4

Conjugation of mid-sequence dienyl modified oligonucleotides to Tat peptide *via* Diels-Alder cycloaddition

4.1 Introduction

Mid-sequence modification of oligonucleotides is achieved by nucleoside modification with the desired functionality; the modified nucleoside can then be incorporated into oligonucleotide synthesis by standard phosphoramidite chemistry. Nucleoside modification can be performed on either the heterocycle or the sugar. For the purposes of this work, this report will focus on modification of the heterocycle.

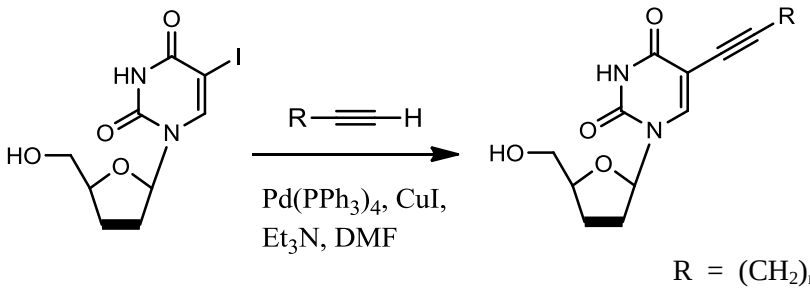
A common approach to nucleoside modification involves palladium catalysed addition or substitution reactions. The Heck reaction [(1)] (coupling of an aryl iodide or an arylmercuryhalide to an activated alkene in the presence of an organopalladium catalyst) was first reported by Bergstrom *et. al* for palladium catalysed coupling of nucleosides with allylic halides, [(2)] allylic alcohols [(3)] and allylic acetates. [(4)] Since then, various other palladium catalysed coupling reactions have been reported for nucleoside modification, including the Sonogashira reaction. [(5)]

4.1.1 Sonogashira reaction for nucleoside modification

The Sonogashira reaction is the palladium catalysed reaction of terminal alkynes, in the presence of a CuI co-catalyst, with organic halides to produce disubstituted alkynes *via* the formation of a new carbon-carbon bond. The reaction has been reported often in the literature for nucleoside modification by palladium-catalysed cross coupling to pyrimidine nucleosides at the C-4, C-5 and C-6 positions.[see (6) for review]

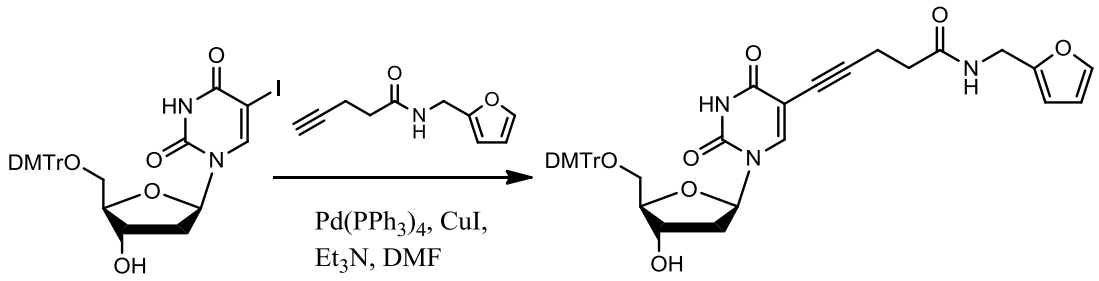
This work will focus on modification of pyrimidines at the C-5 position. Nucleoside analogues substituted at the 5- position of the pyrimidine base, in particular on 2'-deoxyuridines, have been synthesised for their biological activity as anti-cancer [(6)] and anti-viral agents. [(7)] In terms of bioanalysis, the Sonogashira reaction has been reported for functionalisation of chain-terminating 2', 3'-dideoxyuridines at the 5- position with 3-(acylamino)propynes for the attachment of fluorescent labels, for use in DNA sequencing (**Scheme 4.1**). [(8)]

2'-deoxyuridines have also been functionalised with a spaced furan at the 5-position by Sonogashira methodology, for attachment of maleimido modified fluorescent labels by the Diels-Alder reaction for bioanalytical applications (**Scheme 4.2**). [(9)]



R = $(CH_2)_nNHCOCF_3$

Scheme 4.1 – *Nucleoside functionalisation with 3-(acylamino)propynes by the Sonogashira reaction, for coupling to fluorescent labels, as reported by Prober et. al* [(8)]



Scheme 4.2 – *Nucleoside functionalisation with furan by the Sonogashira reaction, for coupling to fluorescent labels by the Diels-Alder reaction, as reported by Graham et. al* [9]

For mid-sequence dienyl modification of oligonucleotides for the purposes of this research, a protected nucleoside phosphoramidite must be synthesised for incorporation into an oligonucleotide sequence by standard phosphoramidite chemistry. This will be achieved, as aforementioned, by dienyl functionalisation of a 2'-deoxyuridine nucleotide (**Fig. 4.1**).

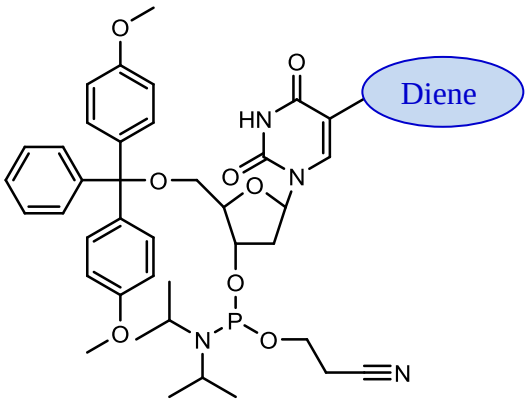


Figure 4.1 – *Modified nucleoside phosphoramidite for mid-sequence dienyl modification of oligonucleotides*

4.2 Results and discussion

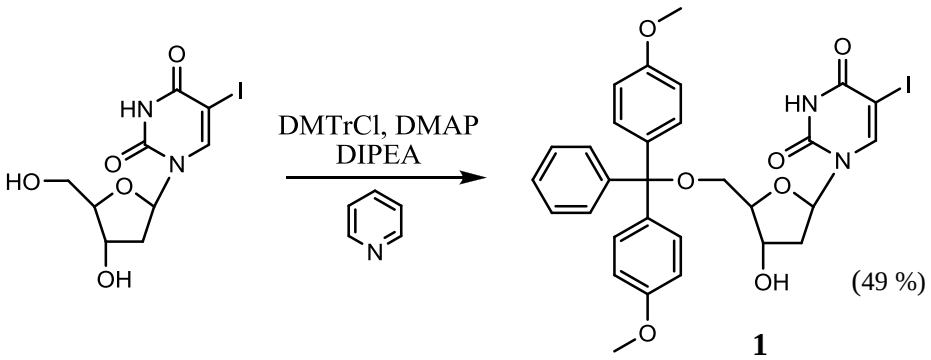
3.2.1 Synthesis of mid-sequence dienyl modified oligonucleotides

3.2.1.1 Synthesis of mid-sequence furanyl modified oligonucleotides

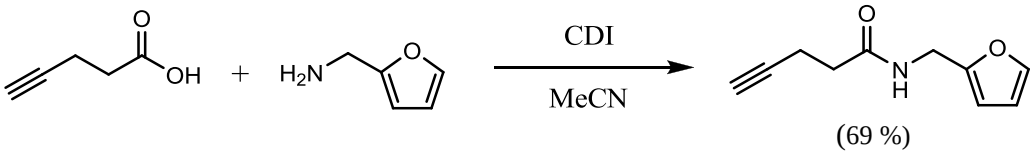
Mid-sequence dienyl modified oligonucleotides were synthesised using Sonogashira methodology. [5] An established procedure was developed through optimisation of synthesis of mid-sequence furanyl modified oligonucleotides, since starting materials for this chemistry were to hand.

In the primary approach to synthesis of mid-sequence furanyl modified oligonucleotide, the first step taken was protection of the 5'- hydroxyl functionality of 5-iodo-2'-deoxyuridine (**Scheme 4.3**).

