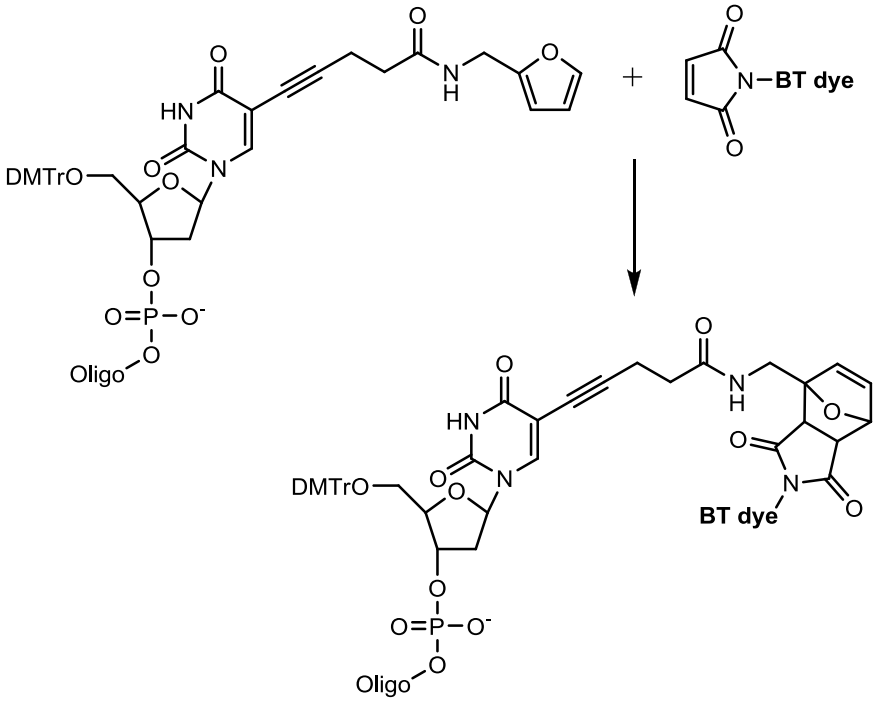
3.1.1.1 Diels-Alder cycloaddition for oligonucleotide bioconjugation to dye labels

Oligonucleotides conjugated to dye labels allow specific sequence detection following a biological assay. Previous methods for the bioconjugation of dyes to oligonucleotides to be used as DNA probes in this capacity have involved 5'- or 3'-terminus modification with a primary alkyl amine, followed by post-synthetic reaction with an activated dye, [4] or involved the use of pre-labelled phosphoramidites. [5] However, the post-synthetic route is low yielding and can potentially suffer side reactions with oligonucleotide functionalities [3] and the phosphoramidite route involves complex synthetic chemistry and expensive monomers.

Graham *et al*. [6] report the synthesis of oligonucleotide dye conjugates *via* both 5'-modification (**Scheme 3.5**), and mid-sequence modification (**Scheme 3.6**) of oligonucleotides with furan. Dyes were linked to the oligonucleotides by conjugation, *via* Diels-Alder cycloaddition, with both maleimido modified benzotriazole (BT) dyes for Surface Enhanced Resonance Raman Spectroscopy (SERRS) analysis, and commercially available fluorescent dyes containing a maleimido moiety.



Scheme 3.5 – *Diels-Alder cycloaddition of 5'-furanyl modified oligonucleotide to a maleimide BT dye, as reported by Graham et. al* [6]



Scheme 3.6 – *Diels-Alder cycloaddition of mid-sequence furanyl modified oligonucleotide to a maleimide BT dye, as reported by Graham et. al* [6]

The Diels-Alder route for oligonucleotide labelling rules out several of the complexities associated with the labelling methods detailed previously; oligonucleotide modification with dienes and maleimide derivitisation of dye labels with maleimide require only simple synthetic chemistry, the stereospecificity of the Diels-Alder reaction is such that there is no risk of side reactions with other oligonucleotide functionalities and the reaction takes place in aqueous media with no need for the addition of activating agents, thereby simplifying purification of the oligonucleotide-dye conjugate.

3.1.1.2 Diels-Alder cycloaddition for oligonucleotide bioconjugation to peptides

Diels-Alder cycloaddition has also more recently been reported by Marchán *et. al* to be effective for oligonucleotide peptide bioconjugation. [7] Cycloadditions, in aqueous media, of 5'- 1, 3-hexadienyl modified thymine dimers to a small series of maleimido modified dipeptides, and of 5'- 1, 3-hexadienyl modified 8-mer and 15-

mer oligonucleotide sequences to simple maleimido modified 8-mer and 20-mer peptide sequences, were reported in an investigation into the effect of sequence length on conjugate yield, using Diels-Alder cycloaddition.

The peptide sequences used in the study by Marchán *et al*. were not highly charged, as is the case for cell penetrating peptide sequences, which are generally highly cationic and are notoriously difficult peptide sequences to handle. This work investigates the use of the Diels-Alder reaction, as an alternative to linkages described in the literature before now (see 1.3.3 – *Oligonucleotide conjugation to Tat peptide*), for the conjugation of 5'-dienyl modified oligonucleotides to the highly cationic Tat peptide.

The properties of the Diels-Alder reaction make it a suitable method for the bioconjugation of oligonucleotides to peptides because it is fast and efficient in aqueous media and thereby conducive to biological moieties, and the chemoselective reaction of the diene and the dienophile is such that no additional protection of the oligonucleotide or the peptide is required.

3.2 Results and discussion

3.2.1 Synthesis of an unmodified oligonucleotide control sequence

An unmodified oligonucleotide sequence (**1**) was synthesised to be used for comparison in analysis of synthesised dienyl modified oligonucleotides.

The sequence selected for use in the synthesis of oligonucleotide peptide conjugates was a basic 12-mer sequence incorporating equal amounts of each of the four nitrogenous bases.

Oligonucleotide sequence 1: 5'- CGC ATT CAG GAT -3'

Oligonucleotide sequence (**1**) was synthesised on a CPG solid support pre-modified with the first base (T), using standard phosphoramidite chemistry. After synthesis on an automated DNA synthesiser, the oligonucleotide was cleaved from the solid support and the base protecting groups by treatment with aqueous conc. ammonium hydroxide solution. The sequence was purified from its failure sequences by ion-exchange HPLC; HPLC buffer salts were removed by size exclusion chromatography (desalting). Synthesis of oligonucleotide sequence (**1**) was confirmed by MALDI-TOF mass spectrometry analysis (**Table 3.1**).

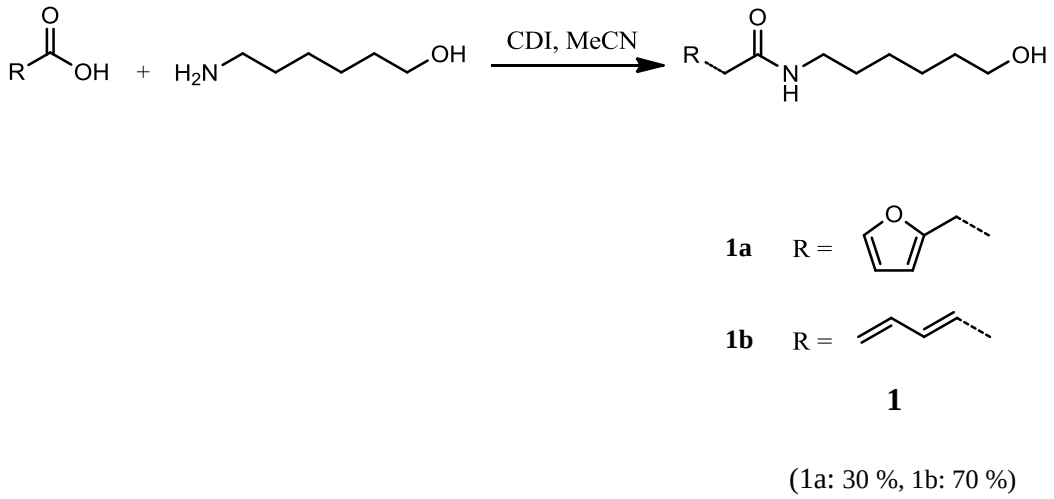
Calc'd M	**MALDI-TOF MS Found [M + H]** $^{+}$
3646.3	3647.6

Table 3.1 – *Mass spectroscopic characterisation of unmodified oligonucleotide sequence (**1**)*

3.2.2 Synthesis of 5'-dienyl modified oligo-nucleotides

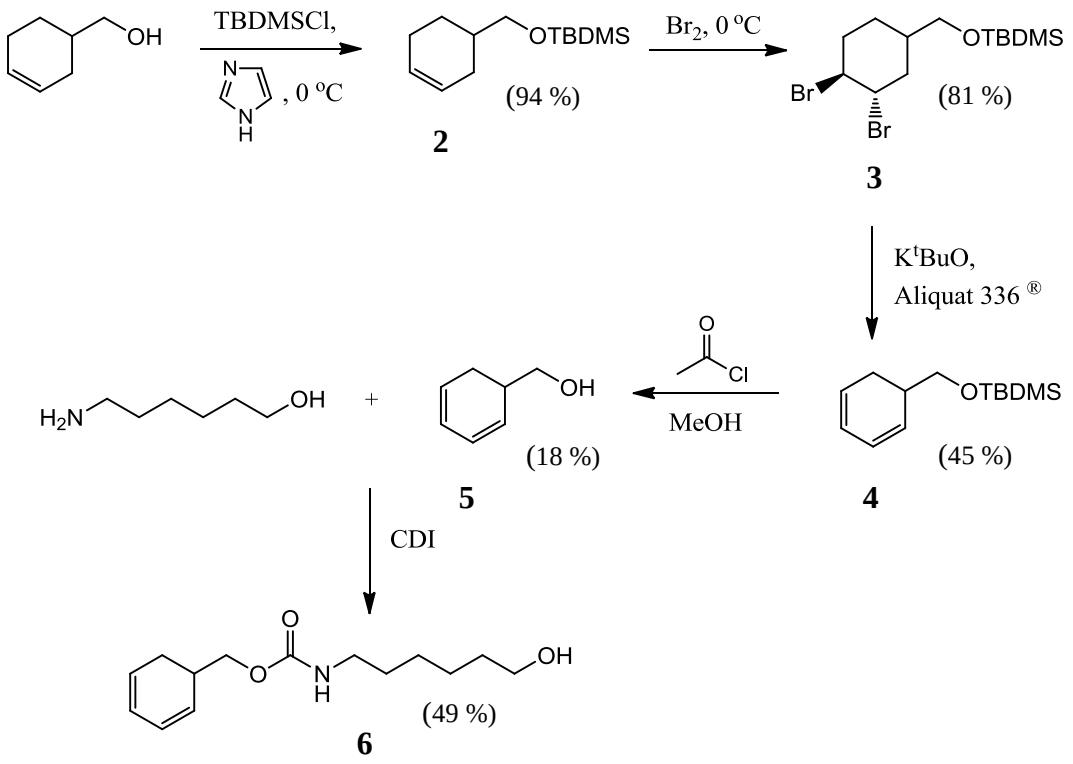
A series of three different 5'-dienyl modified oligonucleotide sequences was generated *via* synthesis of unique phosphoramidites for use in the final cycle in automated DNA synthesis. The dienyl functionalisations selected for this purpose were 1, 3-butadiene (reported for the first time here), furan [8] and cyclohexadiene. [3]

In order to synthesise a phosphoramidite for 5'-modification, a molecule containing a hydroxyl group for phosphitylation is required. In the cases of the 1, 3-butadienyl and the furanyl modifications this was achieved by coupling the respective dienyl acids with 6-amino-1-hexanol (**Scheme 3.7**), which also allowed for incorporation of a spacer of at least six carbon units between the diene and the rest of the oligonucleotide sequence. It was anticipated that this would contribute towards minimising steric hindrance of the Diels-Alder cycloadditions, by distancing the reactive group from the rest of the oligonucleotide.



Scheme 3.7 – *First step in synthesis of 1, 3-butadienyl and furanyl moieties for 5'-dienyl oligonucleotide modification*

This reaction was first performed for the furanyl modification. 3-(2-Furyl) propanoic acid was activated with CDI at 40 ^{o}C, before reaction with 6-amino-1-hexanol at room temperature. The reaction was first performed using anhydrous DMF as the solvent, but later anhydrous acetonitrile was the solvent of choice. This was found to improve the outcome of the reaction; ^{1}H NMR analysis post work up showed only trace impurities, such that no further purification was required, leading to an increased yield of amide (**1**) (70 % compared with 58 % obtained with DMF). For 1, 3-butadienyl modification the reaction conditions were almost identical except that activation of sorbic acid with CDI was performed at room temperature rather than at 40 ^{o}C. This reaction was found to proceed equally well at this lower temperature; analysis by thin layer chromatography (TLC) showed complete conversion to the activated acid. However, the coupling of the activated sorbic acid with 6-amino-1-hexanol to form amide (**1**) was found to be significantly lower yielding (30 %) than that observed for 3-(2-furyl) propanoic acid. It was thought that this could be due to partial degradation of the 6-amino-1-hexanol, indicated by its slightly yellowish colour.



Scheme 3.8 – *First steps in synthesis of cyclohexadienyl moiety for 5'-dienyl oligonucleotide modification*

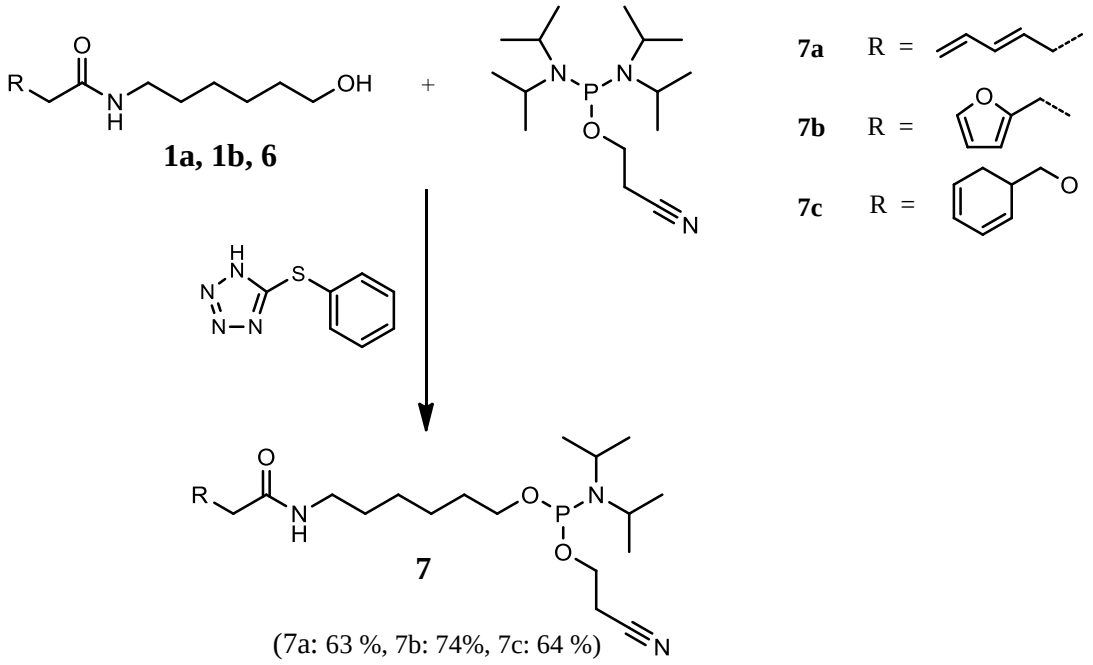
Synthesis of a hydroxylated molecule containing a cyclohexadiene ring was more complex since a carboxylic acid functionalised cyclohexadiene was not commercially available. Synthesis of carbamate (**6**) was achieved following a method described by Hill *et al*. [3] (**Scheme 3.8**).