Protection of the uridine was achieved by reaction with dimethoxytrityl chloride (DMTrCl); this was purified first by recrystallisation from 1 % acetyl chloride in hexane. The reaction was first performed using a catalytic amount of DMAP only, but low yields were obtained. Yield of the tritylated product (**1**) was improved by addition of DIPEA to the reaction mixture, as recommended by Gait. [10]



Scheme 4.3 – *5'- OH protection of 5-iodo-2'-deoxyuridine by tritylation*



(69 %)

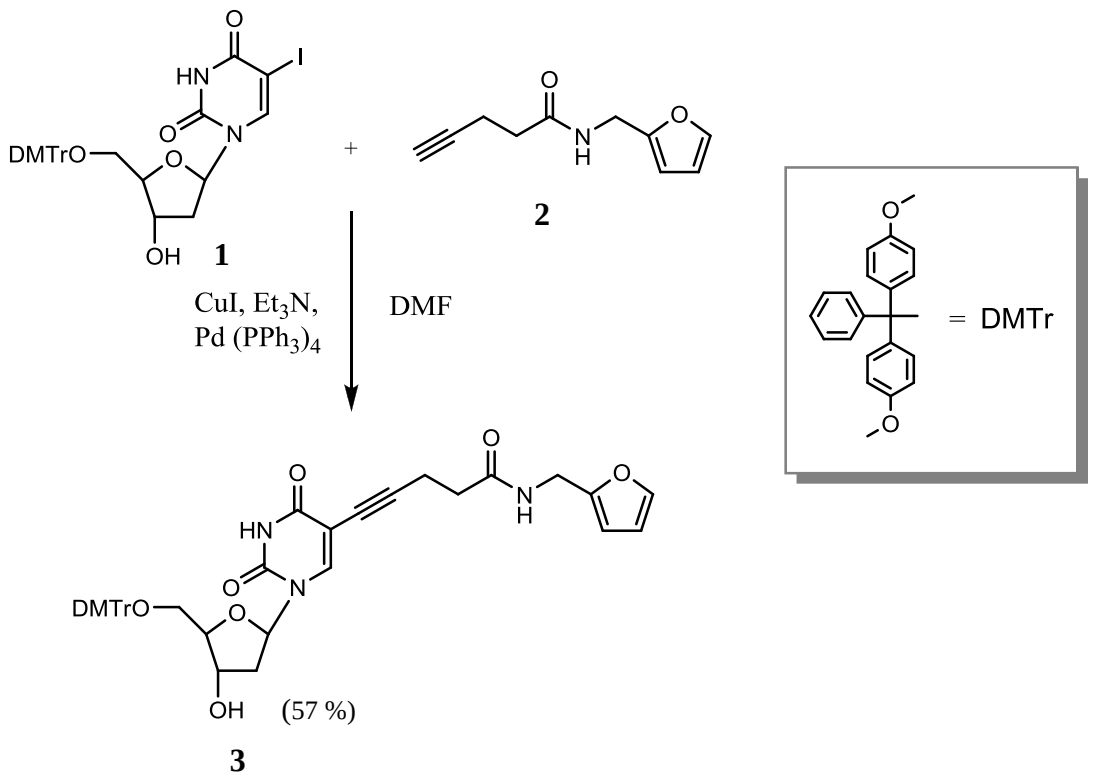
2

Scheme 4.4 – *Synthesis of a furanyl derived acetylene moiety for use in Sonogashira methodology*

For furanyl modification of tritylated uridine (**1**), it was now necessary to synthesise a furanyl derived acetylene to be used in a Sonogashira reaction. This was made possible by reaction of commercially available 4-pentynoic acid and furfuryl amine, using coupling agent CDI, for amide formation to afford the desired acetylene (**2**) in good yield (**Scheme 4.4**).

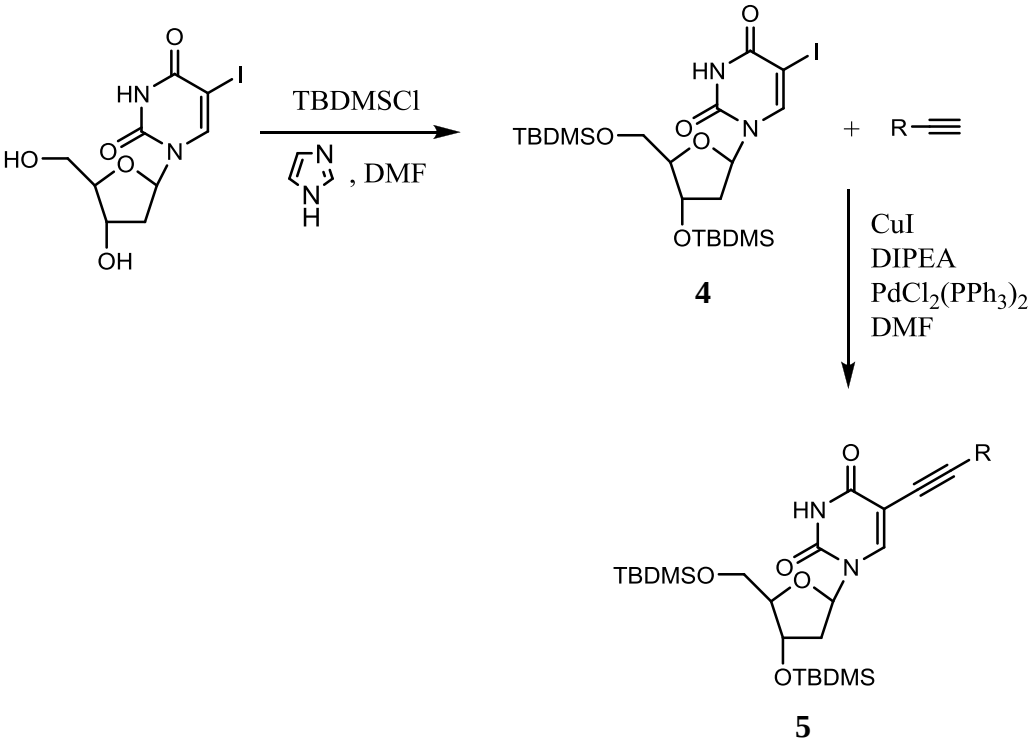
With a furanyl derived acetylene in hand it was now possible to functionalise tritylated uridine (**1**) with the diene, using palladium chemistry. A Sonogashira reaction was performed for linkage of the acetylene and the protected nucleoside *via* a carbon-carbon bond (**Scheme 4.5**).

The reaction was performed using methodology reported by Graham, [(11)] wherein the importance of the order of addition of reagents was noted. To tritylated uridine (**1**) was added copper iodide, triethylamine, acetylene (**2**) and tetrakis (triphenylphosphine) palladium (0), in that order. On first attempt at this reaction, the desired product (**3**) was obtained successfully, in relatively good yield. However, no synthesis thereafter produced the desired product. The reaction was repeated several times, varying the reaction stoichiometry, the base used and the oxidation state of the palladium catalyst. The reaction was also attempted under microwave conditions but the desired product was not obtained.



Scheme 4.5 – *Synthesis of a furanyl modified nucleoside by Sonogashira methodology*

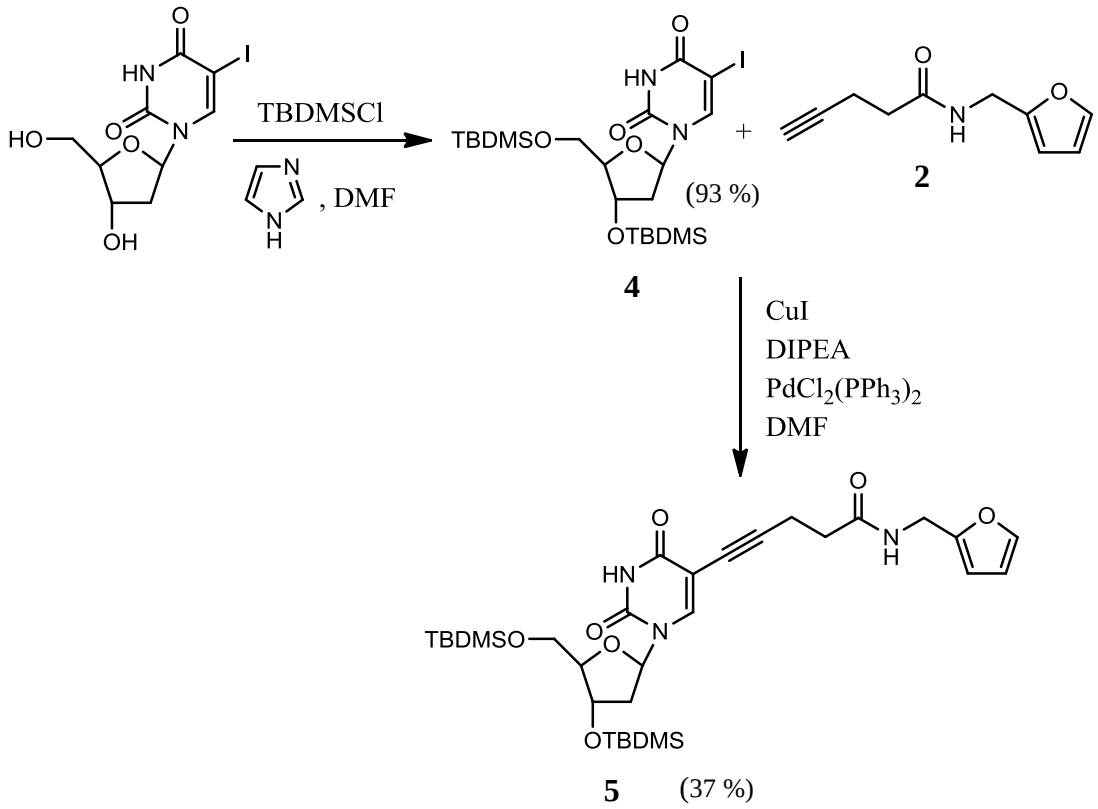
Further research into the literature uncovered a report by Gierlich *et. al* [12] in which the Sonogashira reaction was successfully used for alkynyl modification of 5-iodo-2-deoxyuridine in an approach to labelling DNA *via* a 'click chemistry', Cu(I)-mediated, Huisgen cycloaddition reaction. The reported methodology described the first step as protection of both the 3'- and 5'- hydroxyl functionalities of 5-iodo-2'-deoxyuridine by silylation, before performing a Sonogashira reaction using a Pd (II) catalyst for alkynyl functionalisation of the protected nucleoside (**Scheme 4.6**).