The hydroxyl functionality of 3-cyclohexene-1-methanol was first protected by silylation. TBDMSCl was reacted with imidazole at 0 °C before addition of the alcohol to form silyl ether (**2**) in excellent yield. Treatment of (**2**) with one equivalent of molecular bromine at 0 °C afforded a diastereomeric mixture of dibromides (**3**) on which a double elimination was performed, by reaction with potassium *tert*-butoxide in the presence of a catalytic amount of Aliquat 336 ®, to generate diene (**4**) in moderate yield (45 %). Aliquat 336 ® is a quartenary ammonium salt, used here as a phase transfer catalyst [9] to enhance the solubility of

the inorganic potassium *tert*-butoxide salt in the organic tetrahydrofuran (THF) reaction solvent.

The penultimate step in the synthesis of carbamate (**6**) was cleavage of the protective silyl ether to afford dienyl alcohol (**5**). Hill *et al*. note the instability of the cyclohexadiene ring under the normal desilylation conditions; treatment with tetra-*n*-butylammonium fluoride (TBAF) solution in THF. [10] As such, it was decided to use an alternative, milder desilylation procedure reported by Khan and Mondal. [11] This involved treatment of the silyl ether with a catalytic amount of acetyl chloride in anhydrous methanol and was effective in quickly generating the dienyl alcohol, although in relatively low yield. The final step in this synthesis was incorporation of the hydroxylated C6 spacer molecule, achieved by activation of alcohol (**5**) with CDI at room temperature in acetonitrile, before reaction with a slight excess of 6-amino-1-hexanol to afford carbamate (**6**) in moderate yield (49 %).

With three hydroxylated dienyl moieties in hand it was possible to synthesise a series of dienyl phosphoramidites for use in DNA synthesis (**Scheme 3.9)**. Each of the alcohols (**1a**), (**1b**) and (**6**) was treated with one equivalent of tetraisopropyl phosphitylating reagent in the presence of 5-benzylthio-1*H*-tetrazole activator. All three phosphoramidites were obtained in excellent yield – over 60 %. Successful synthesis of the phosphoramidites was confirmed by P (III) signals in ^{31} P NMR.



Scheme 3.9 – *Synthesis of dienyl modified phosphoramidites for use in solid phase oligonucleotide synthesis*

The phosphoramidites were dissolved in an amount of anhydrous acetonitrile to a requisite concentration and used in the final cycle of oligonucleotide synthesis, utilising standard phosphoramidite methodology, to generate oligonucleotide sequence (**2**):

Oligonucleotide sequence 2: 5'- X CGC ATT CAG GAT -3'

Where X is the 1, 3- butadienyl, furanyl or cyclohexadienyl modification.

Cleavage of the modified oligonucleotides from the solid support, and of the base and phosphate protecting groups, was achieved by treatment with conc. aqueous ammonium hydroxide. Analysis of the modified oligonucleotides was performed by anion-exchange HPLC, where separation of the oligonucleotide components is by virtue of the anionic charge on their phosphate backbones. Addition of the dienyl modifier results in an increase of one phosphate group in the sequence, hence an increase in anionic charge. This should lead to a visible separation of the dienyl modified oligonucleotide from its unmodified counterpart when using an ion-

exchange buffer system. For all three dienyl modifications this was observed (**Fig. 3.2**).

The modified sequences were separated from their failure sequences by anion-exchange HPLC; HPLC buffer salts were removed by desalting. Synthesis of the dienyl modified oligonucleotides was confirmed by MALDI-TOF mass spectrometry analysis (**Table 3.2**).

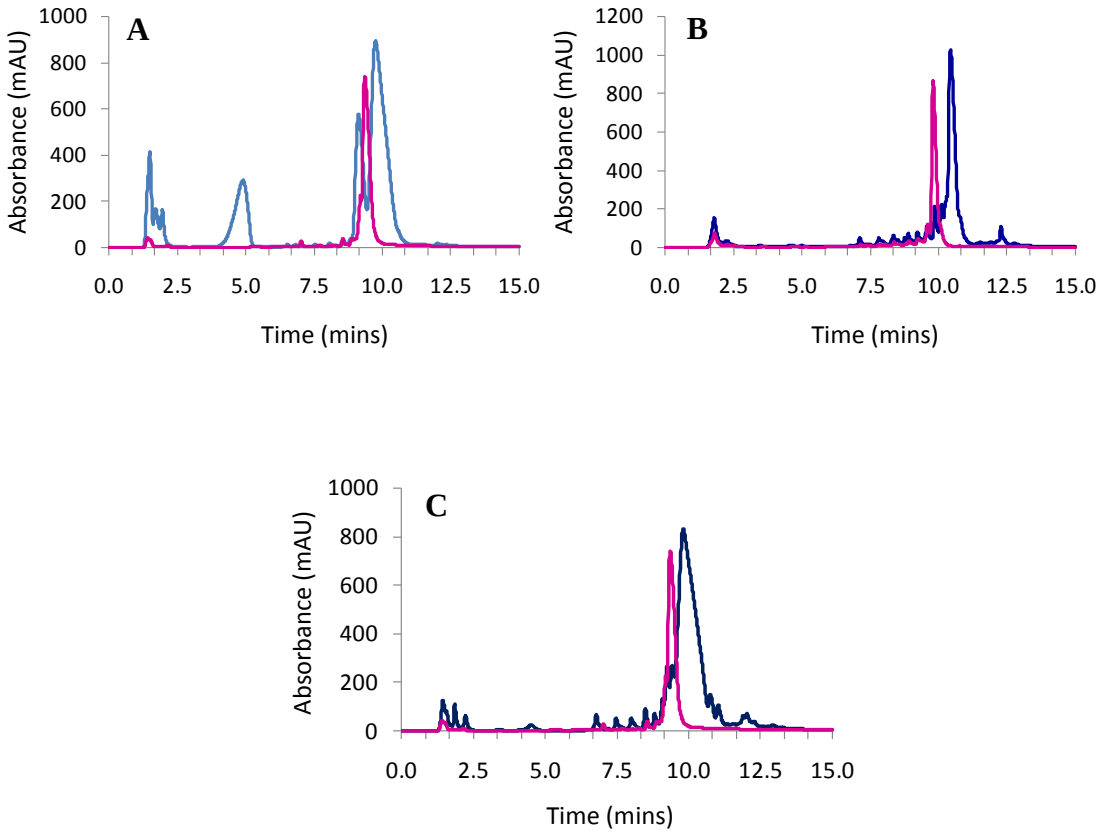


Figure 3.2 – *Ion-exchange HPLC traces at 260 nm of unmodified (pink) and 5'-dienyl modified (blue) oligonucleotide sequence (**2**), 5'- X CGC ATT CAG GAT, where (**A**) X = butadiene, (**B**) X = furan and (**C**) X = cyclohexadiene*

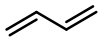
5'-Modification (X)	Calc'd *M*	MALDI-TOF MS Found $[M - H]^-$
	3918.6	3917.9
	3946.6	3946.7
	3960.6	3959.8

Table 3.2 – *Mass spectroscopic characterisation data of dienyl modified oligonucleotides, 5' - X CGC ATT CGA GAT*

3.2.2.1 Evaluation of 5'-dienyl modified oligonucleotide stability

In order to evaluate the stability of the synthesised 5'-dienyl modified oligonucleotides, a series of DNA melting experiments was performed**.**

Oligonucleotide sequence (**3**), complementary to 5'-dienyl modified oligonucleotide sequence (**2**), was synthesised, analysed and purified as decribed previously, and characterised by MALDI-TOF mass spectrometry (**Table 3.3**).

Oligonucleotide sequence 3: 5'- ATC CTG AAT GCG -3'

Calc'd M	MALDI-TOF MS Found $[M - H]^-$
3645.3	3641.8

Table 3.3 – *Mass spectroscopic characterisation of oligonucleotide sequence (**3**), complementary to 5'-dienyl modified sequence (**2**)*

Samples of each of the 5'-dienyl modified oligonucleotides were incubated with equimolar amounts of the complementary sequence (**3**) at room temperature, overnight. The DNA hybrids were then heated to 90 ^{o}C at 1 ^{o}C min^{-1}, measuring the UV absorbance at 260 nm (λ_{max} of single stranded DNA). From the resulting melting curves (**Fig. 3.3**), melting temperatures were determined (**Table 3.4**).

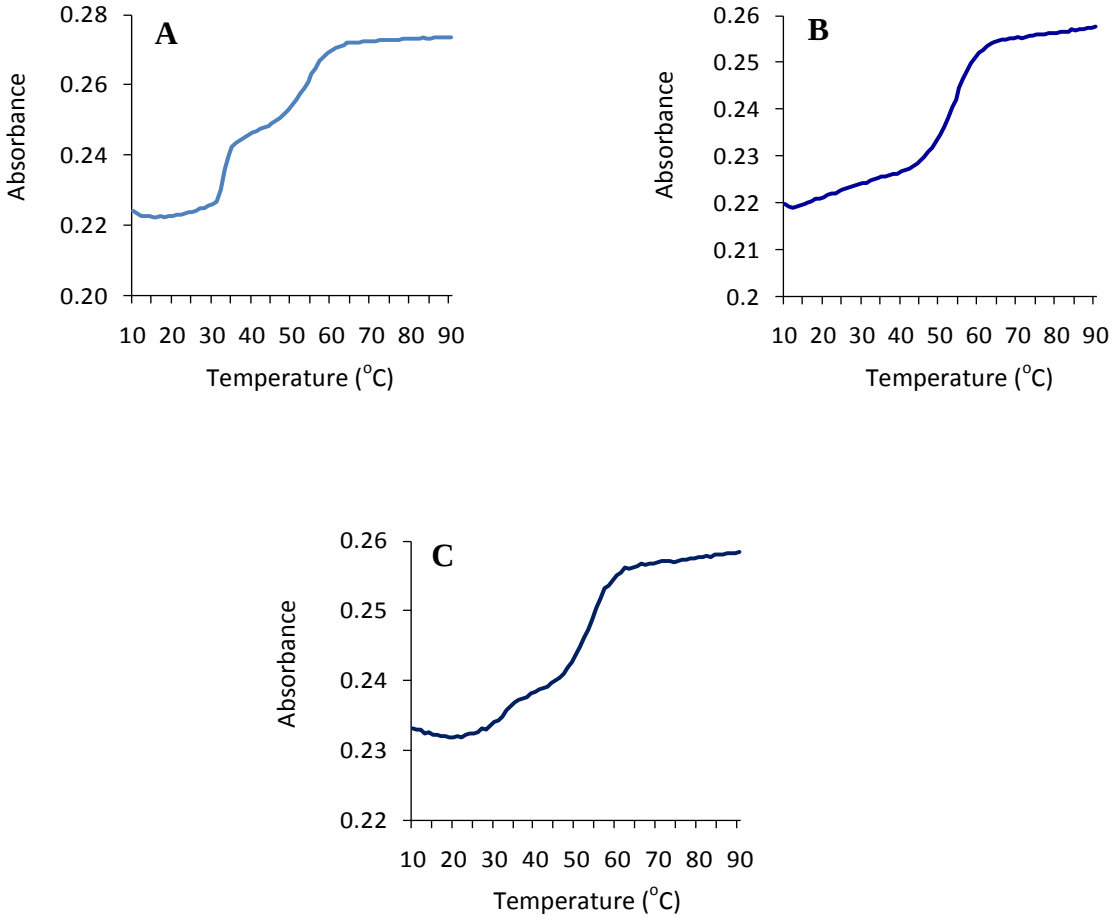


Figure 3.3 – *UV melts at 260 nm of oligonucleotide sequence (**2**), 5'- X CGC ATT CAG GAT, where (**A**) X = butadiene, (**B**) X = furan and (**C**) X = cyclohexadiene*

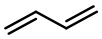
5'-Modification (X)	T_m Found (^{0}C)
	33.0, 54.0
	54.0
	53.2

Table 3.4 – *Melting temperatures (T_m) of dienyl modified oligonucleotide sequence (**2**), 5'- X CGC ATT CAG GAT, determined by UV analysis at 260 nm*

The expected T_m of oligonucleotide sequence (**2**) was estimated to be 57.7 ^{o}C ± 1.4 ^{o}C, using oligonucleotide prediction software. For each of the 5'-dienyl modified oligonucleotides the T_m found was determined to be in the region of 53 – 54 ^{o}C. As such, it was concluded that the 5'-dienyl modification has a negligible effect on oligonucleotide stability. However, an anomaly in the T_m determination of 5'- 1, 3-butadienyl modified oligonucleotide sequence (**2**) was noted, with the observation of an earlier increase in absorbance at 260 nm, resulting in an additional T_m of 33 ^{o}C. A possible explanation for this could be as a result of an intramolecular Diels-Alder [4 + 2] cycloaddition of the butadiene functionality with the thymidine C5-C6 double bond; thymidine dimers are known to form by photochemically-induced [2 + 2] cycloadditions. The first, unexpected transition in the UV melt could then be explained by a retro Diels-Alder reaction, brought about by the increase in temperature. This would result in release of the thymidine bases and a subsequent increase in absorbance at 260 nm, brought about by reformation of their conjugated π-electron systems. A similar anomaly, although to a much lesser extent was also noted for 5'-cyclohexadienyl modified oligonucleotide sequence (**2**). It should be noted however that no evidence was found in the literature to support such a cycloaddition and this is only a theoretical explanation for this phenomenon.