Scheme 4.6 – *Nucleoside modification by Sonogashira methodology using a TBDMS protected uridine, as reported by Gierlich et. al* [12]

This methodology was applied for furanyl modification of 5-iodo-2'-deoxyuridine. TBDMSCl was first reacted with imidazole in DMF at 0 ^{o}C, before addition of 5-iodo-2'-deoxyuridine to afford the disilyl-protected uridine (**4**) (**Scheme 4.7**). The protected uridine was then reacted with furanyl acetylene (**2**) using CuI, thoroughly degassed DIPEA and $PdCl_2(PPh_3)_2$ in DMF, to afford modified uridine (**5**) in moderate yield (37 %). Importantly however, this result was reproducible – with all repeat syntheses producing the desired product.

The observation that the Sonogashira reaction proceeded successfully, and reproducibly, using the silylation method developed by Gierlich *et al.* could indicate that the issues with the original methodology attempted may have lain with coordination of the Pd (0) catalyst to the free 3'- hydroxyl functionality of tritylated uridine (**1**).



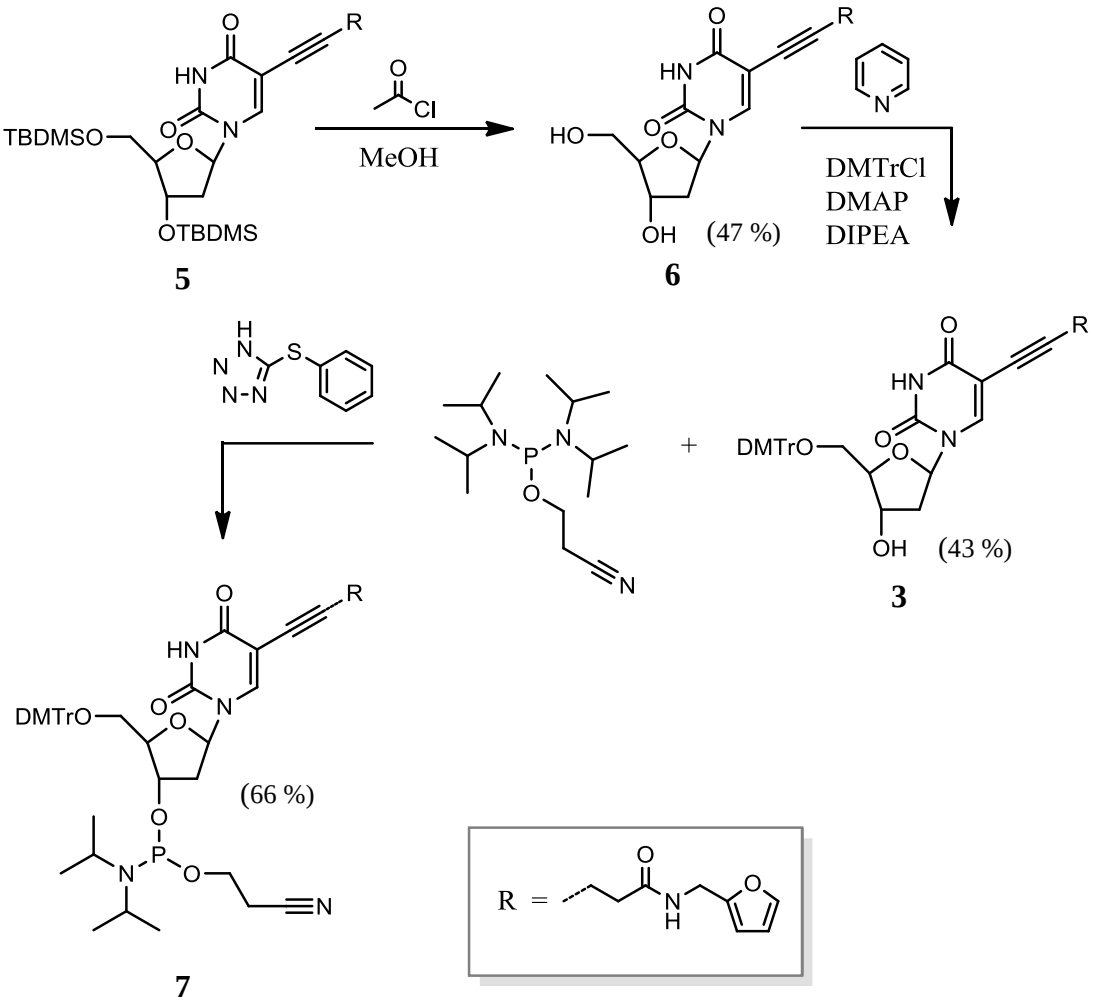
Scheme 4.7 – *Furanyl nucleoside modification, using methodology as reported by Gierlich et. al* [12]

In order to carry Sonogashira product (**5**) through to a phosphoramidite to be used in DNA synthesis the silyl protecting groups were cleaved, before tritylation to protect the 5'- hydroxyl functionality of the sugar (**Scheme 4.8**).

Desilylation was attempted, as reported by Gierlich *et al.*, by treatment with TBAF in THF. However, even after purification by column chromatography, followed by washing over Dowex resin, the product was found by ^{1}H NMR to still contain a considerable amount of TBAF. Thus, based on the success of Khan and Mondal's methodology [13] described previously, the decision was taken to attempt desilylation by reaction with acetyl chloride in anhydrous methanol. The reaction had to be stirred overnight for complete conversion to (**6**); this was not unexpected since there were two bulky silyl ethers present undergoing cleavage.

Using this procedure, pure product (**6**) was obtained, in relatively good yield (47 %). Tritylation to afford protected nucleoside (**7**) was performed using the same conditions as described in **Scheme 4.3**. Finally, for synthesis of phosphoramidite

(**8**), the trityl-protected nucleoside was treated with tetraisopropyl phosphitylating reagent and 5- benzylthio-1*H*-tetrazole activator, as described previously. Formation of active phosphoramidite (**8**) was confirmed by ^{31} P NMR, by the presence of a peak showing P $^{(III)}$.



5 **6** **3** **7**

Scheme 4.8 – *Desilylation followed by tritylation and phosphitylation to produce a furanyl modified nucleoside phosphoramidite*

Oligonucleotide sequence (**1**) was written for automated DNA synthesis, replacing a mid-sequence thymine (T) with the furanyl modified phosphoramidite (U*):

Oligonucleotide sequence 1: 5'- CGC ATU* CAG GAT -3'

The phosphoramidite was made up to the requisite concentration in anhydrous acetonitrile and used in an interim cycle of standard oligonucleotide synthesis. Cleavage and deprotection of the mid-sequence furanyl modified oligonucleotide was achieved by treatment with conc. aqueous ammonia. As for 5'- dienyl modified oligonucleotides (see 3.2.2 – *Synthesis of 5'-dienyl modified oligonucleotides*), analysis of the modified oligonucleotides was by anion-exchange HPLC (**Fig. 4.2**), using a DNAPac PA200 column. A slight increase in retention time of the furanyl modified oligonucleotide (blue trace) compared to the unmodified oligonucleotide (pink trace) was observed. This was unexpected, since the mid-sequence dienyl modification results in no formal increase in anionic charge on the oligonucleotide sequence. The increased retention time was attributed to the slightly hydrophobic nature of the column's stationary phase and, as such, its increased interaction with the mid-sequence furanyl modified sequence, compared with the unmodified sequence.

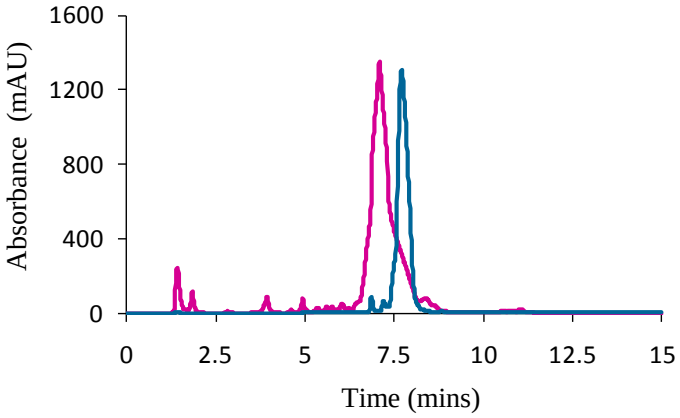


Figure 4.2 – *Ion-exchange HPLC traces at 260 nm of unmodified (pink) and mid-sequence furanyl modified (blue) oligonucleotide sequence (**1**), 5'- CGC ATU* CAG GAT, where U* is furanyl modified uridine*

The modified sequence was purified from its failure sequences by ion-exchange HPLC; HPLC buffer salts were removed by desalting. Synthesis of mid-sequence furanyl modified oligonucleotide sequence (**1**) was confirmed by MALDI-TOF mass spectrometry analysis (**Table 4.1**).

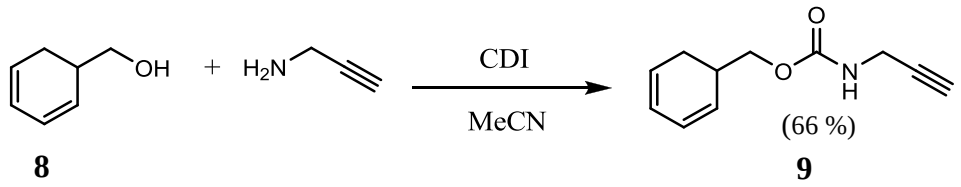
Calc'd M	MALDI- TOF MS Found $[M + H]^+$
3795.4	3796.2

Table 4.1 – *Mass spectroscopic characterisation data of mid-sequence furanyl modified oligonucleotide sequence (**1**), 5' - CGC ATU* CGA GAT*

3.2.1.2 Synthesis of mid-sequence cyclohexadienyl modified oligonucleotides

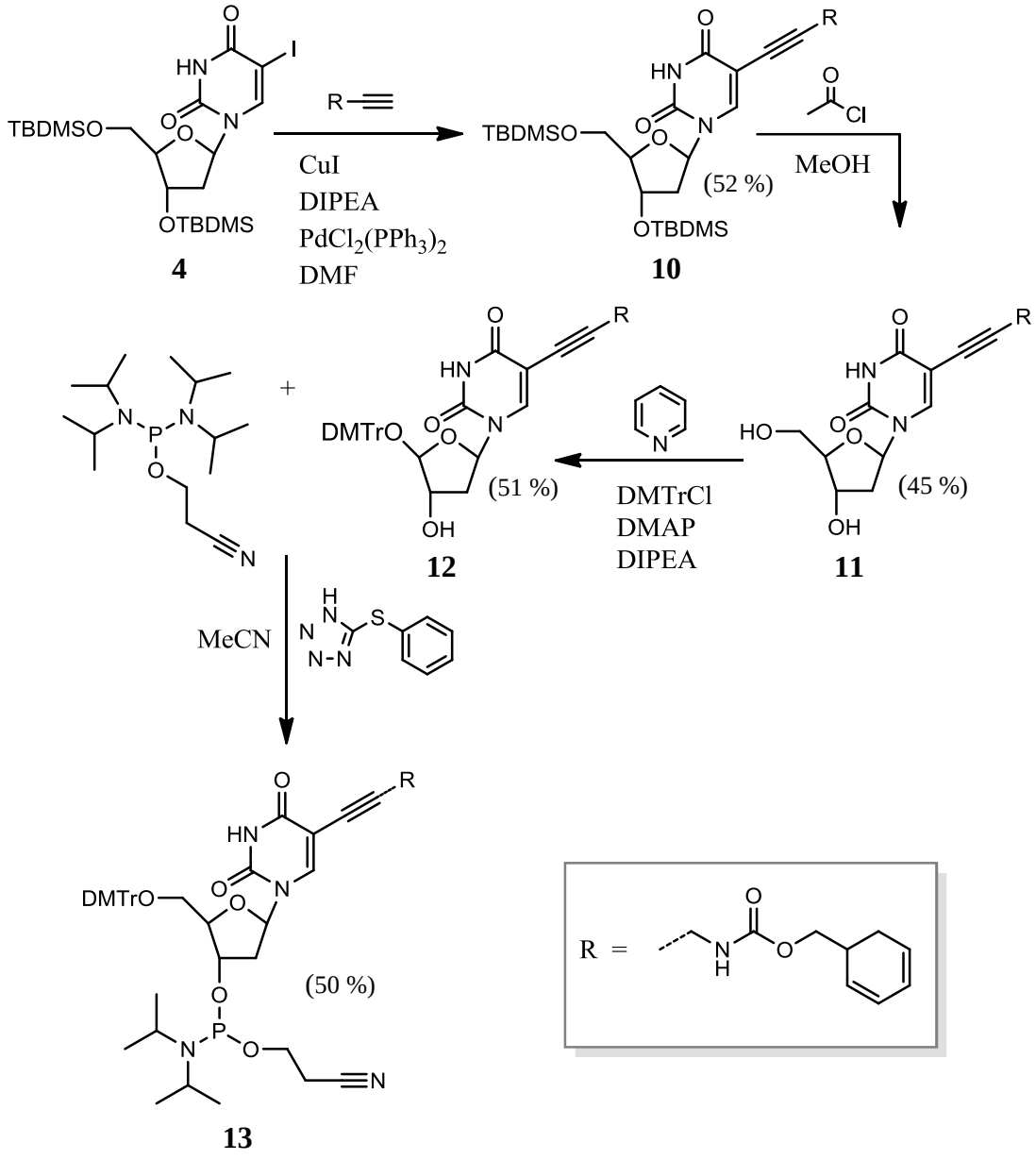
Having established a successful methodology for mid-sequence dienyl modification of oligonucleotides, and following on from the observation that cyclohexadiene was the most effective diene for Tat peptide conjugation to 5'- dienyl modified oligonucleotides *via* Diels-Alder cycloaddition, it was decided to apply the methodology to synthesis of mid-sequence cyclohexadienyl oligonucleotides. Of interest was the possibility of improved yields in maleimido modified Tat peptide conjugation to a labelled oligonucleotide containing the cyclohexadienyl modification mid-sequence, compared to at the 5'- terminus.

Before performing a Sonogashira reaction for uridine functionalisation with cyclohexadiene, cyclohexadienyl alcohol (**8**), synthesised as described previously for 5'- cyclohexadienyl modification (see 3.2.2 – *Synthesis of 5'- dienyl modified oligonucleotides*), was modified to incorporate an acetylene functionality. This was achieved by activation of alcohol (**8**) with CDI, followed by reaction with propargyl amine to afford acetylene (**9**) in good yield (66 %, **Scheme 4.9**).



8 **9**

Scheme 4.9 – *Synthesis of a cyclohexadienyl derived acetylene moiety for use in Sonogashira methodology*



4 **10** **12** **11** **13**

Scheme 4.10 – *Synthesis of a cyclohexadienyl phosphoramidite by Sonogashira methodology*

After protection of the hydroxyl groups of 5- iodo- 2'- deoxyuridine by disilylation (Scheme **4.6**, previously), it was possible to synthesise a cyclohexadienyl modified uridine phosphoramidite, as per the method used for synthesis of furanyl modified uridine (Scheme **4.10**). Using this synthetic method the phosphoramidite (**13**) was obtained in relatively good yield (50 %), taking into consideration the multi-step reaction procedure undertaken. Formation of the active phosphoramidite was confirmed by ^{31}P NMR.

For oligonucleotide synthesis, sequence (**1**) was used once again, replacing a mid-sequence thymine (T) with the cyclohexadienyl modified phosphoramidite (U*):

Oligonucleotide sequence 1: 5'- CGC ATU* CAG GAT -3'

Cleavage and deprotection of the modified oligonucleotides was achieved by treatment with conc. aqueous ammonia. Analysis of the modified oligonucleotides was performed, as before, by anion-exchange HPLC (**Fig. 4.3**).

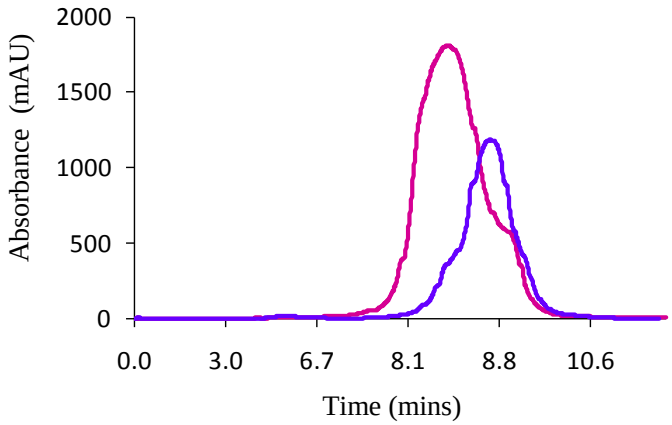


Figure 4.3 – *Ion-exchange HPLC traces at 260 nm of unmodified (pink) and mid-sequence cyclohexadienyl modified (blue) oligonucleotide sequence (**1**), 5'- CGC ATU* CAG GAT, where U* cyclohexadienyl modified uridine*

As for mid-sequence furanyl modified oligonucleotide, a slight increase in retention time of the mid-sequence cyclohexadienyl modified product was observed which was attributed to use of the DNAPac PA200 column for HPLC analysis. Considerable broadening of the peaks produced in the HPLC trace was noted. This was thought to be due to degradation of the column brought about as a result of its prolonged use.

The modified sequence was purified from its failure sequences by anion-exchange HPLC; HPLC buffer salts were removed by desalting. Synthesis of mid-sequence cyclohexadienyl modified oligonucleotide sequence (**1**) was confirmed by MALDI-TOF mass spectrometry analysis (**Table 4.2**).

As was the case for 5'- cyclohexadienyl modified oligonucleotides, in order to assess cellular uptake of Tat peptide conjugates of mid-sequence cyclohexadienyl modified oligonucleotides, it was necessary to incorporate a fluorescent label into the sequence. This was achieved by labelling with FAM at the 3'- terminus to afford oligonucleotide sequence (**2**):

Oligonucleotide sequence 2: 5'- CGC ATU* CAG GAT - FAM -3'

Where U* is the cyclohexadienyl modified uridine. Synthesis, analysis and purification of labelled oligonucleotide sequence (**2**) was performed as described previously for unlabelled sequence (**1**). Synthesis of the mid-sequence cyclohexadienyl modified sequence was confirmed by MALDI-TOF mass spectrometry (**Table 4.2**).