With the 5'- dienyl modifications confirmed by MALDI-TOF analysis and little observed effect on the ability of the dienyl modified DNA to hybridise under

standard conditions, it was decided to continue with this investigation and perform Diels-Alder cycloadditions with the modified sequences.

3.2.3 Diels-Alder Cycloadditions

3.2.3.1 Optimisation of Cycloaddition Reaction Conditions

In order to optimise the reaction conditions for Diels-Alder cycloadditions of the 5'- dienyl modified oligonucleotides with maleimido modified Tat peptide, the decision was taken to first attempt cycloadditions with 5'- furanyl modified oligonucleotide sequence (**2**) and the less expensive fluorescein-5-maleimide (**Fig. 3.5**).

The high extinction coefficient of this maleimide derived dye allowed for easy judgement of the success of the cycloaddition reactions, both visually, by the yellow colour of the oligonucleotide-dye conjugate, and by HPLC analysis.

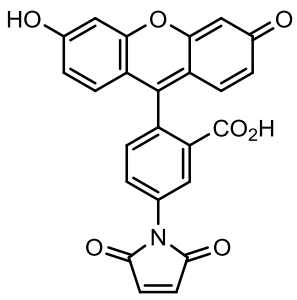


Figure 3.5 – *Molecular structure of fluorescein-5-maleimide*

Research into the literature afforded a protocol, designed by Leuck and Wolter, for Diels-Alder cycloadditions of dienyl modified oligonucleotides and maleimido modified dyes. [12] This protocol uses ten equivalents of the dienophile modified dye as part of a concentrated mixture of the components of the cycloaddition reaction in the following proportions:

20 μl dienyl modified oligonucleotide (1 mM),

50 μl sodium acetate buffer (100 mM, pH 4.5),

25 μl distilled water,

5 μl maleimido dye stock solution (40 mM in DMF).

Leuck and Wolter also recommend heating the cycloaddition reactions at 60 ^{o}C for 4 hours and using crude dienyl modified oligonucleotides; this could be because the suggested method for purification from the oligonucleotide failure sequences, by reverse-phase (RP) HPLC, would be made easier following conjugation to bulky, organic dyes.

Following this protocol, an attempt was made to conjugate crude 5'-furanyl modified oligonucleotide sequence (**2**) to fluorescein maleimide *via* the Diels-Alder reaction. The reaction was performed at 60 ^{o}C for 4 hours, as recommended, followed by removal of the excess dye by size exclusion chromatography. HPLC analysis of the conjugation reaction was performed using anion-exchange buffers and a DNAPac PA200 column (Dionex). The type of methacrylate monomer used as the solid phase in this column is such that, as well as separation of oligonucleotide chains by length of the anionic phosphate backbone, there is some separation of oligonucleotides by virtue of hydrophobic interactions with the bases, any base modifications or any residual protecting groups. [13] As such, conjugation of the 5'-furanyl modified oligonucleotide to the largely hydrophobic fluorescein dye should have a lengthening effect on the retention time when compared to the unconjugated starting material. This was confirmed in the HPLC analysis (**Fig. 3.6**) which showed a new peak with longer retention time than that of the oligonucleotide starting material, indicating formation of the oligonucleotide-dye conjugate. HPLC analysis was performed at both 260 nm (λ_{max} of DNA) and 492 nm (λ_{max} of fluorescein).

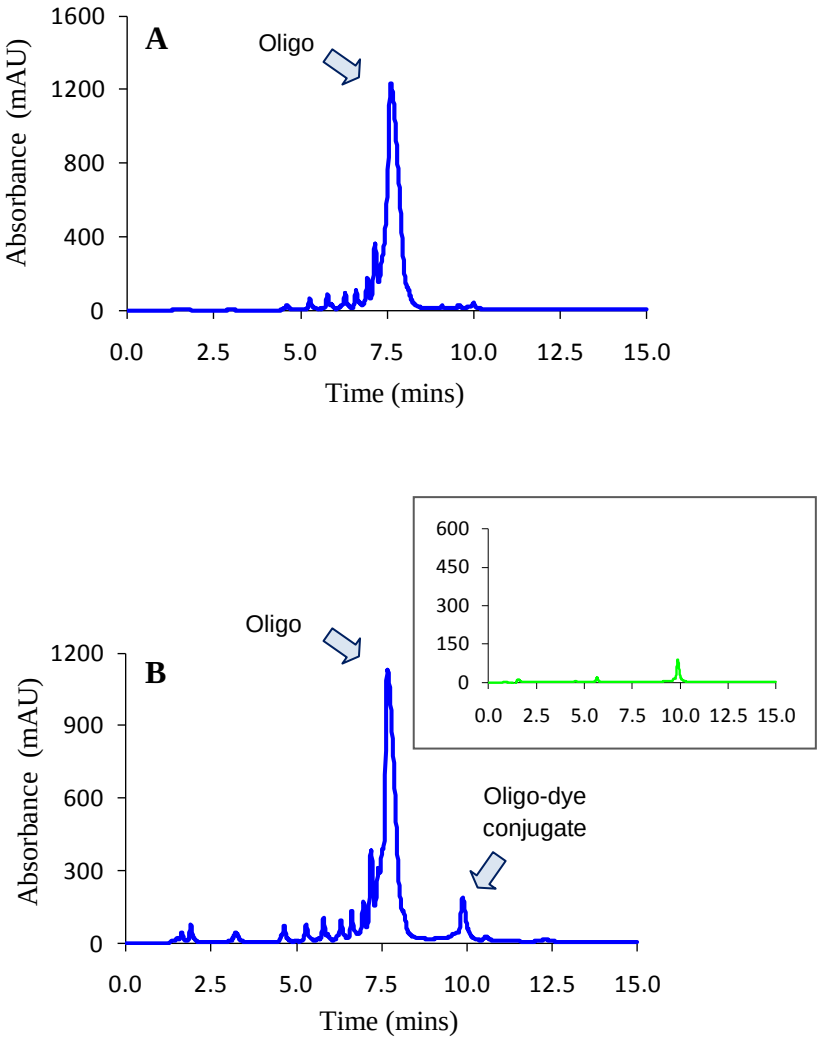


Figure 3.6 – *Ion-exchange HPLC traces of 5'-furanyl modified oligonucleotide and oligonucleotide-fluorescein conjugate, following heating at 60 ^{o}C for 4 hours. (**A**) starting material 5'- X CGC ATT CAG GAT at 260 nm; (**B**) Diels-Alder reaction mixture at 260 nm and 492 nm (**B**, insert)*

Formation of the oligonucleotide-dye conjugate was reaffirmed by observation of a peak at 492 nm with the same retention time as that observed for the conjugate in the trace at 260 nm.

However, the yield of this cycloaddition reaction was low – only 15 % based on the ratio of peak areas. Work by Grondin *et al.*, [14] using Diels-Alder cycloaddition for conjugation of benzotriazole maleimide to a series of dienes, had shown that cycloadditions involving a furanyl moiety were improved by the addition of copper

(II) nitrate, a Lewis acid. Based on this observation, Leuck and Wolter's original cycloaddition protocol was modified by replacement of distilled water with the same amount of 0.01 M copper (II) nitrate. Conjugation of 5'-furanyl modified oligonucleotide to fluorescein maleimide was repeated using this new protocol, at 60 ^{o}C for 4 hours. HPLC analysis of the cycloaddition reaction (**Fig. 3.7**) showed a significant increase in yield of the conjugate.

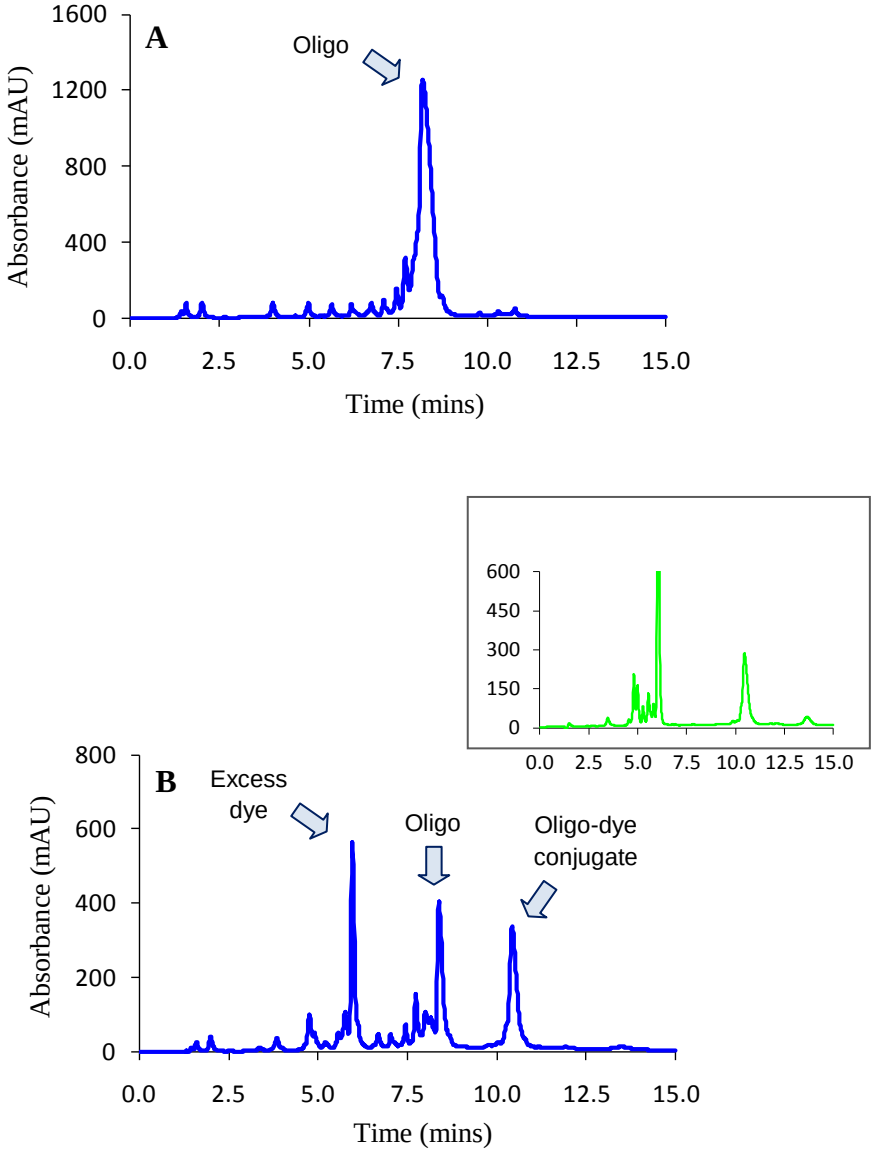


Figure 3.7 – *Ion-exchange HPLC traces of 5'-furanyl modified oligonucleotide and oligonucleotide-fluorescein conjugate, following addition of $Cu(NO_3)_2$ and heating at 60 ^{o}C for 4 hours.* ***(A)*** *starting material 5'- X CGC ATT CAG GAT at 260 nm;* ***(B)*** *Diels-Alder reaction mixture at 260 nm and (**B**, insert) 492 nm*

On addition of copper (II) nitrate to the cycloaddition reaction mixture, the yield of oligonucleotide-dye conjugate formed increased significantly from 15 % to 57 %, based on the area of peak ratios.

Following the improved outcome on addition of copper (II) nitrate, it was decided to attempt conjugation of 5'-furanyl modified oligonucleotide to fluorescein maleimide at 40 ^{o}C, since this lower temperature would be better suited to oligonucleotide conjugation to Tat peptide. The reaction was heated at 40 ^{o}C overnight. HPLC analysis showed formation of the oligonucleotide-dye conjugate but also, unexpectedly, showed a further increase in conjugate yield to 83 %, based on the area of peak ratios (**Fig. 3.8**).

As such, it was concluded that optimal reaction conditions for conjugation of 5'-dienyl modified oligonucleotides to a maleimide derivative *via* Diels-Alder cycloaddition are heating at 40 ^{o}C overnight, with the addition excess copper (II) nitrate for Lewis acid catalysis. These conditions were carried through to cycloaddition reactions for the conjugation of 5'-dienyl modified oligonucleotides to maleimido modified Tat peptide.

It is worth noting that in all the aforementioned cycloadditions of the 5'-dienyl modified oligonucleotides and fluorescein maleimide, the HPLC traces of the reaction mixtures showed a much greater extent of oligonucleotide impurity than the traces of the oligonucleotide starting materials. This may be due to degradation as a result of incubation with Cu^{2+}; in Cu (I) catalysed Huisgen 1, 3- dipolar cycloadditions of azido modified oligonucleotides, a copper stabilising ligand has been used to prevent this. [15] It should be noted that no control sample, resulting from incubation of the 5'-dienyl modified oligonucleotides and copper (II) nitrate only, was analysed to confirm whether or not this was the case.