Sequence No.	Calc'd M	MALDI-TOF MS Found $[M - H]^-$
1	3822.6	3820.6
2	4390.4	4388.8

Table 4.2 – *Mass spectroscopic characterisation data of mid-sequence cyclohexadienyl modified oligonucleotide sequences (**1**) and (**2**), 5'- CGC ATU* CGA GAT, where U* cyclohexadienyl modified uridine*

4.2.2 Diels-Alder cycloadditions

4.2.2.1 Mid-sequence modified oligonucleotide conjugation to Tat peptide

Conjugation, *via* Diels-Alder cycloaddition, of unlabelled mid-sequence cyclohexadienyl modified oligonucleotide sequence (**2**) to the same maleimido modified Tat peptide derivative used previously (see 3.2.3.2 – *Oligonucleotide conjugation to Tat peptide*) was performed using conditions described previously for cycloadditions with 5'-dienyl modified oligonucleotides. The reaction was heated at 40 ^{o}C, overnight, using a solution of crude oligonucleotide; purification was performed after covalent linkage to the Tat peptide derivative. Analysis of the cycloaddition reaction mixture was by anion exchange HPLC, using a Resource Q column and eluents containing formamide. Analysis showed formation of a new peak with shorter retention time than that of the unconjugated oligonucleotide (**Fig. 4.4**), indicating formation of the oligonucleotide Tat peptide conjugate.

HPLC analysis of the cycloaddition mixture showed only 42 % conversion to the oligonucleotide Tat peptide conjugate, based on the ratio of peak areas. This is significantly less than the result achieved for the same 5'- cyclohexadienyl modified oligonucleotide. It was thought that the reduced yield could be due to steric reasons, brought about as a result of the short linkage between the cyclohexadiene ring and the pyrimidine. As such, the diene is held in close proximity to the oligonucleotide sequence, possibly hindering access by the maleimido modified Tat peptide derivative.

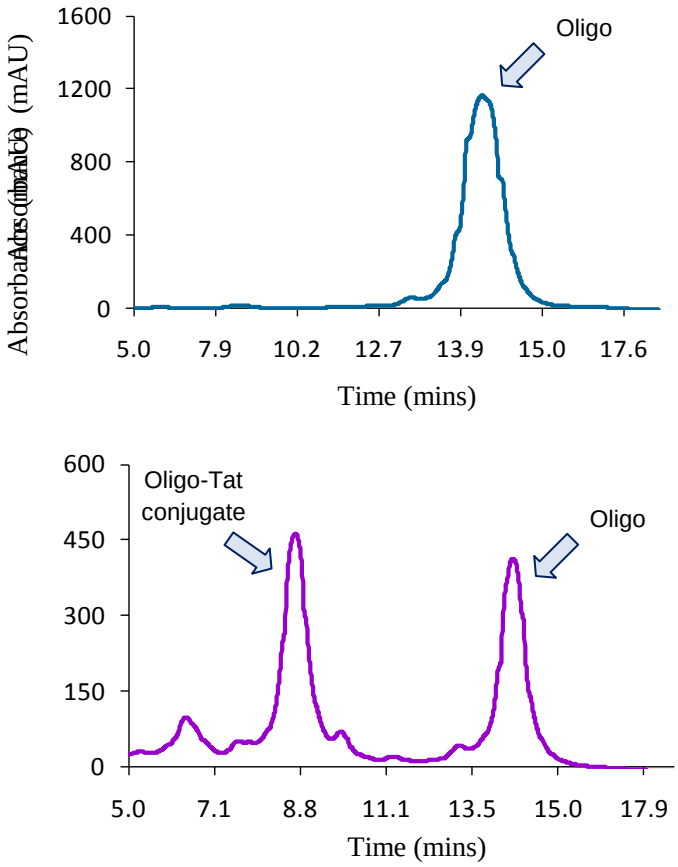


Figure 4.4 – *Ion-exchange HPLC traces of mid-sequence cyclohexadienyl modified oligonucleotide and oligonucleotide TAT peptide conjugate at 260 nm. (**A**) Starting material oligonucleotide sequence (**2**); (**B**) Diels- Alder reaction mixture*

Purification of the oligonucleotide Tat peptide conjugate was achieved by anion exchange HPLC. MALDI-TOF mass analysis of the purified product confirmed successful synthesis of the conjugate (**Table 4.3**).

Calc'd M	MALDI-TOF MS Found $[M - H]^-$
5581.6	5581.2

Table 4.3 – *Mass spectroscopic characterisation data of malemido Tat peptide oligonucleotide conjugate, 5'- CGC ATU* (Tat) CGA GAT -3', where U* is cyclohexadienyl modified uridine*

4.2.2.2 Conjugation of labelled mid-sequence modified oligonucleotides to Tat peptide

Tat peptide conjugation to labelled mid-sequence cyclohexadienyl modified oligonucleotide sequence (**3**) was also attempted, to see if an improved yield of labelled oligonucleotide Tat peptide conjugate, over that observed for 5'-cyclohexadienyl modified oligonucleotides, could be achieved.

3'-FAM labelled oligonucleotide sequence (**3**) was heated at 40 ^{o}C, overnight, with maleimido modified Tat peptide using the denaturing reaction conditions described previously. However, as observed for 5'- dienyl modified oligonucleotides, anion-exchange HPLC analysis of the reaction mixture showed only a very small peak with shorter retention time than the unconjugated oligonucleotide (**Fig. 4.5**).

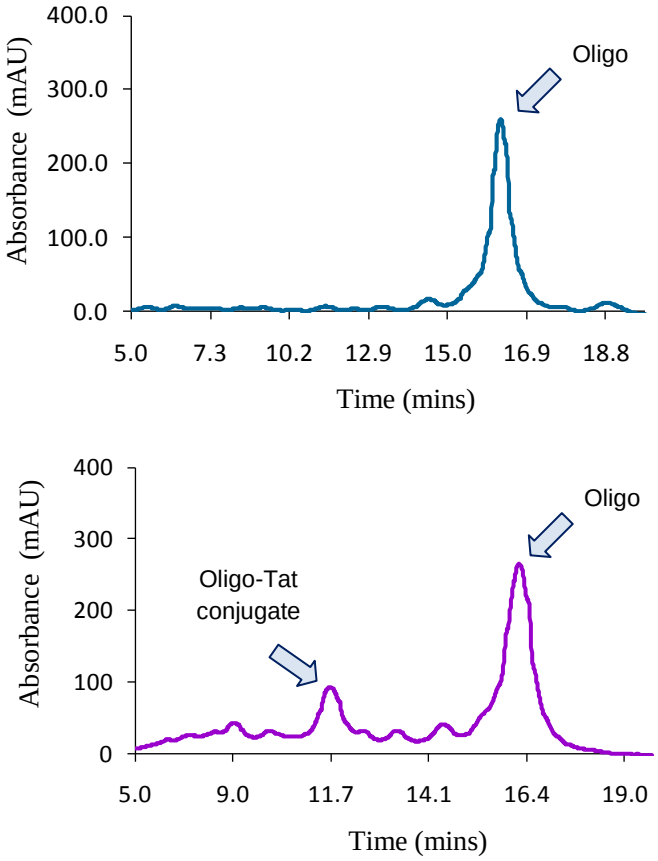


Figure 4.5 – *Ion-exchange HPLC traces at 260 nm of 3'- FAM labelled, mid-sequence cyclohexadienyl modified oligonucleotide and labelled oligonucleotide Tat peptide conjugate. (**A**) Starting material oligonucleotide sequence (**3**); (**B**) Diels-Alder reaction mixture*

Calc'd M	MALDI-TOF MS Found $[M - H]^-$
6149.4	6149.5

Table 4.4 – *Mass spectroscopic characterisation data of FAM labelled malemido Tat peptide oligonucleotide conjugate, 5' - CGC ATU* (Tat) CGA GAT - FAM - 3', where U* is cyclohexadienyl modified uridine*

Purification of the oligonucleotide Tat peptide conjugate was achieved by anion exchange HPLC. MALDI-TOF mass analysis of the purified product confirmed successful synthesis of the conjugate (**Table 4.4**).

However, HPLC analysis showed only 16 % conversion to the labelled oligonucleotide Tat peptide conjugate, based on the ratio of peak areas. As such, it was concluded that altering the position of the cyclohexadienyl modification within an oligonucleotide sequence, from the 5'-terminus to a mid- sequence position did not increase the viability of Diels-Alder cycloaddition for the synthesis of labelled oligonucleotide Tat peptide conjugates.

4.3 Conclusions

A methodology for mid-sequence dienyl modification, using Sonogashira chemistry, was established by way of synthesis of mid-sequence furanyl modified oligonucleotide. It was noted that, in order to achieve reproducible results for furanyl modification of 5'-iodo-2-deoxyuridine, to be used in oligonucleotide synthesis, silyl protection of both the 3'- and 5'- hydroxyl functionalities of the uridine was necessary as a first step, prior to performing the Sonogashira reaction. This was thought to improve the outcome of the Sonogashira reaction by preventing coordination of the Pd catalyst to the free 3'- OH of the nucleoside.

Using the established methodology, both labelled and unlabelled mid-sequence cyclohexadienyl modified oligonucleotide sequences were synthesised. Conjugation, *via* Diels-Alder cycloaddition, of unlabelled mid-sequence cyclohexadienyl modified oligonucleotide to maleimido modified Tat peptide afforded the desired

oligonucleotide peptide conjugate, but in a lower yield than that achieved previously for 5'- cyclohexadienyl modified oligonucleotides. This was attributed to steric hindrance brought about by the short linkage between the cyclohexadiene modification and the rest of the oligonucleotide sequence.