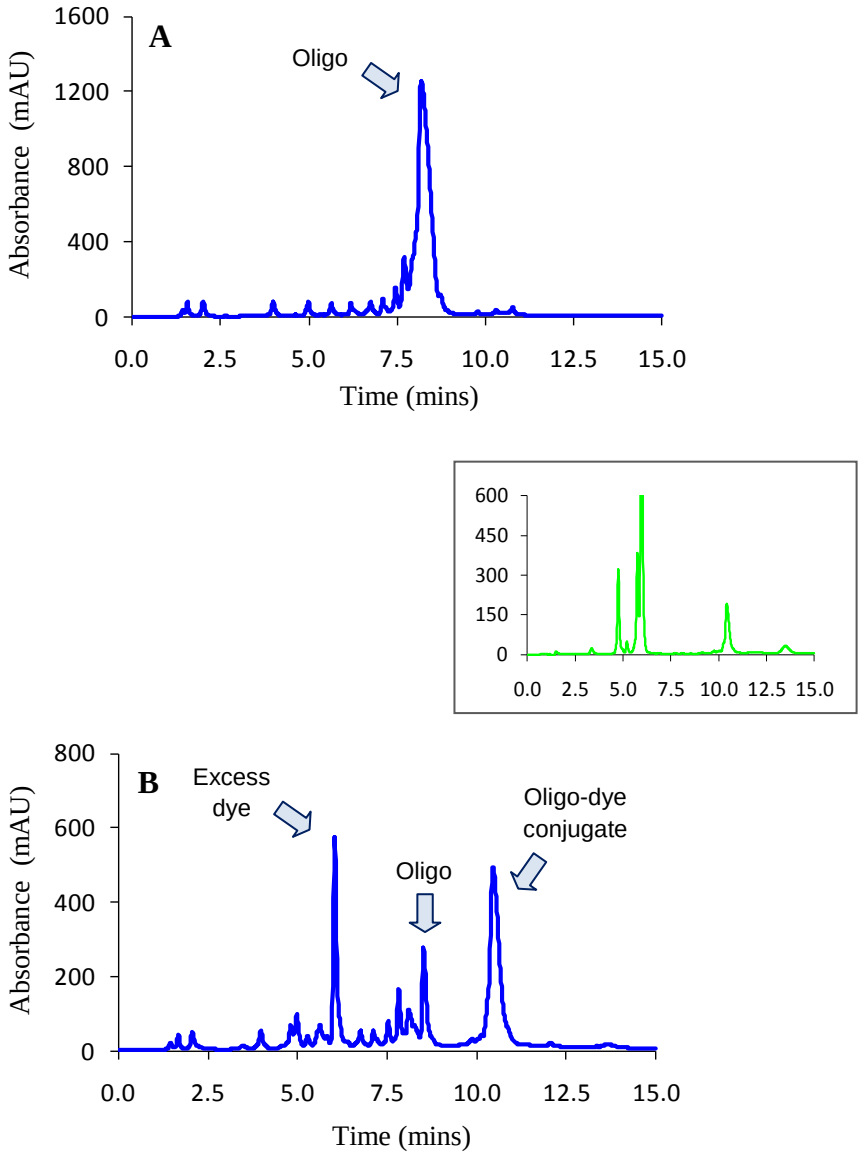


Figure 3.8 – *Ion-exchange HPLC traces of 5'-furanyl modified oligonucleotide and oligonucleotide-fluorescein maleimide conjugate, following addition of $Cu(NO_3)_2$ and heating at 40 ^oC overnight. (**A**) starting material 5'- X CGC ATT CAG GAT at (**A**) 260; (**B**) Diels-Alder reaction mixture at 260 nm and (**B**, insert) 492 nm*

3.2.3.2 Oligonucleotide conjugation to Tat peptide

The peptide sequence to be conjugated to the dienyl modified oligonucleotide sequences was a maleimido modified derivative of Tat peptide, residues 47 - 57 (**Fig. 3.9**). The peptide derivative was modified with maleimide at the *N*-terminus using

the heterobifunctional crosslinking reagent 3-maleimidobenzoic acid *N*-hydroxy-succinimide (MBS).

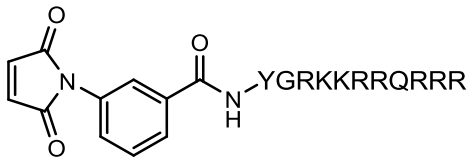


Figure 3.9 – *Maleimido modified Tat peptide derivative*

Before performing the bioconjugation reaction, it was necessary to make a further modification to the cycloaddition protocol. Due to the highly anionic nature of the oligonucleotide sequences, attributed to the polyphosphate backbone, and the highly cationic nature of the Tat peptide derivative, attributed to the majority number of arginine and lysine residues, addition of one to the other would result in binding of the two through ionic interactions as opposed to the desired covalent linkage brought about by the Diels-Alder cycloaddition. Turner *et al.* [16] recommend the use of formamide as a denaturing agent, due to its high availability for hydrogen bonding, in the conjugations of oligonucleotides to highly cationic residues. Based on this recommendation and the previous observation that addition of copper (II) nitrate was necessary for high cycloaddition yields, the protocol for conjugation of dienyl modified oligonucleotides to maleimido modified Tat peptide became:

20 μl dienyl modified oligonucleotide (1 mM),
50 μl sodium acetate buffer (100 mM, pH 4.5),
25 μl copper (II) nitrate (0.01 M),
60 μl formamide (neat),
5 μl maleimido dye stock solution (40 mM in DMF).

Cycloaddition reactions were also attempted without formamide as a component of the reaction mixture, without success, reaffirming Turner's report that a denaturing agent is necessary in bioconjugations involving cationic peptides.

Conjugations of 5'-dienyl modified oligonucleotides to the maleimido modified Tat peptide derivative were performed at 40 ^{o}C overnight, using solutions of crude oligonucleotides; purification was performed after covalent linkage to the Tat peptide derivative. The cycloaddition reaction mixtures were analysed directly, by anion exchange HPLC. Anion exchange HPLC was the chosen method for analysis of the cycloaddition reaction mixtures due to the highly cationic nature of the Tat peptide derivative; its conjugation to the anionic charge-carrying oligonucleotide would result in a decrease in the overall anionic charge compared to the starting oligonucleotide, bringing about a decrease in retention time on the HPLC trace. A Resource Q column has been reported to be the most suitable column for analysis of oligonucleotides conjugated specifically to Tat peptide. [16] This type of column was used in conjunction with eluents containing formamide to ensure denaturation of any ionic interactions between the oligonucleotide and Tat peptide which could potentially lead to false positive results.

The Diels-Alder cycloaddition for oligonucleotide conjugation to Tat peptide was first performed using the 1, 3-butadienyl modification. Following heating overnight at 40 ^{o}C, HPLC analysis of the conjugation mixture showed formation of a new peak (**Fig. 3.10 B**) with shorter retention time than that of the unconjugated oligonucleotide (**Fig. 3.10 A**), indicating formation of the oligonucleotide Tat peptide conjugate.

Characterisation of the conjugation product was achieved by MALDI-TOF mass spectrometry (**Table 3.5**, page 64). It was found that, prior to mass spectroscopy, the conjugate product had to be purified using ZipTip ™ C_{18} purification pipette tips in order to observe the desired mass. Optimal conditions for characterisation of the oligonucleotide Tat peptide conjugate were found to be performing the mass analysis in positive mode, using a matrix commonly used for MALDI-TOF analysis of peptides. [17] Analysis of the reaction product, isolated by HPLC, showed the mass of the desired conjugate. However, the yield of this reaction was found to be low, with an observed conversion of only 23 % (based on the ratio of peak areas) of oligonucleotide sequence (**2**) to the Tat peptide conjugate.

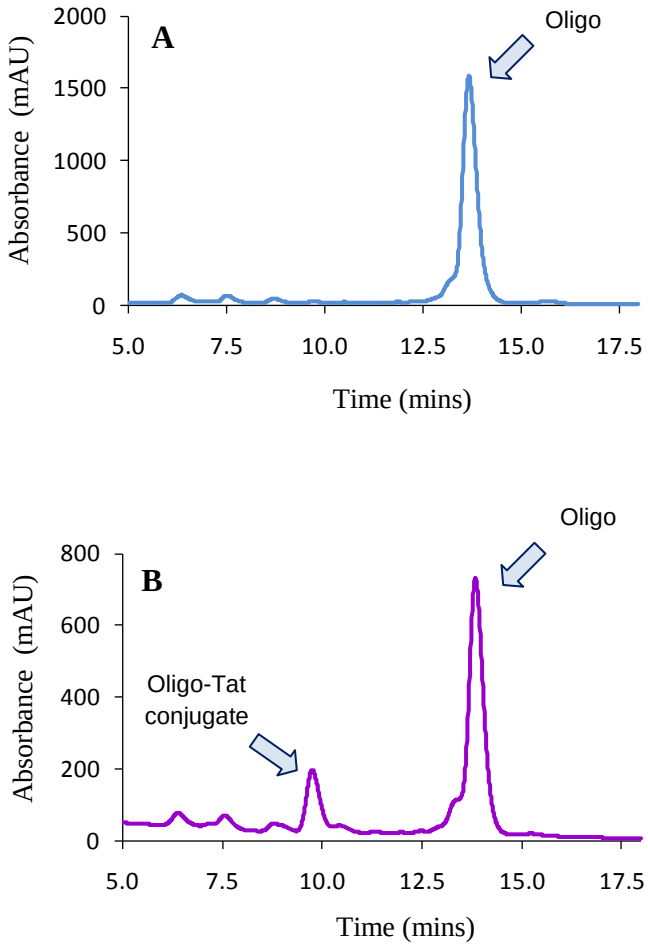


Figure 3.10 – *Ion-exchange HPLC traces of 5'-butadienyl modified oligonucleotide and oligonucleotide Tat peptide conjugate at 260 nm.* ***(A)*** *starting material 5'- X CGC ATT CAG GAT,* ***(B)*** *Diels-Alder reaction mixture.*

The conjugation reaction was repeated using 5'-furanyl modified oligonucleotide sequence (**2**), under the same conditions. HPLC analysis of the conjugation mixture showed an improved reaction yield, with conversion to the oligonucleotide peptide conjugate increasing to 33 %, based on the ratio of peak areas (**Figure 3.11**).

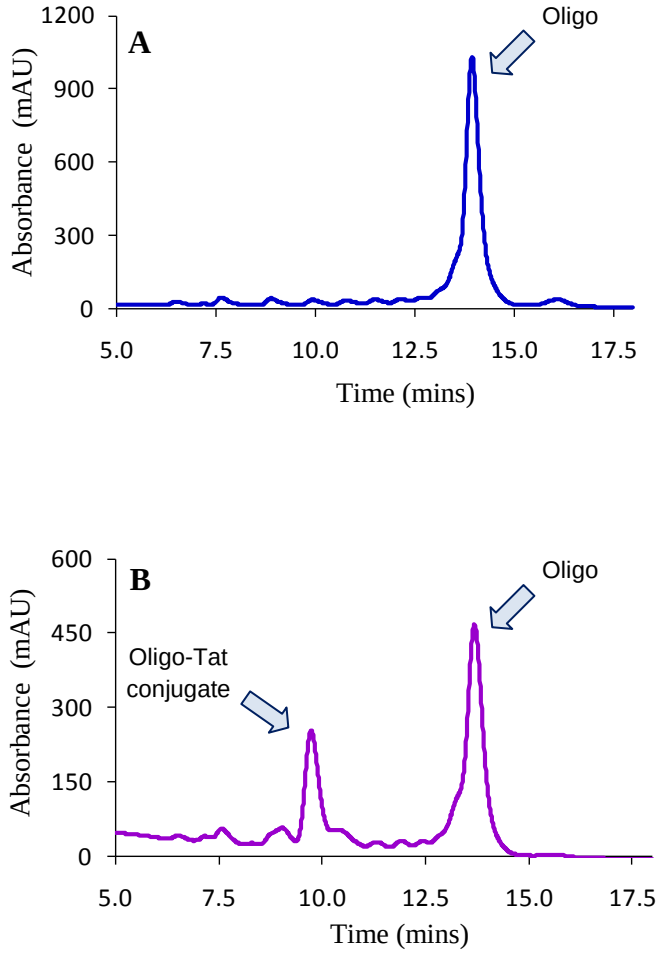


Figure 3.11 – *Ion-exchange HPLC traces of 5'-furanyl modified oligonucleotide and oligonucleotide Tat peptide conjugate at 260 nm. (**B**) starting material 5'- X CGC ATT CAG GAT, (**B**) Diels-Alder reaction mixture.*

Analysis of the reaction product by MALDI-TOF mass spectrometry showed the desired mass of the oligonucleotide peptide conjugate (**Table 3.5**, page 64).

Furans are often used in Diels-Alder reactions because of their high reactivity; this has been attributed to the fixed *s-cis*-conformation of the diene, by virtue of its cyclic structure, and the electron donating capacity of the furanyl oxygen. It was therefore concluded that this was feasible reasoning for the improved outcome of conjugation to the maleimido modified Tat peptide using the 5'-furanyl modified oligonucleotide sequence **(2)** versus the 5'- 1, 3-butadienyl modified sequence.

Conjugation of 5'-cyclohexadienyl modified sequence **(2)** to the maleimido modified Tat peptide derivative was then attempted (**Fig. 3.12**).

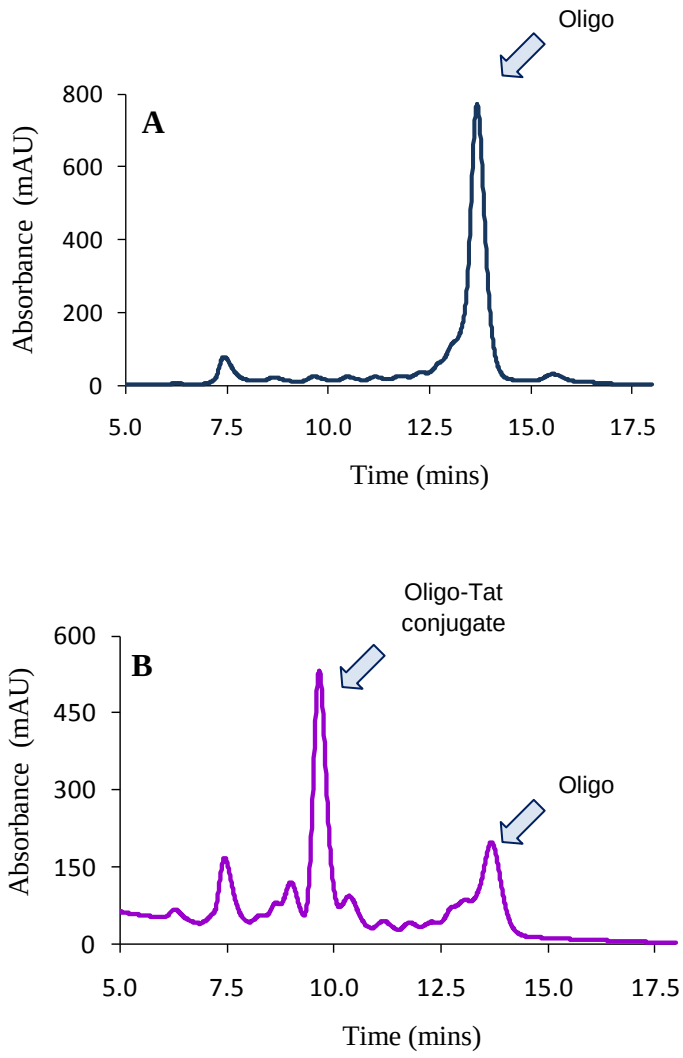


Figure 3.12 – *Ion-exchange HPLC traces of 5'-cyclohexadienyl modified oligonucleotide and oligonucleotide Tat peptide conjugate at 260 nm. (**A**) starting material 5'- X CGC ATT CAG GAT, (**B**) Diels-Alder reaction mixture.*

As per the furanyl modification, the diene is held firmly in a fixed *s-cis* - conformation; however, there is no electron donating group present on the ring. As such, a lower yield of oligonucleotide Tat peptide conjugate was expected from the Diels-Alder cycloaddition with cyclohexadiene c.f., furan. Surprisingly though, an increase in conjugate yield was apparent, with observation by, ion-exchange HPLC analysis, of a significantly larger peak with shorter retention time than that of the oligonucleotide starting material. A significant increase from 33 % to 72 % conversion to the oligonucleotide Tat peptide conjugate was calculated, based on the ratio of peak areas. MALDI-TOF mass analysis of the reaction product showed the desired mass of the oligonucleotide peptide conjugate (**Table 3.5,** page 64).