Conjugation of 3'-FAM labelled, mid-sequence cyclohexadienyl modified oligonucleotide to maleimido modified Tat peptide was attempted. As observed for 5'-cyclohexadienyl modified oligonucleotides, yield of the Tat peptide conjugate was low. As such, Diels Alder cycloaddition of mid-sequence cyclohexadienyl modified oligonucleotides was considered no more viable than cycloaddition of 5'-cyclohexadienyl modified oligonucleotides as a method for synthesis of a labelled oligonucleotide Tat peptide conjugate for visualisation in cell samples.

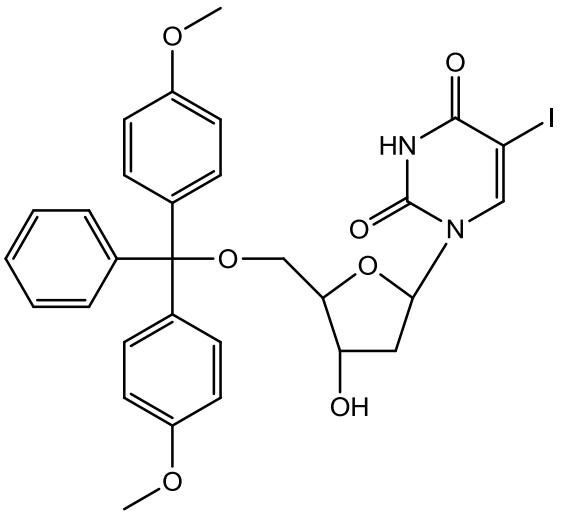
4.4 Experimental

3. 4. 1 General

As for Chapter 2 – *Conjugation of 5'- dienyl modified oligonucleotides to Tat peptide via Diels-Alder cycloaddition,* except that anhydrous pyridine and Et_3N were distilled over CaH_2. $Pd(PPh_3)_4$ for use in Sonogashira reactions was supplied by Strem.

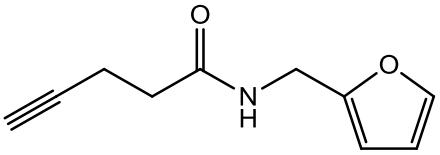
3. 4. 2 Chemical Synthesis

5'-*O*-[4, 4'-Dimethoxytrityl]-5-iodo-2'-deoxyuridine (**1**)



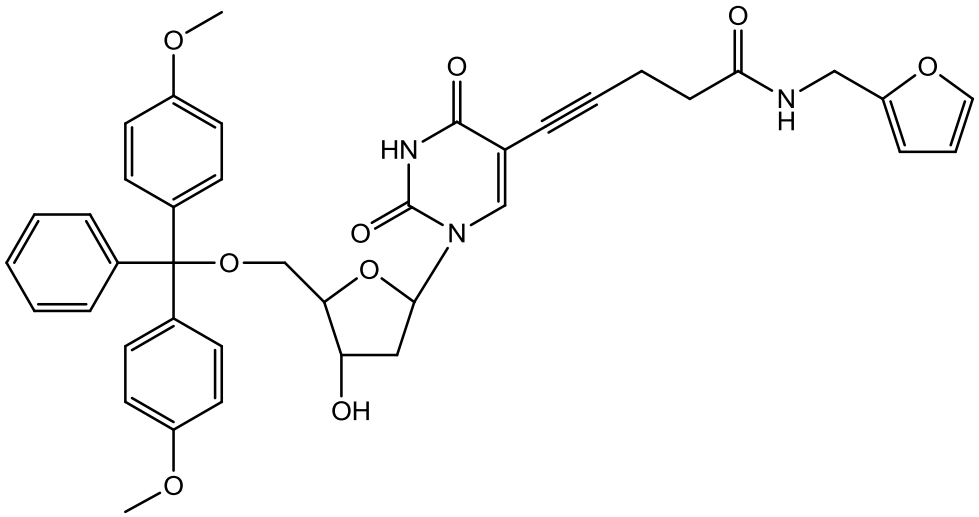
5-Iodo-2'-deoxyuridine (1.00 g, 2.8 mmol) was co evaporated with freshly distilled pyridine (x 3), dried overnight under vacuum, then dissolved in freshly distilled pyridine (20 ml). To this solution, under nitrogen, was added DMAP (69 mg, 0.6 mmol) and DIPEA (689 µl, 4.0 mmol). 4, 4'-dimethoxytrityl chloride (1.148 g, 3.4 mmol) was added, portionwise, over 2 hours. The reaction mixture was stirred at room temperature, overnight then quenched by addition of methanol (20 ml). All solvents were removed under reduced pressure. The green, oily residue was taken up in DCM and washed with saturated KCl solution, 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 wet flash column chromatography, eluting on a gradient of 0 % to 3 % MeOH in DCM, over silica pre- equilibrated with Et_3N, to afford the title compound as a white crystalline foam (1.211 g, 67 %). (Found: C, 54.7; H, 4.4; N, 4.4; I, 21.6. $C_{30}H_{29}N_2O_7I$ requires C, 54.9; H, 4.5; N, 4.3; I, 19.3 %); δ_H (400 MHz, d_6-acetone) 2.37 - 2.40 (2H, m, 2' IdU), 3.32 - 3.40 (2H, m, 5' IdU), 3.79 (6H, s, 2 x C*H*$_3$O), 4.09 (1H, q, *J* 3.1, 4' IdU), 4.56 (1H, quintet, *J* 2.9, 3' IdU), 6.27 (1H, t, *J* 6.8, 1' IdU), 6.85 - 6.92 (4H, m, Ar*H*), 7.21 - 7.52 (9H, m, Ar*H*), 8.14 (1H, s, 6 IdU), ; δ_C (100.6 MHz, d_6-acetone) 41.8, 55.8, 64.9, 69.1, 72.6, 86.4, 87.7, 114.3, 127.9, 129.1, 131.2, 136.9, 145.5, 146.2, 151.1, 160.0, 161.0; *m/z* 679.0917 ([M + Na^+] $C_{30}H_{29}N_2O_7I$ requires 679.0912); mp 144 ^oC.

N-(Furan-2-ylmethyl)pent-4-ynamide (2)



To a solution of 4-pentynoic acid (350 mg, 3.6 mmol) in anhydrous MeCN (10 ml) was added CDI (694 mg, 4.3 mmol). The formation of CO_2 was observed. The mixture was stirred at 40 ^oC for 1 hr. Furfuryl amine (381 mg, 3.9 mmol) was added. The mixture was stirred at 40 ^oC for a further 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), water and saturated NaCl solution. The organic phase was dried over Na_2SO_4. After filtration, EtOAc was removed under reduced pressure. Purification was by trituration of the crude product, dissolved in the minimum amount of DCM, with hexane to afford the title compound as an off-white powder (437 mg, 69 %). (Found: C, 67.7; H, 6.3; N, 7.8. $C_{10}H_{11}O_2N$ requires C, 67.8; H, 6.3; N, 7.9 %); δ_H (400 MHz; $CDCl_3$) 2.00 (1H, t, *J* 2.6, C≡C*H*), 2.44 (2H, t, *J* 7.0, CH_2C≡CH), 2.56 (2H, m, CH_2CH_2), 4.47 (2H, d, *J* 5.5, CH_2NH), 5.90 (1H, s br, N*H*), 6.25 (1H, dd, *J* 3.2, 2.5, furfuryl), 6.33 (1H, dd, *J* 1.8, 1.0, furfuryl), 7.36 (1H, dd, *J* 1.8, 1.0. furfuryl) ; δ_C (100.6 MHz; $CDCl_3$) 170.6, 151.0 142.1, 110.4, 107.5, 82.8, 69.3, 36.5, 35.1, 14.7; *m/z* 178.0862 ([M + H^+]; $C_{10}H_{11}$ NO_2 requires 178.0863); mp 56 ^oC.

5'-*O*-(4,4'-Dimethoxytrityl)-5-((*N*-furan-2-ylmethyl)-pent-4-ynamid-5-yl)-2'-deoxyuridine (3)



Method A

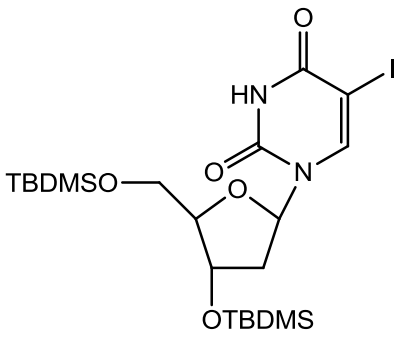
To a thoroughly degassed solution of 5'-*O*-[4,4'-dimethoxytrityl]-5-iodo-2'-deoxyuridine (**1**) (100 mg, 0.2 mmol) in anhydrous DMF (4 ml) was added CuI (59 mg, 0.3 mmol), anhydrous Et_3N (1 ml), *N*-(furan-2-ylmethyl)pent-4-ynamide (**2**) (83 mg, 0.5 mmol) and $Pd(PPh_3)_4$ (90 mg, 0.1 mmol), in that order. The mixture was stirred at room temperature for 2 days. The solvent was removed under reduced pressure. The residue was suspended in DCM and washed with saturated KCl solution and EDTA solution (5 %). The organic phase was dried over Na_2SO_4. After filtration, DCM was removed under reduced pressure. Purification was by wet flash column chromatography, eluting with 0 - 3 % MeOH in DCM, over silica pre-equilibrated with Et3N, to afford the title compound as a brown foam (63 mg, 57 %).