Further research into the literature revealed that Diels-Alder adducts of furan and maleimide have been shown to be thermally labile and dissociate readily into their original components; in fact, Diels-Alder reactions with furan have been used as a strategy for the protection of maleimides. [18] Contradictory to the theory that, by kinetic control, the *endo* adduct is the major product of Diels-Alder reactions, Berson and Swidler found that a mixture of both the *endo* and *exo* isomers was formed in reactions of furan with maleimide. [19] It has since been observed that the *endo* adduct formed from furan and maleimide at 20 ^{o}C dissociates at elevated temperatures, bringing about conversion of the kinetically controlled adduct to the thermodynamically more stable *exo* isomer. [20] Rulišek and co workers [21] propose that the propensity of maleimide-furan adducts to undergo dissociation *via* a retro Diels-Alder reaction is as a result of a loss of aromaticity of the furan ring as a component of the cycloadduct. These observations could thereby account for the lower yield calculated for the Diels-Alder cycloaddition of the 5'-furanyl modified oligonucleotide with maleimido modified Tat peptide c.f., that which was calculated for the 5'-cyclohexadienyl modified oligonucleotide.

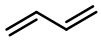
5'-Modification (X)	Calc'd *M*	MALDI-TOF MS Found $[M + H]^+$
	5676.9	5676.0
	5704.9	5706.5
	5718.9	5719.9

Table 3.5 – *Mass spectroscopic characterisation data of malemido Tat oligonucleotide peptide conjugates, Tat 5'- X CGC ATT CGA GAT.*

As such, it was concluded that the cyclohexadiene modification was the optimum dienyl modification to use for oligonucleotide conjugation to a maleimido modified Tat peptide *via* the Diels-Alder reaction. [22]

As discussed earlier, the Diels-Alder cycloaddition is usually reversible, although the retro reaction typically occurs at higher temperatures than the forward reaction. Oligonucleotide conjugation to Tat peptide was performed at 40 ^{o}C, but the reaction mixtures were analysed after cooling to room temperature. As such, a retro Diels-Alder reaction at elevated temperatures could be a possibility. Bearing in mind that use of an oligonucleotide Tat conjugate probe in cell assays would more than likely involve incubation at 37 ^{o}C (body temperature), it was deemed necessary to investigate whether cleavage of the oligonucleotide from the peptide *via* the retro Diels-Alder reaction could be a possibility at this temperature. A sample of Tat 5'-X CGC ATT CAG GAT, where X = cyclohexadiene was heated over a period of 5 hours at 37 ^{o}C and monitored by ion-exchange HPLC analysis at hourly intervals. No obvious evidence of cleavage was observed (**Fig. 3.13**); however, the emergence of a shoulder on the conjugate peak was apparent.

In order to ensure that this shoulder was not indicative of cleavage, a sample of the oligonucleotide peptide conjugate, following heating at 37 ^{o}C for 5 hours, was mixed with a sample of unconjugated 5'-cyclohexadienyl modified oligonucleotide sequence **(2)** for HPLC analysis (**Fig 3.13**, insert). Two peaks, with a significant

difference in retention time, were clearly visible, thereby confirming that the oligonucleotide Tat conjugate is not broken down into its individual components *via* a retro Diels-Alder reaction at 37 ^{o}C. A possible explanation then for the formation of the peak shoulder could be hydrolysis of the maleimide component of the Diels-Alder linkage as a result of the prolonged heating in aqueous media.

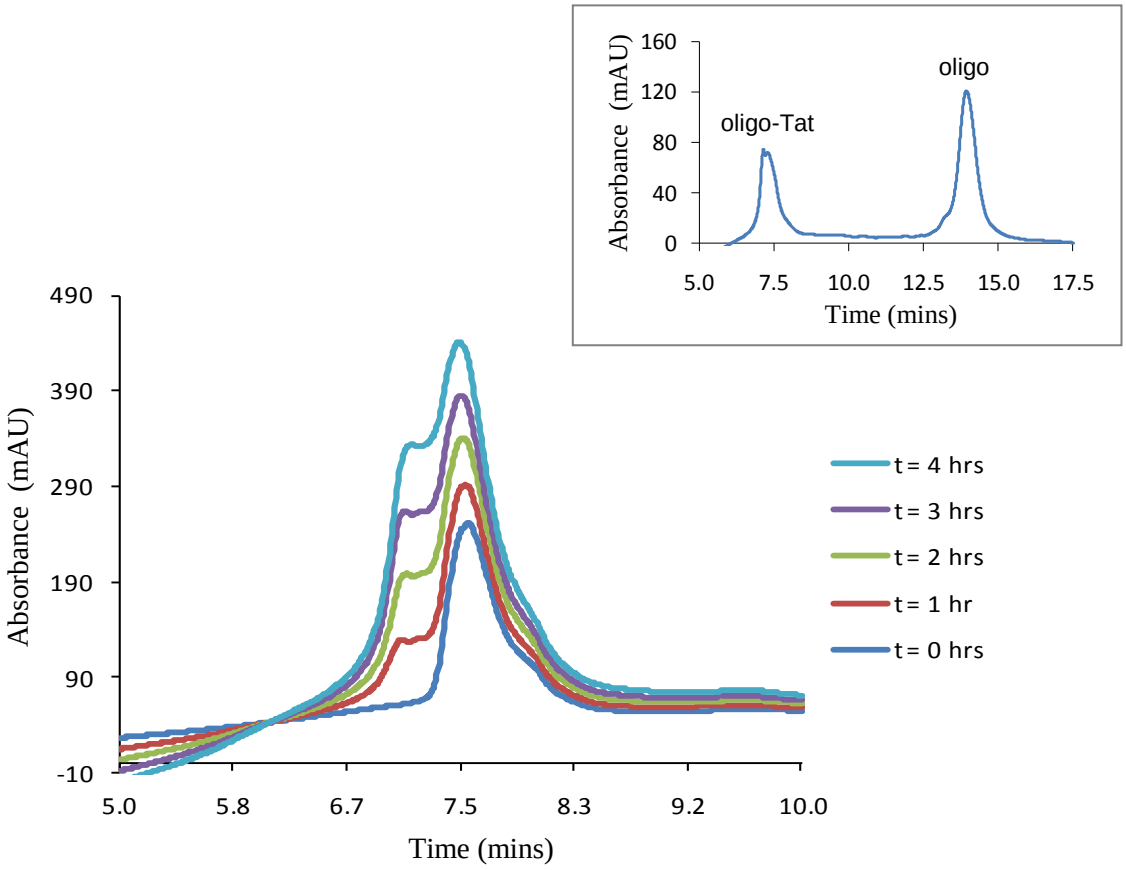


Figure 3.13 – *Ion-exchange HPLC traces at 260 nm of Tat 5'- X CGC ATT CAG GAT, where X = cyclohexadiene, heated at 37 ^{o}C over 5 hours.* ***(Insert** – ion-exchange HPLC trace at 260 nm of oligonucleotide Tat conjugate, post heating at 37 ^{o}C for 5 hours, mixed with unconjugated oligonucleotide)*

3.2.3.3 Conjugation of labelled oligonucleotides to Tat peptide

It was necessary to incorporate a visualisation element into the oligonucleotide Tat peptide conjugates, in order to effectively assess their cellular uptake. This was

made possible by using oligonucleotide sequences labelled with a fluorescent dye at the 3'-terminus. Previous experiments had shown that the cyclohexadiene modification gave the best yields for oligonucleotide conjugation to Tat peptide *via* Diels-Alder cycloaddition. As such, this was the diene of choice for oligonucleotide modification at the 5'-terminus. Dye labelled sequences were synthesised on an automated DNA synthesiser as described previously (See 3.2.2 – *Synthesis of 5'-dienyl modified oligonucleotides*), but using a CPG column pre-modified with either 6-FAM or TAMRA fluorescent labels (**Fig. 3.14**).

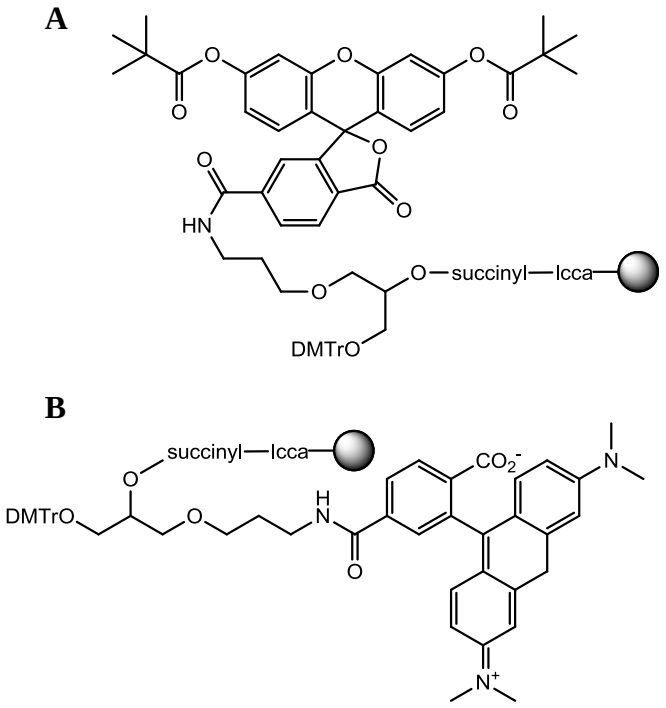


Figure 3.14 – *Molecular structures of (**A**) 6-FAM and (**B**) TAMRA modified CPG for fluorescent oligonucleotide labelling at the 3'-terminus*

Two labelled sequences, modified with cyclohexadiene at the 5'-terminus were synthesised then analysed and purified by ion-exchange HPLC, as described previously. Analysis of the oligonucleotide products by MALDI-TOF mass spectrometry confirmed synthesis of the desired sequences (**Table 3.6**).

Oligonucleotide Sequence No.	Sequence	Calc'd *M*	MALDI-TOF MS Found $[M - H]^-$
4	5' - X CGC ATT CAG GAT FAM - 3'	4529.5	4526.0
5	5' - X CGC ATT CAG GAT TAMRA -3'	4548.6	4536.2

Table 3.6 – *Mass spectroscopic characterisation data of 3'- FAM / TAMRA labelled oligonucleotide sequences, modified with cyclohexadiene at the 5'- terminus*

Tat peptide conjugation to oligonucleotide sequence (**4**) was attempted. The FAM-labelled sequence was reacted with maleimido modified Tat peptide using the reaction conditions described previously (See *3.2.3.2 – Oligonucleotide conjugation to Tat peptide*); heating at 40 ^{o}C overnight, with the addition of formamide to the reaction mixture. Unexpectedly, ion-exchange HPLC analysis of the cycloaddition reaction mixture showed the yield of the labelled oligonucleotide peptide conjugate to be very low (**Fig. 3.15**), with observation of only a very small peak with shorter retention time than the oligonucleotide starting material. It was found that using the FAM labelled sequence **(4)**, the yield of the cycloaddition was reduced to 20 % (based on the ratio of peak areas) *c.f.*, unlabelled 5'-cyclohexadienyl modified sequence **(2)**.

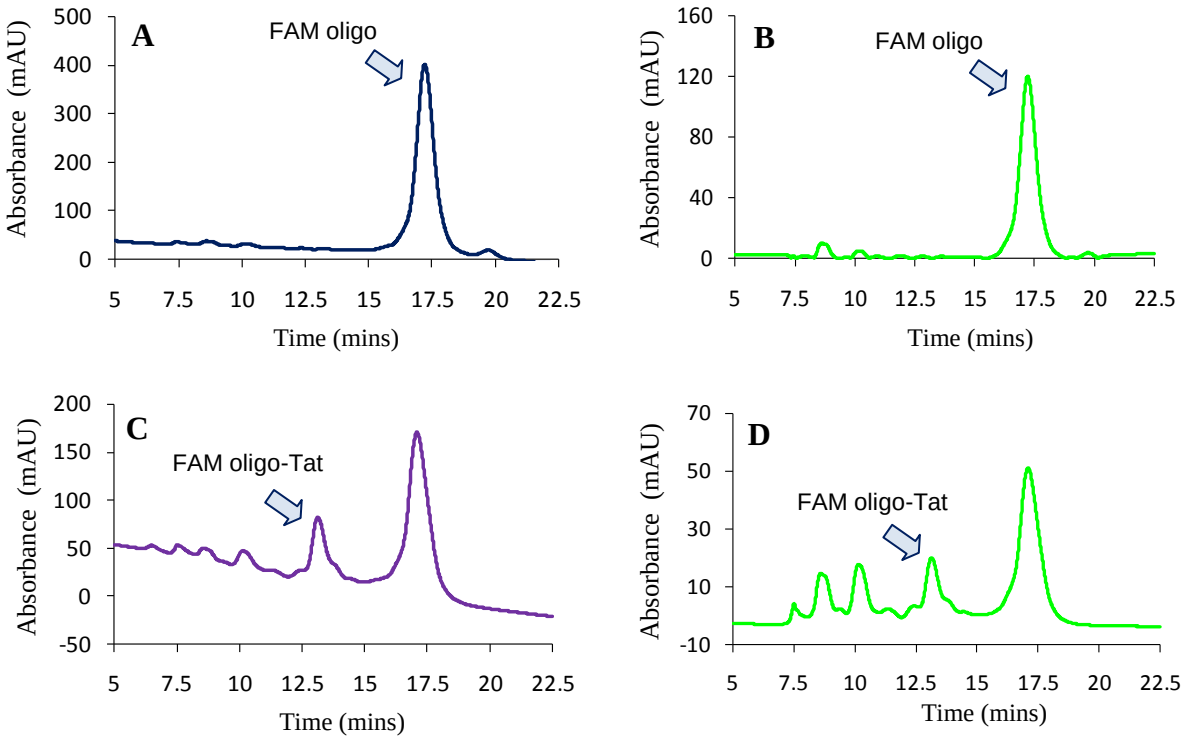


Figure 3.16 – *Ion-exchange HPLC traces of 3'- FAM labelled, 5'-cyclohexadienyl modified oligonucleotide and labelled oligonucleotide Tat peptide conjugate. Starting material 5'- X CGC ATT CAG GAT - FAM- 3' at (**A**) 260 nm and (**B**) 492 nm; Diels-Alder reaction mixture at (**C**) 260 nm and (**D**) 492 nm*

The only modification to the procedure used for conjugation of 5'-cyclohexadienyl modified oligonucleotide to maleimido modified Tat peptide was the inclusion of the FAM label at the 3'-terminus of the oligonucleotide sequence. As such it was decided to attempt the cycloaddition again, but using a different fluorescent label. The cycloaddition reaction was repeated using TAMRA labelled oligonucleotide sequence (**5**). However, HPLC analysis of the cycloaddition reaction mixture showed that the yield of this reaction was also low (**Fig. 3.17**). Using the TAMRA labelled sequence, the yield of the cycloaddition reaction with maleimido modified Tat peptide was calculated to be only 12 % (based on the ratio of peak areas).

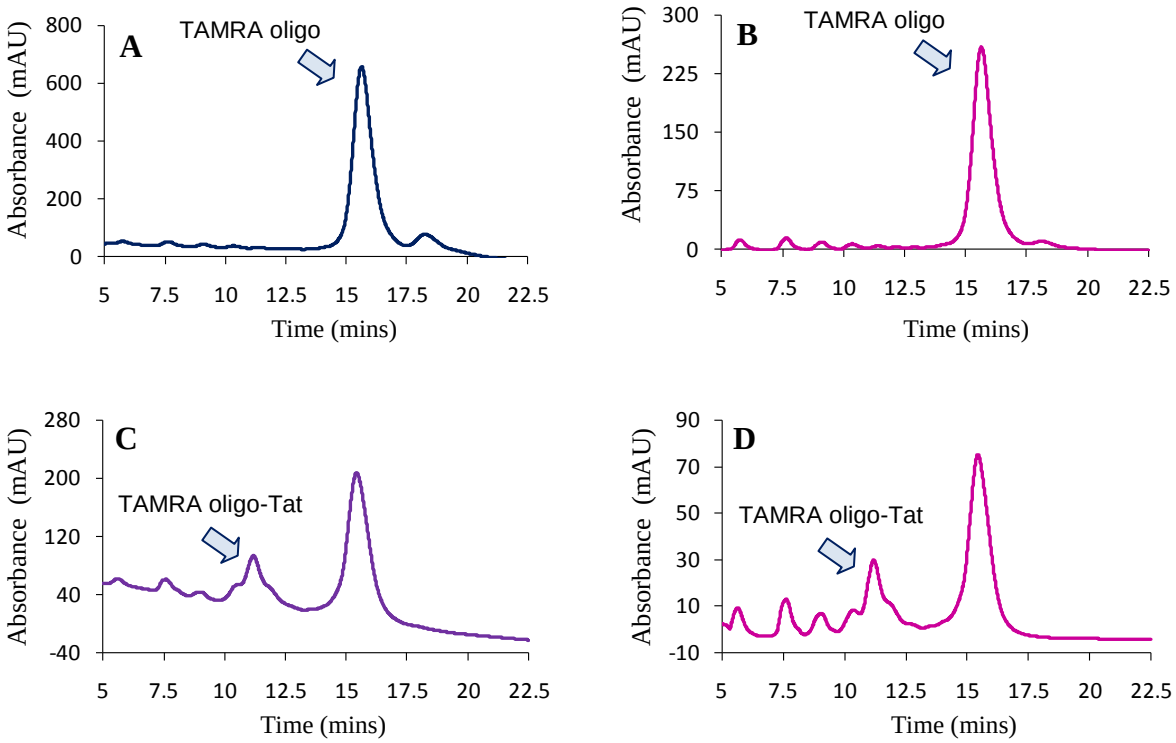


Figure 3.17 – *Ion-exchange HPLC traces of 3'- TAMRA labelled, 5'-cyclohexadienyl modified oligonucleotide and labelled oligonucleotide Tat peptide conjugate. Starting material 5'- X CGC ATT CAG GAT - TAMRA -3' at (**A**) 260 nm and (**B**) 565 nm; Diels-Alder reaction mixture at (**C**) 260 nm and (**D**) 565 nm*

The low yields for Tat peptide conjugation to labelled, 5'- cyclohexadienyl modified oligonucleotides were unexpected. A possible explanation for this could be that, in the case of the labelled sequences, the conjugated diene is involved in π-stacking interactions with the aromatic rings of the fluorescent dyes, thereby reducing its reactivity in the Diels-Alder reaction.

Once again, it should be noted that HPLC analysis of the cycloaddition mixtures appears to show a greater degree of oligonucleotide impurity when compared with the oligonucleotide starting material that could be due to degradation as a result of incubation with Cu^{2+}.

Several attempts were made to improve the yield of cycloadditions with labelled oligonucleotides; the proportion of denaturant present in the reaction mixture was increased up to 80 % formamide; the proportion of copper (II) nitrate Lewis acid present in the reaction mixture was increased; the pH of the reaction was varied by way of a different buffer system; the temperature at which the reaction was performed was increased up to 85 ^{o}C and a solid-phase cycloaddition reaction was attempted using FAM labelled sequence **(4)** attached to CPG. However, none of these alterations to the reaction conditions resulted in an improved yield of the desired oligonucleotide peptide conjugate. As such, it was concluded that the Diels-Alder reaction was not a viable method for conjugation of 5'- dienyl oligonucleotides to maleimido modified Tat peptide, if the oligonucleotide sequences in question incorporates a fluorescent label at the 3'- terminus.

3.3 Conclusions

A series of 5'-dienyl modified oligonucleotides have been generated *via* synthesis of 1, 3-butadienyl, furanyl and cyclohexadienyl modified phosphoramidites. Diels-Alder cycloadditions of a 5'-furanyl modified 12-mer oligonucleotide sequence with a fluorescein maleimide dye showed, through ion-exchange HPLC analysis of the reaction mixtures, that the highest yield of oligonucleotide-dye conjugate were achieved by heating the reaction at 40 ^{o}C overnight at pH 4.5, with the addition of a Lewis acid; in this case copper (II) nitrate.

Using these optimum conditions, Diels-Alder cycloadditions of 1, 3-butadienyl, furanyl and cyclohexadienyl modified 12-mer oligonucleotide sequences with a maleimido modified derivative of Tat peptide were performed. It was found that it was necessary to add formamide to the reaction mixture as a denaturant; in its absence no product was afforded. Ion-exchange HPLC analysis of the reaction mixtures showed that the highest yield of oligonucleotide Tat peptide conjugate was achieved using 5'-cyclohexadienyl modified oligonucleotide.

To assess the possibility of oligonucleotide cleavage from the cell penetrating peptide *via* a retro Diels-Alder reaction, a sample of oligonucleotide Tat peptide conjugate was heated over a period of 5 hours at 37 ^{o}C and monitored by ion-exchange HPLC. No evidence of cleavage was observed, although there was some evidence to suggest conjugate decomposition.

In order to assess the cellular uptake of oligonucleotide Tat peptide conjugates synthesised *via* Diels-Alder cycloaddition, it was necessary to incorporate a tool for visualisation as a component of the conjugate. This was attempted by cycloaddition of a 5'-cyclohexadienyl modified 12-mer oligonucleotide sequence, labelled at the 3'-terminus with a fluorescent (FAM) dye, with the maleimido modified Tat peptide derivative. However, it was found that the conjugate yield was dramatically reduced when using a labelled oligonucleotide sequence, from 72 % to 20 %. Diels-Alder cycloadditions were attempted with a 12-mer oligonucleotide sequence labelled with an alternative fluorescent (TAMRA) dye; however no increase in yield was observed. Cycloadditions were also attempted at different pH, increased temperature, with an increased proportion of formamide and on the solid phase, but no improvement in conjugate yield was evident.

As such, it was concluded that, while Diels-Alder cycloaddition is an excellent method for bioconjugation of unlabelled oligonucleotides to a cell penetrating (Tat) peptide, it is not viable for the conjugation of labelled oligonucleotides, and as such would not be suitable for use in the synthesis of oligonucleotide Tat peptide conjugates as biosensors.

3.4 Experimental

3. 4. 1 General

Solvents used were of laboratory grade. Anhydrous DMF and anhydrous MeOH were supplied in SureSeal ™ bottles by Sigma-Aldrich; anhydrous MeCN was supplied in a sealed bottle by Link Technologies.

All reagents were supplied by Sigma-Aldrich; maleimido modified Tat peptide derivative was supplied by CSS Albachem, off resin and of > 95 % purity by HPLC analysis.

Thin layer chromatography was carried out on silica gel, pore size 60 Å, 0.2 mm layer backed with aluminium (Merck). Purification by wet flash column chromatography was performed using silica gel, pore size 60 Å (Sigma-Aldrich).

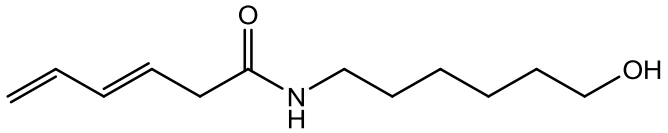
^{1}H NMR, ^{13}C NMR and ^{31}P NMR were recorded on a Brüker DPX 400 MHz spectrometer with the appropriate solvent as reference. *J* values are quoted in Hertz. Mass spectrometry was carried out as a service either by the university or by the EPSRC National Mass Spectrometry Centre, Swansea. MALDI-TOF mass spectrometry was performed on a Shimadzu Axima-CFR system. Analytical and preparative HPLC were performed on a Dionex UVD170U detector, operating Chromeleon software, fitted with a P680 pump, through either a DNA Pac PA200 column (Dionex) on a Resource Q (1 ml) column (Amersham Biosciences). Desalting HPLC was performed on a Dionex UVD 170U detector fitted, operating Chromeleon software, with a P680 pump and a CD20 Conductivity Detector, using a HiTrap (5 ml) size exclusion column. UV analysis was performed on a Cary 300 Bio UV/Visible spectrometer.

Oligonucleotides were synthesized on a MerMade 6 Nucleic Acid Synthesiser. Oligonucleotide synthesis reagents were purchased from Link Technologies. Estimated loading of columns with functionalised CPG was 1.0 µmol.

Tube-O-Dialyzers were purchased from G-Biosciences. Zip Tip ™ C_{18} purification pipette tips were purchased from Millipore Corp.

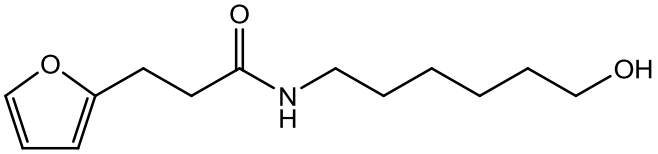
3. 4. 2 Chemical Synthesis

(3-E/Z)-N-(6-hydroxyhexyl) hexa-3, 5-dienamide (1a)



To a solution of sorbic acid (500 mg, 4.5 mmol) in anhydrous MeCN (10 ml) was added, under dark conditions, CDI (868 mg, 5.4 mmol). Formation of CO_2 gas was observed. The mixture was stirred at room temperature for 3 hrs, after which time 6-amino-1-hexanol (784 mg, 6.6 mmol) was added. The mixture was stirred at room temperature overnight. The solvent was removed under reduced pressure. The residue was dissolved in EtOAc and washed with citric acid solution (10 % m/v), NaOH solution (10 % m/v), saturated NaCl solution and water. The organic phase was dried over Na_2SO_4. After filtration, EtOAc was removed under reduced pressure to afford the title compound as a white solid (284 mg, 30 %). (Found: C, 68.2; H, 9.8; N, 6.6. $C_{12}H_{21}NO_2$ requires C, 68.2; H, 10.0; N, 6.6%); δ_H (400 MHz; $CDCl_3$) 1.25 – 1.61 (8H, m, 4 x C*H*$_2$), 1.84 (2H, d, *J* 5.8, C*H*$_2$CO), 3.34 (2H, q *J* 6.7, NHC*H*$_2$), 3.64 (2H, t, *J* 6.5, C*H*$_2$OH), 5.48 (1H, s br, N*H*), 5.70 (1H, s, C*H*$_2$=CH), 5.74 (1H, s C*H*$_2$=CH), 6.04 – 6.19 (2H, m, C*H*-C*H*), 7.16 -7.22 (1H, m, CH=C*H*); δ_C (100.6 MHz; d_6-acetone) 14.4, 18.5, 26.3, 27.5, 33.7, 39.7, 62.2, 124.0, 131.0. 136.7, 140.2, 166.5; *m/z* 212.1648 ([M + H^+]) $C_{12}H_{21}NO_2$ requires 212.1651); mp 115 ^oC.

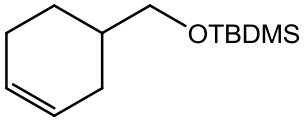
3-furan-2-yl-N-(6-hydroxyhexyl) propanamide (1b)



To a solution of 3-(2-furyl) propanoic acid (252 mg, 1.8 mmol) in anhydrous MeCN (5 ml) was added CDI (350 mg, 2.2 mmol). The formation of CO_2 gas was observed. The mixture was stirred at 40 ^oC for 1 hr 30 mins, after which time 6-amino-1-

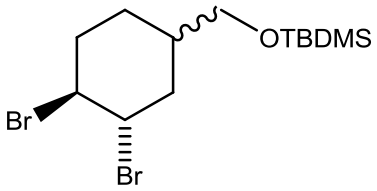
hexanol was added. The mixture was stirred at room temperature for 2 hrs. The solvent was removed under reduced pressure. The residue was dissolved in EtOAc and washed with citric acid solution (10 % m/v), NaOH solution (10 % m/v), saturated NaCl solution and water. The organic phase was dried over Na_2SO_4. After filtration, EtOAc was removed under reduced pressure to afford the title compound as a pale yellow solid (302 mg, 70 %). (Found: C, 65.3; H, 8.8; N, 5.8. $C_{13}H_{21}NO_3$ requires C, 65.3; H, 8.8; N, 5.9 %); δH (400 MHz, $CDCl_3$) 1.26 – 1.60 (8H, m, 4 x C*H*$_2$), 2.49 (2H, t, *J* 7.5, C*H*$_2$CO), 2.99 (2H, t, *J* 6.5, furan C*H*$_2$), 3.24 (2H, t, *J* 6.5, NHC*H*$_2$), 3.64 (2H, t, *J* 6.5, C*H*$_2$OH), 5.44 (1H, s br, N*H*), 6.04 (1H, dd, *J* 1.8, 0.8, furfuryl), 6.28 (1H, dd, *J* 3.1, 1.9, furfuryl), 7.31 (1H, dd, *J* 1.8, 0.8, furfuryl); δ_C (100.6 MHz, $CDCl_3$) 24.2, 25.2, 26.4, 29.6, 32.5, 35.1, 39.3, 62.7, 105.6, 110.3, 141.2, 154.4, 171.7; *m/z* 240.1594 ([M + H^+]) $C_{13}H_{21}NO_3$ requires 240.1594; mp 75 ^oC.