Method B

5-((*N*-Furan-2-ylmethyl)-pent-4-ynamid-5-yl)-2'-deoxyuridine (**6**), (1.00 g, 2.5 mmol) was co evaporated in freshly distilled pyridine (x 3), then dissolved in freshly distilled pyridine (30 ml). To this solution was added DMAP (61 mg, 0.5 mmol) and DIPEA (0.6 ml, 3.5 mmol). The mixture was placed under a blanket of nitrogen. 4, 4'-dimethoxytrityl chloride was added, portionwise, over 1 hr. The mixture was

stirred at room temperature overnight. The reaction was quenched by addition of methanol. All solvents were removed under reduced pressure. The residue was dissolved in DCM and washed with saturated KCl solution. The organic phase was dried over Na_2SO_4. After filtration, DCM was removed under reduced pressure. Purification was by wet flash column chromatography, eluting with 0 - 3 % MeOH in DCM, over silica pre-equilibrated with Et_3N, to afford the title compound as a pale orange foam (755 mg, 43 %). (Found: C, 66.4; H, 5.3; N, 5.7. $C_{40}H_{39}N_3O_9$ requires C, 68.1; H, 5.6; N, 5.9 %); δ_H (400 MHz; d_6-acetone) 2.20 - 2.24 (2H, m, 2' IdU), 2.38 (2H, t, *J* 6.8 CH_2CH_2CO), 2.42 (2H, t, J 6.7 CH_2CH_2CO), 3.29 - 3.40 (2H, m, 5' IdU), 3.79 (6H, s, 2 x CH_3O), 4.09 (1H, m, 4' IdU), 4.42 (2H, d, *J* 5.7, CH_2NH), 4.61 (1H, m, 3' IdU), 5.40 (1H, s br, N*H*), 6.23 (1H, dd, *J* 3.2, 2.4, furfuryl), 6.28 (1H, t, *J* 6.8, 1' IdU), 6.32 (1H, dd, *J* 3.1, 1.3, furfuryl), 6.89 - 6.92 (4H, m, Ar*H*), 7.22 - 7.51 (9H, m, Ar*H*), 7.53 (1H, dd, *J* 3.8, 1.3, furfuryl), 7.98 (1H, s, 6 IdU); δ_C (100.6 MHz; d_6-acetone) 16.7, 35.9, 37.1, 42.1, 56.0, 65.1, 72.8, 73.8, 86.6, 87.9, 88.0, 93.7, 101.1, 108.0, 111.6, 114.5, 128.0, 129.2, 131.4, 137.0, 137.3, 143.2, 146.4, 150.7, 154.0, 160.1, 162.7, 171.4 ; *m/z* 706.2765 ([M + H^+] $C_{40}H_{39}N_3O_9$ requires 706.2686).

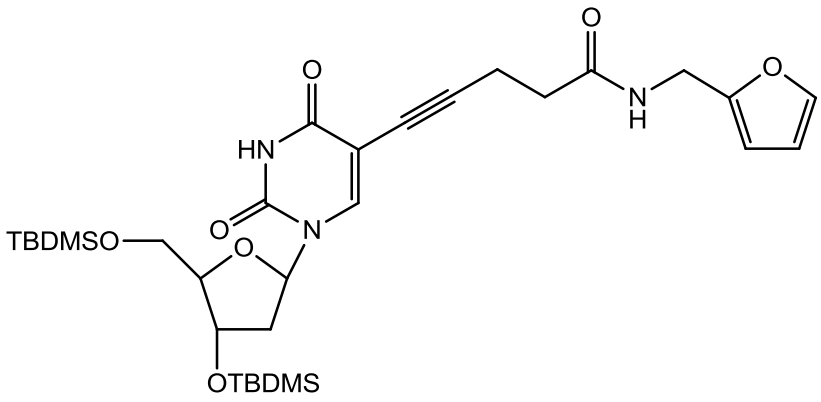
5',3'-Bis-(*O*-*tert*-butyldimethylsilyl)- 2'-deoxyuridine (**4**)



A solution of imidazole (1.923 g, 28.2 mmol) in anhydrous DMF (8 ml) was cooled to 0 oC. *tert*-Butyldimethylsilyl chloride (2.554 g, 16.9 mmol) was added. The mixture was stirred at 0 ^oC for 20 mins. 5-iodo-2'-deoxyuridine (2.00 g, 5.7 mmol) was added at 0 ^oC then the mixture was allowed to warm to room temperature. The mixture was stirred at room temperature for 4 hrs. The reaction was quenched by addition of water then the mixture was extracted with copious Et_2O. The organic phase was washed with water and saturated NaCl then dried over

Na_2SO_4. After filtration, Et_2O was removed under reduced pressure. Purification was by wet flash column chromatography, eluting with 20 % EtOAc in hexane, to afford the title compound as a white foam (3.333 g, 93 %). (Found: C, 43.6; H, 7.1; N, 4.8. $C_{21}H_{39}N_2O_5Si_2I$ requires C, 43.3; H, 6.8; N, 4.8 %); δ_H (400 MHz; d_6-acetone) 0.13 (6H, s, $(CH_3)_2Si$), 0.19 (6H, s, $(CH_3)_2Si$), 0.92 (9H, s, $C(CH_3)_3$), 2.21 - 2.28 (2H, m, 2' IdU), 3.87 - 3.93 (m, 2H, 5'IdU), 3.99 (1H, dd, *J* 5.7, 2.6, 4' IdU), 4.54 (1H, quintet, *J* 2.7, 3' IdU), 6.24 (1H, t, *J* 6.2, 1' IdU), 8.08 (1H, s, 6 IdU); δ_C (100.6 MHz; d_6-acetone) -5.2, -5.1, -4.8, -4.7, 18.4, 18.9, 26.0, 26.4, 41.7, 63.7, 68.8, 73.4, 86.0, 88.7, 145.1, 150.7, 160.5; *m/z* 583.1521 ($[M + H^+]$ $C_{21}H_{39}N_2O_5Si_2I$ requires 583.1442).

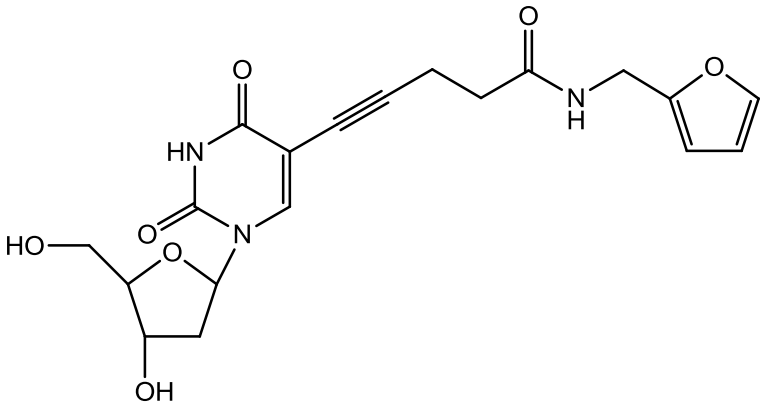
5',3'-Bis-(*O*-*tert*-butyldimethylsilyl)-5-((*N*-furan-2-ylmethyl)-pent-4-ynamid-5-yl)-2'-deoxyuridine (**5**)



A solution of 5',3'-bis-(*O*-*tert*-butyldimethylsilyl)-2'-deoxyuridine (**4**) (1.611 g, 2.8 mmol) in anhydrous DMF (10 ml) was placed in a flame-dried flask under a blanket of nitrogen. CuI (105 mg, 0.6 mmol) and $PdCl_2(PPh_3)_2$ (194 mg, 0.3 mmol) were added, under dark conditions. The mixture was thoroughly degassed then degassed DIPEA (2.40 ml, 13.8 mmol) was added. The mixture was stirred at room temperature for 30 mins. A thoroughly degassed solution of *N*-(furan-2-ylmethyl)pent-4-ynamide (**2**) (735 mg, 14.1 mmol), in DMF (1 ml), was added, portionwise, over 1 hr. The mixture was stirred at room temperature overnight. The reaction was quenched by addition of water 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, Et_2O was removed under

reduced pressure. Purification was by wet flash column chromatography, eluting with 50 % EtOAc in hexane, to afford the title compound as a pale brown foam (639 mg, 37 %). (Found: C, 58.2; H, 7.7; N, 6.4. $C_{31}H_{49}N_3O_7Si_2$ requires C, 58.9; H, 7.8; N, 6.7 %); δ_H (400 MHz; d_6-acetone) 0.13 (6H, s, $(CH_3)_2Si$), 0.18 (6H, s, $(CH_3)_2Si$), 0.92 (9H, s, $C(CH_3)_3$), 0.96 (9H, s, $C(CH_3)_3$), 2.25 - 2.29 (2H, m, 2' IdU), 2.47 (2H, t, *J* 7.5, CH_2CH_2CO), 2.66 (2H, t, *J* 7.6, CH_2CH_2CO), 3.87 - 3.96 (2H, m, 5' IdU), 3.99 (1H, q, *J* 2.8, 4' IdU), 4.55 (1H, quintet, *J* 2.8, 3' IdU), 4.64 (2H, d, *J* 5.7, CH_2NH), 6.24 (1H, dd, *J* 3.2, 2.5, furfuryl), 6.27 (1H, t, *J* 6.8, 1' IdU), 6.33 (1H, dd J 3.1, 1.2, furfuryl), 7.42 (1H, dd, J 3.8, 1.0, furfuryl), 7.49 (1H, s br, NH), 7.92 (1H, s, 6 IdU); *m/z* 632.3188 ($[M + H^+]$. $C_{31}H_{49}N_3O_7Si_2$ requires 632.3182).