4-(*tert*-Butyldimethylsilyloxymethyl) cyclohex-1-ene (2)



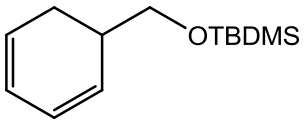
A solution of imidazole (2.43 g, 35.7 mmol) in anhydrous DMF (8 ml) was cooled to 0 ^oC. *tert*-Butyldimethylsilyl chloride (3.23 g, 21.4 mmol) was added and the mixture was stirred at 0 ^oC for 20 mins. 3-Cyclohexene-1-methanol (2.00 g, 17.8 mmol) was added at 0 ^oC then the mixture was stirred at room temperature for 1 hr. Water was added to quench the reaction, and then the mixture was extracted with copious Et_2O. The combined organic phases were washed with water and saturated NaCl solution then dried over Na_2SO_4. After filtration, the solvent was removed under reduced pressure to afford the crude title compound as a clear liquid (3.80 g, 94 %) without further purification. δ_H (400 MHz; $CDCl_3$) 0.05 (6H, s, Si(C*H*$_3$)$_2$), 0.91 (9H, s, C(C*H*$_3$)$_3$), 1.22 – 1.28 (1H, m, cyclohexenyl), 1.74 – 1.82 (3H, m, cyclohexenyl), 2.04 – 2.11 (3H, m, cyclohexenyl), 3.48 (1H, d, *J* 3.7, C*H*$_2$O), 3.49 (1H, d, *J* 3.6, C*H*$_2$O), 5.64 – 5.75 (2H, m, C*H*=C*H*); δ_C (100.6 MHz, $CDCl_3$) -5.2, 18.6, 25.6, 25.9, 26.2, 28.4, 36.5, 68.1, 126.4, 127.2.

S-*Trans*-1, 2- dibromo-3-(*tert*-butyldimethylsilyloxymethyl)-cyclohexane (3)



A solution of crude 4-(*tert*-butyldimethylsilyloxy) cyclohex-1-ene (**2**) (3.80 g, 16.8 mmol) in DCM (30 ml) was placed in a flask filled with nitrogen and cooled to 0 ^{o}C. To bromine (2.68 g, 16.8 mmol) was added enough DCM to bring the total volume of bromine solution to 2 ml. This solution was added dropwise to the solution of (**2**). At first the solution of (**2**) remained clear on addition of bromine, but later turned red-brown. The reaction mixture was stirred at room temperature for 1 hr. Further DCM (40 ml) was added to the reaction mixture. The mixture was washed with $Na_2S_2O_3$ (10 % m/v) and water. The organic phase was dried over Na_2SO_4. After filtration the solvent was removed under reduced pressure to afford the crude title compound as a brown liquid (5.22 g, 81 %) without further purification. δ_H (400 MHz; $CDCl_3$) 0.05 (6H, s, Si(C*H*$_3$)$_2$), 0.89 (9H, s, C(C*H*$_3$)$_3$), 1.93 – 1.99 (5H, m, cyclohexenyl), 2.40 – 2.50 (2H, m, cyclohexenyl), 3.48 (2H, d, *J* 5.8, C*H*$_2$O), 4.66 – 4.72 (2H, m, 2 x C*H*Br); δ_C (100.6 MHz; $CDCl_3$) -5.4, 18.3, 23.4, 25.8, 28.2, 31.5, 34.4, 53.4, 53.6, 67.4.

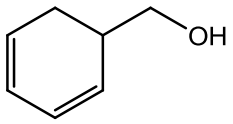
5-(*tert*-butyldimethylsilyloxymethyl) cyclohexa-1, 3-diene (4)



To a solution of crude *s-trans*-1,2-dibromo-3-(*tert*-butyldimethylsilyloxymethyl)-cyclohexane (**3**) (5.22g, 13.5 mmol) in anhydrous THF (40 ml) was added Aliquat 336® (109 mg, 0.27 mmol). The mixture was cooled to 0 ^{o}C and potassium *tert*-butoxide (3.34 g, 29.7 mmol) was added, all at once. Immediately a yellow-brown

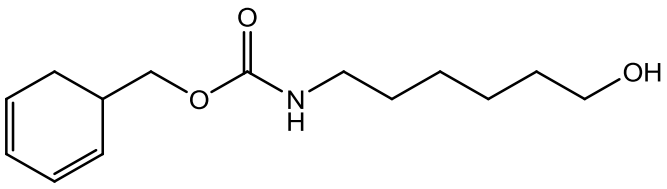
precipitate formed. The mixture was stirred at 0 ^{o}C for 5 mins, then at room temperature for 1 hr. The mixture was poured into a separating funnel, with traces of the mixture being washed from the reaction flask into the funnel with petroleum ether (30 ^{o}C - 40 ^{o}C). The combined organic layers were washed with saturated NH_4Cl solution and water before being dried over Na_2SO_4. After filtration, the solvent was removed under reduced pressure to afford the crude title compound as a brown liquid (3.25 g, 45 %) without further purification. δ_H (400 MHz; $CDCl_3$) 0.05 (6H, s, Si(CH_3)$_2$), 0.90 (9H, s, C(CH_3)$_3$), 2.10 – 2.30 (2H, m, CH_2CH), 2.37 – 2.48 (1H, m, CH_2CH), 3.54 (2H, d, *J* 5.8, CH_2O), 5.85 – 5.95 (4H, m, 2 x C*H*=C*H*); δ_C (100.6 MHz; $CDCl_3$) -5.1, 18.6, 26.2, 28.4, 36.2, 65.2, 124.1, 124.9, 125.9, 128.0.

(Cyclohexa-2, 4-dien-1-yl) methanol (**5**)



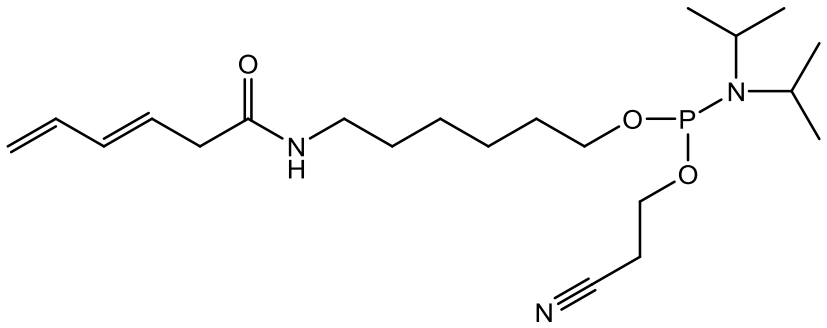
To a solution of crude 5-(*tert*-butyldimethylsilyloxymethyl) cyclohexa-1, 3-diene (**4**) (3.25 g, 14.3 mmol) in anhydrous MeOH (25 ml) was added a catalytic amount of acetyl chloride (153 μl, 2.15 mmol). The mixture was stirred at room temperature for 10 mins. DCM (40 ml) was added to quench the reaction then all solvents and acetyl chloride were removed under reduced pressure. Purification was by wet flash column chromatography, eluting with 10 % EtOAc in petroleum ether (30 ^{o}C - 40 ^{o}C) to afford the title compound as a yellow liquid (291 mg, 18 %). δ_H (400 MHz; d_6-acetone) 2.04 – 2.26 (2H, m, CH_2CH), 2.40 – 2.44 (1H, m, CH_2CH), 3.47 (2H, d, *J* 8.9, CH_2O), 5.71 – 5.92 (4H, m, 2 x C*H*=C*H*); δ_C (100.6 MHz; $CDCl_3$) 25.4, 35.9, 65.5, 124.0, 124.9, 125.7, 127.0.

(Cyclohexa-2, 4-dien-1-yl) methyl (6-hydroxyhexyl) carbamate (6)



To a solution of (cyclohexa-2,4-dien-1-yl) methanol (**6**) (274 mg, 2.5 mmol) in anhydrous MeCN (8 ml) was added CDI (484 mg, 3.0 mmol), under dark conditions. The mixture was stirred at room temperature for 1 hr. 6-Amino-1-hexanol (350 mg, 3.0 mmol) was added and the mixture was stirred at room temperature overnight. The solvent was removed under reduced pressure. The residue was dissolved in DCM and washed with water and saturated NaCl solution. The organic phase was dried over Na_2SO_4. After filtration, the solvent was removed under reduced pressure. Purification was by trituration of the crude product, dissolved in the minimum amount of DCM, with petroleum ether (60 ^{o}C – 80 ^{o}C) to afford the title compound as a pale pink powder (311 mg, 49 %). (Found: C, 64.9; H, 9.1; N, 5.5. $C_{14}H_{23}NO_3$ requires C, 66.4; H, 9.1; N, 5.6 %); δ_H (400 MHz; d_6-acetone) 1.31 – 1.55 (8H, m, 4 x C*H*$_2$), 2.21 – 2.25 (2H, m, C*H*$_2$CH), 2.56 – 2.57 (1H, m, C*H*$_2$CH), 3.10 (2H, t, *J* 6.4, C*H*$_2$NH), 3.52 (2H, dd, *J* 11.7, 6.4 C*H*$_2$OH), 3.91 (2H, d, *J* 7.9, C*H*$_2$O), 5.64 – 5.70 (2H, m, C*H*=C*H*), 5.87 (2H, m, C*H*=C*H*); δ_C (100.6 MHz, d_6-acetone) 25.6, 26.2, 27.2, 31.1, 33.5, 33.8, 41.3, 62.1, 66.0, 124.6, 125.8, 126.0, 127.5, 157.5; *m/z* 254.1752 ([M + H $^{+}$] $C_{14}H_{23}NO_3$ requires 254.1756.

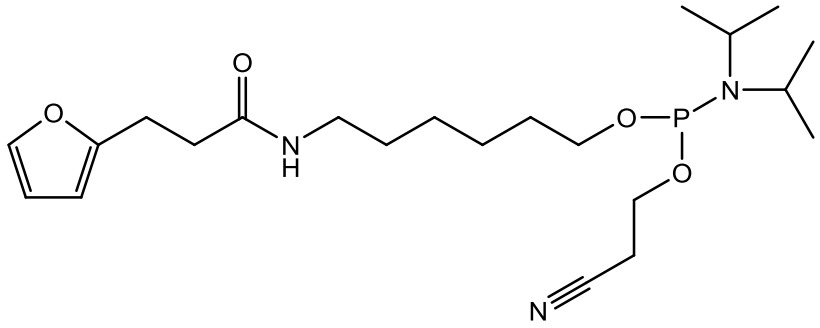
2-Cyanoethyl-6-[(3-E/Z)-3, 5-hexadienoylamino] hexyldiisopropyl - amidophosphite (7a)



(3- *E*/*Z*)-*N*- (6-Hydroxyhexyl) hexa-3, 5-dienamide (**1a**) (150mg, 0.7 mmol) was dried overnight over P_2O_5, under vacuum. To a suspension of (**1a**) in anhydrous MeCN (10 ml) was added (2-cyanoethoxy) bis (*N*, *N*- diisopropylamino) phosphine (321 mg, 1.1 mmol). 5- Benzylthio-1*H*-tetrazole (0.3 M in MeCN, 3.5 ml, 1.1 mmol) was added slowly *via* syringe. The mixture was stirred at room temperature

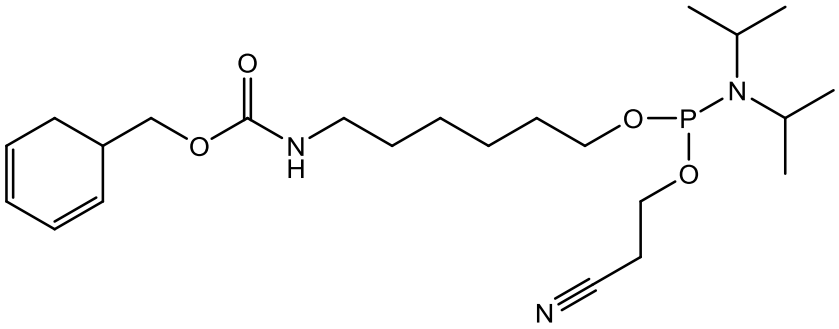
for 1 hr. The solvent was removed under reduced pressure, with the flask being opened to N_2 on removal from the rotary evaporator. Purification was by wet flash column chromatography, eluting with 100 % EtOAc, over silica pre-equilibrated with Et_3N. The product was co evaporated with anhydrous MeCN (x 3) to afford the title compound as a yellow oil (184 mg, 63 %). δ_P (162.0 MHz; d_8-THF) 145.5.

2-Cyanoethyl-6-{[3-(2-furyl)propanoyl]amino} hexyl diisopropyl-amidophosphite (7b)



3-Furan-2-yl-*N*-(6-hydroxyhexyl) propanamide (**1b**) (150 mg, 0.6 mmol) was dried overnight over P_2O_5, under vacuum. To a solution of (**1b**) in anhydrous MeCN (10 ml) was added (2-cyanoethoxy) bis (*N, N*-diisopropylamino) phosphine (189 mg, 0.6 mmol). 5- Benzylthio-1*H*-tetrazole (0.3 M in MeCN, 2.5 ml, 0.7 mmol) was added slowly *via* syringe. The mixture was stirred at room temperature for 2 hrs 30 mins. The solvent was removed under reduced pressure with the flask being opened up to N_2 on removal from the rotary evaporator. Purification was by wet flash column chromatography, eluting with 100 % EtOAc, over silica pre-equilibrated with Et_3N. The product was co evaporated with anhydrous MeCN (x 3) to afford the title compound as a yellow oil (204 mg, 74 %). δ_P (162.0 MHz; d_8-THF) 146.20.

(2, 4- Cyclohexadienyl-1-yl)methyl-6-{[(2-cyanoethoxy)(diisopropyl-amino) phosphino]oxy}hexyl carbamate (7c)



(Cyclohexa-2,4-dien-1-yl)methyl(6-hydroxyhexyl)carbamate (**6**) (150 mg, 0.6 mmol) was dried overnight over P_2O_5, under vacuum. To a solution of (**6**) in anhydrous MeCN (10 ml) was added (2-cyanoethoxy) bis (*N*, *N*- diisopropylamino) phosphine (276 mg, 0.9 mmol). 5- Benzylthio-1*H*-tetrazole (0.3 M in MeCN, 2.9 ml, 0.9 mmol) was added slowly *via* syringe. The mixture was stirred at room temperature for 1 hr. The solvent was removed under reduced pressure with the flask being opened up to N_2 on removal from the rotary evaporator. Purification was by wet flash column chromatography, eluting with EtOAc over silica pre-equilibrated with Et_3N. The product was co evaporated with anhydrous MeCN (x 3) to afford the title compound as a yellow oil (172 mg, 64 %). δ_P (162.0 MHz; d_8-THF) 147.19.

3. 4. 3 Oligonucleotide Synthesis

3.4.3.1 Solid phase synthesis of unmodified oligonucleotide

The following sequences were synthesised on an automated DNA synthesiser:

Oligonucleotide sequence 1: 5'- CGC ATT CAG GAT -3'

Oligonucleotide sequence 3: 5'- ATC CTG AAT GCG -3'

Synthesis was performed using a column containing thymine (T) modified CPG solid support (1 µmol). 0.1 M solutions, in anhydrous MeCN, of phosphoramidites of each of the bases A, C, G and T were used. The synthesised oligonucleotide was cleaved from the solid support by treatment with conc. NH_4OH solution (1 ml) at room temperature for 1 hr. Deprotection of the oligonucleotide was achieved by incubation in conc. NH_4OH solution (1 ml) at 40 ^oC overnight. NH_4OH was removed under reduced pressure and the remaining oligonucleotide was re-dissolved in distilled H_2O (1 ml).

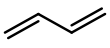
Purification was by ion-exchange HPLC, using HPLC method (1). Buffer salts were removed by size exclusion chromatography (HPLC desalt method). The pure oligonucleotide retrieved was re-dissolved in distilled H_2O (1 ml). Synthesis of the product was confirmed by MALDI-TOF mass spectrometry, using a matrix of 50 mg ml^{-1} 3-hydroxypicolinic acid in MeCN / H_2O (1:1 v/v), 50 mg ml^{-1} picolinic acid in MeCN / H_2O (1:1 v/v) and 50 mg ml^{-1} dihydrogen ammonium citrate in MeCN / H_2O (1:1 v/v), mixed 50:1:1 respectively. ZipTip™ purification was performed prior to MALDI-TOF analysis. MALDI-TOF: CGC ATT CAG GAT requires 3647.3; found 3647.6.

3.4.3.2 Solid phase synthesis of 5'-dienyl modified oligonucleotides

The following sequence was synthesised on an automated DNA synthesiser:

Oligonucleotide sequence 2: 5'- X CGC ATT CAG GAT -3'

Where X is the pre-synthesised dienyl modified phosphoramidite (**7a**), (**7b**) or (**7c**), made up to a concentration of 0.1 M, in anhydrous MeCN. Oligonucleotide synthesis, purification and MALDI-TOF analysis were as described for oligonucleotide sequence (**1**).

X =  MALDI-TOF: $C_{12}H_{22}NO_2$ CGC ATT CAG GAT requires 3918.6; found 3917.9.

X = MALDI-TOF: $C_{13}H_{20}NO_2$ CGC ATT CAG GAT requires 3946.6; found 3946.7.

X = MALDI-TOF: $C_{14}H_{22}NO_2$ CGC ATT CAG GAT requires 3960.6; found 3959.8.

3.4.3.3 Solid phase synthesis of labelled 5'-cyclohexadienyl modified oligonucleotides

The following sequences were synthesised on an automated DNA synthesiser:

Oligonucleotide sequence 4: 5'- CGC ATT CAG GAT - FAM -3'

Oligonucleotide sequence 5: 5'- CGC ATT CAG GAT - TAMRA -5'

Where X is the pre-synthesised dienyl modified phosphoramidite (**7c**), made up to a concentration of 0.1 M, in anhydrous MeCN. Synthesis, cleavage and deprotection of 3'-FAM labelled oligonucleotide sequence (**4**) was performed as described for oligonucleotide sequence **(2)**, but using a column containing (6-FAM) modified CPG solid support. Synthesis of 3'-TAMRA labelled oligonucleotide sequence **(5)** was performed as described for oligonucleotide sequence **(2)**, but using a column containing TAMRA modified CPG solid support and DNA UltraMILD synthesis reagents. Cleavage from the solid support and deprotection of the TAMRA labelled oligonucleotide was achieved by treatment with 0.05 M K_2CO_3/MeOH (1 ml) at room temperature for 4 hrs. The carbonate solution was neutralised by the addition of acetic acid (3 μl), then concentrated under reduced pressure. The remaining oligonucleotide was re-dissolved in distilled H_2O (1ml). Salts were removed by size exclusion chromatography (HPLC desalt method). All labelled sequences were used unpurified in Diels-Alder cycloadditions.

Purification for MALDI-TOF analysis and MALDI-TOF analysis conditions were as described for sequence **(2)**, using HPLC method (2) for FAM-labelled sequences (**4**) and HPLC method 3 for TAMRA-labelled sequence (**5**).

Oligonucleotide sequence 4: MALDI-TOF: $C_{41}H_{46}N_2O_{11}$ CGC ATT CAG GAT requires 4529.5; found 4526.0

Oligonucleotide sequence 5: MALDI-TOF: $C_{47}H_{60}N_4O_8$ CGC ATT CAG GAT requires 4548.6; found 4536.2

3.4.3.4 Concentration determination of oligonucleotide stock solutions

Concentrations of oligonucleotide stock solutions were determined by UV spectroscopy. Samples were generally diluted by a factor of 50. Absorption was measured at a wavelength of 260 nm (λ_{max} for DNA bases). Concentration of the sample was calculated using the Beer-Lambert Law:

$A = a\ b\ c$ where A = absorbance
a = absorption coefficient
b = path length (cm)
c = concentration (mol l^{-1})

The absorption coefficient is the cumulative sum of the absorption coefficients for each of the bases in the oligonucleotide sequence. For labelled oligonucleotides the absorption coefficient of the dye used also has to be taken into account. The values used are shown below:

dA:	15, 200	dC:	7, 050
dG:	12, 010	dT:	8, 400
FAM:	21, 000	TAMRA:	32, 300

The absorption coefficient is calculated using the following equation:

$$a = \{ (dA \times n) + (dG \times n) + (dC \times n) + (dT \times n) + (dye) \} \times 0.9$$

Where 0.9 is the hypochromicity factor. Concentrations of oligonucleotide solutions were generally found to be in the region of 1 x 10^{-4} mol l^{-1}.

3.4.3.5 Melting temperature (T_m) determination of 5'- dienyl modified oligonucleotides

To samples of sequence (2) (100 μl, 10 μM) was added complementary oligonucleotide sequence (**3**) (100 μl, 10 μM), NaCl (150 μl, 2M), phosphate buffer (250 μl, 60 mM, pH 7.6) and Milli-Q water (900 μl). The mixture was incubated at room temperature overnight to allow hybridisation. The samples were then heated from 10 - 90 ^{o}C, at 1 ^{o}C min^{-1} over 4 cycles, monitoring the absorbance at 260 nm.

3. 4. 4 Diels-Alder Cycloadditions

3.4.4.1 Optimisation of cycloaddition reaction conditions

A stock solution of unpurified 5'-furanyl modified oligonucleotide sequence **(2)** was made up to a concentration of 1 mM in distilled water. A stock solution of fluorescein maleimide was made up to a concentration of 40 mM in DMF (5 mg in 293 μl).

Cycloaddition Method 1

To 20 μl oligonucleotide stock solution, in an Eppendorf tube, was added 50 μl NaOAc buffer (0.1 M, pH 4.8), 25 μl distilled water and 5 μl fluorescein maleimide stock solution. The reaction was heated at 40 ^{o}C overnight. All organics and excess fluorescein maleimide were removed by size exclusion chromatography (HPLC desalt method). Analysis of the reaction mixture was by ion-exchange chromatography, using HPLC method (2).

Cycloaddition Method 2

To 20 μl oligonucleotide stock solution, in an Eppendorf tube, was added 50 μl NaOAc buffer (0.1 M, pH 4.8), 25 μl $Cu(NO_3)_2$ solution (0.01 M) and 5 μl

fluorescein maleimide stock solution. The reaction was heated at 60 ^{o}C for 4 hours. All organics and excess fluorescein maleimide were removed by size exclusion chromatography (HPLC desalt method). Analysis of the reaction mixture was by ion-exchange chromatography, using HPLC method (2).

Cycloaddition Method (3)

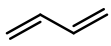
To 20 μl oligonucleotide stock solution, in an Eppendorf tube, was added 50 μl NaOAc buffer (0.1 M, pH 4.8), 25 μl $Cu(NO_3)_2$ solution (0.01 M) and 5 μl fluorescein maleimide stock solution. The reaction was heated at 40 ^{o}C overnight. All organics and excess fluorescein maleimide were removed by size exclusion chromatography (HPLC desalt method). Analysis of the reaction mixture was by ion-exchange chromatography, using HPLC method 2.

3.4.4.2 Oligonucleotide conjugation to Tat peptide

Stock solutions of unpurified 5'- 1, 3-butadienyl, 5'-furanyl and 5'-cyclohexadienyl modified oligonucleotide sequence (**2**) were made up to a concentration of 1 mM in distilled water. A stock solution of maleimido modified Tat peptide derivative was made up to a concentration of 40 mM in DMF (5 mg in 71 μl).

General Cycloaddition Method

To 20 μl oligonucleotide stock solution in an Eppendorf tube was added 50 μl NaOAc buffer (0.1 M, pH 4.8), 25 μl $Cu(NO_3)_2$ solution (0.01 M), 60 μl formamide and 5 μl Tat peptide stock solution. The reaction was heated at 40 ^{o}C overnight. Analysis and purification of the reaction mixture was by ion-exchange chromatography, using HPLC method (4). Buffer salts and formamide were removed by dialysis. Synthesis of the conjugate was confirmed by MALDI-TOF mass spectrometry, using a matrix of 40 mg ml^{-1} dihydroxyacetophenone in MeOH and 80 mg ml^{-1} dihydrogen ammonium citrate in H_2O, mixed in a 1:1 ratio. ZipTip™ purification was performed prior to MALDI-TOF analysis.

X =  MALDI-TOF: Tat $C_{12}H_{22}NO_2$ CGC ATT CAG GAT requires 5676.9; found 5676.0.

X =  MALDI-TOF: Tat $C_{13}H_{20}NO_2$ CGC ATT CAG GAT requires 5704.9; found 5706.5.

X =  MALDI-TOF: Tat $C_{14}H_{22}NO_2$ CGC ATT CAG GAT requires 5718.9; found 5719.9

3.4.4.3 Conjugation of labelled oligonucleotides to Tat peptide

Stock solutions of unpurified, labelled 5'-cyclohexadienyl-modified oligonucleotide sequences **(4)** and **(5)** were made up to a concentration of 1 mM in distilled water. A stock solution of the maleimido modified Tat peptide derivative was made up to a concentration of 40 mM in DMF (5 mg in 71 μl).

General cycloaddition method

To 20 μl oligonucleotide stock solution in an Eppendorf tube was added 50 μl NaOAc buffer (0.1 M, pH 4.8), 25 μl $Cu(NO_3)_2$ solution (0.01 M), 60 μl formamide and 5 μl Tat peptide stock solution. The reaction was heated at 40 ^oC overnight. Analysis of the reaction mixture was by ion-exchange HPLC, using HPLC method (5) for the reaction with FAM labelled sequence **(4)** and HPLC method (6) for reactions involving TAMRA labelled sequence **(5)**.

2. 4. 5 HPLC Methods

Method 1

Ion-exchange chromatography was performed using a Dionex DNA Pac (PA200) column [1 ml min^{-1}, Buffer A: 0.25 M Tris-HCl (pH 8), Buffer B: 0.375 M $NaClO_4$]; gradient 10 % Buffer A; 5 – 85 % Buffer B over 20 min; UV monitoring at 260 and 280 nm.

Method (2)

Ion-exchange chromatography was performed using a Dionex DNA Pac (PA200) column [1 ml min^{-1}, Buffer A: 0.25 M Tris-HCl (pH 8), Buffer B: 0.375 M $NaClO_4$]; gradient 10 % Buffer A; 5 – 85 % Buffer B over 20 min; UV monitoring at 260, 280 and 492 nm.

Method (3)

Ion-exchange chromatography was performed using a Dionex DNA Pac (PA200) column [1 ml min^{-1}, Buffer A: 0.25 M Tris-HCl (pH 8), Buffer B: 0.375 M $NaClO_4$]; gradient 10 % Buffer A; 5 – 85 % Buffer B over 20 min; UV monitoring at 260, 280 and 565 nm.

Method (4)

Ion-exchange chromatography was performed using a 1 ml Resource Q column [1 ml min^{-1}, Buffer A: 20 mM Tris-HCl (pH 6.8); formamide (1:1 v/v), Buffer B: 20 mM Tris-HCl (pH 6.8), 400 mM $NaClO_4$; formamide (1:1 v/v)]; gradient 10 % Buffer A; 0 – 100 % Buffer B over 22 min; UV monitoring at 260 and 280 nm.

Method (5)

Ion-exchange chromatography was performed using a 1 ml Resource Q column [1 ml min^{-1}, Buffer A: 20 mM Tris-HCl (pH 6.8); formamide (1:1 v/v), Buffer B: 20 mM Tris-HCl (pH 6.8), 400 mM $NaClO_4$; formamide (1:1 v/v)]; gradient 10 % Buffer A; 0 – 100 % Buffer B over 22 min; UV monitoring at 260, 280 and 492 nm.

Method (6)

Ion-exchange chromatography was performed using a 1 ml Resource Q column [1 ml min^{-1}, Buffer A: 20 mM Tris-HCl (pH 6.8); formamide (1:1 v/v), Buffer B: 20 mM Tris-HCl (pH 6.8), 400 mM $NaClO_4$; formamide (1:1 v/v)]; gradient 10 % Buffer A; 0 – 100 % Buffer B over 22 min; UV monitoring at 260, 280 and 565 nm.

Desalt Method

Size exclusion chromatography was performed using a 5 ml HiTrap size exclusion column [3 ml min^{-1}, Buffer A: H_2O]; gradient 100 % Buffer A over 6 min; UV monitoring at 260 and 280 nm.

3.5 References

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