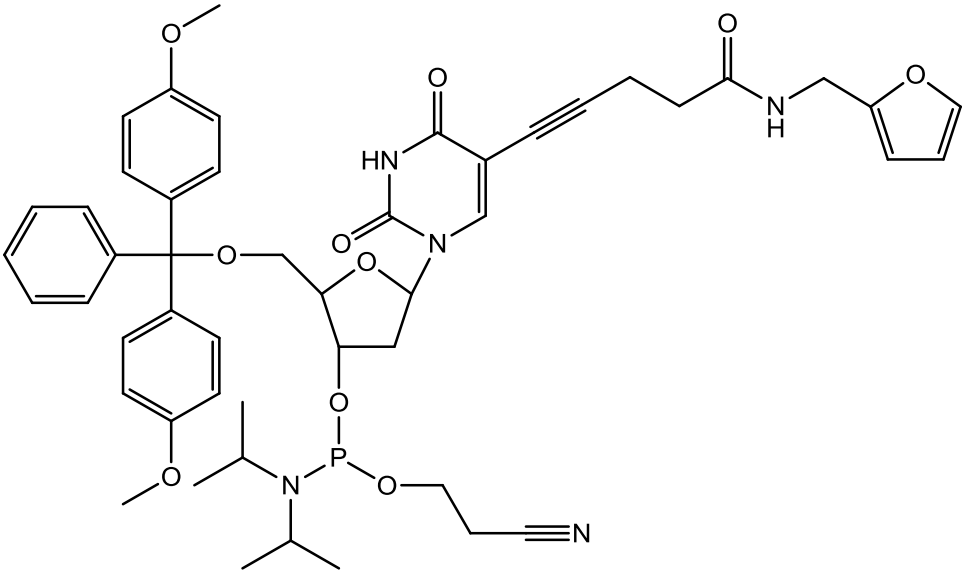
5-((*N*-Furan-2-ylmethyl)-pent-4-ynamid-5-yl)-2'-deoxyuridine (6)



To a solution of 5',3'-bis-(*O*-*tert*-butyldimethylsilyl)-5-((*N*-furan-2-ylmethyl)-pent-4-ynamid-5-yl)-2'-deoxyuridine (**5**) (639 mg, 1.0 mmol) in anhydrous MeOH (5 ml) was added a catalytic amount of acetyl chloride (11 μl, 0.2 mmol). The mixture was stirred at room temperature for 2 hrs. TLC showed both mono-protected and fully deprotected products. Further acetyl chloride (22 μl, 0.3 mmol) was added. The mixture was stirred at room temperature overnight. The reaction was quenched by addition of DCM then all solvents and acetyl chloride were removed under reduced pressure. Purification was by wet flash column chromatography, eluting with 10 % MeOH in DCM, to afford the title compound as a pale brown foam (193 mg, 47 %). (Found: C, 55.1; H, 5.2; N, 9.9. $C_{19}H_{21}N_3O_7$ requires C, 56.6; H, 5.3; N, 10.4 %); δ_H (400 MHz; d_6-acetone) 2.32 - 2.40 (2H, m, 2' IdU), 2.56 (2H, t, *J* 7.2, CH_2CH_2CO), 2.73 (2H, t, *J* 7.3, CH_2CH_2CO), 3.86 - 3.94 (2H, m, 5' IdU), 4.07 (1H, q, *J* 3.0, 4'

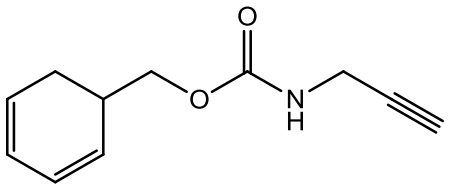
IdU), 4.45 (2H, d, *J* 5.6, C*H*$_2$NH), 4.60 (1H, quintet, *J* 3.02, 3' IdU), 6.33 (1H, dd, J 3.2, 2.4, furfuryl), 6.36 (1H, t, *J* 6.7, 1' IdU), 6.40 (1H, dd, *J* 3.2, 1.3, furfuryl), 7.50 (1H, dd, *J* 1.8, 1.0, furfuryl), 7.75 (1H, s br, N*H*), 8.33 (1H, s, 6 IdU); δ_C (100.6 MHz; DMSO) 15.2, 34.1, 35.3, 39.4, 61.0, 70.2, 72.9, 84.5, 87.5, 92.3, 98.7, 106.7, 110.4, 142.0, 142.8, 149.4, 152.1, 161.6, 170.1; *m/z* 404.1458 ([M + H^+] $C_{19}H_{21}N_3O_7$ requires 404.1380).

3'-*O*-(5'-*O*-(4,4'-Dimethoxytrityl)-5-((*N*-furan-2-ylmethyl)-pent-4-ynamid-5-yl)-2'-deoxyuridine)-yl 2-cyanoethyl diisopropylphosphoramidite (7)



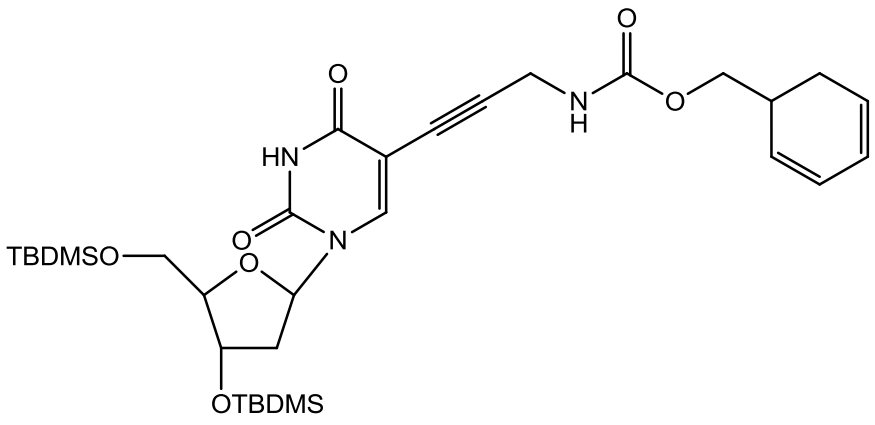
5'-*O*-(4,4'-Dimethoxytrityl)-5-((*N*-furan-2-ylmethyl)-pent-4-ynamid-5-yl)-2'-deoxyuridine (**3**) (150 mg, 0.2 mmol) was dried under vacuum, over P_2O_5, overnight. To a solution of (**3**) in anhydrous MeCN (6 ml) was added (2-cyanoethoxy)bis(*N*,*N*-diisopropylamino)phosphine (66 µl, 0.2 mmol). 5-Benzyl-thio-1*H*-tetrazole (0.3 M in MeCN, 1.1 ml, 0.3 mmol) was added slowly *via* syringe. The mixture was stirred at room temperature for 2 hrs. The solvent was removed under reduced pressure, with the flask being opened to N_2 on removal from the Büchi. 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 (127 mg, 66 %). δ_P (162.0 MHz, d_8-THF) 146.0.

Cyclohexa-2,4-dien-1-ylmethyl prop-2-yn-1-ylcarbamate (9)



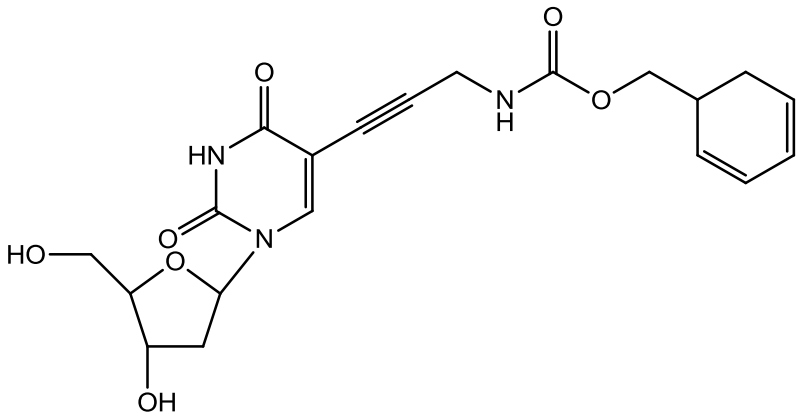
To a solution of (cyclohexa-2, 4-dien-1-yl) methanol (**8**) (3.925 g, 35.6 mmol) in anhydrous acetonitrile (20 ml), was added CDI (6.933 g, 42.8 mmol). Formation of CO_2 gas was observed. The mixture was stirred at room temperature for 1 hr, after which time propargyl amine (2.933 ml, 42.8 mmol) was added. The mixture was stirred at room temperature for 2 days. The solvent was removed under reduced pressure. The residue was dissolved in DCM 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. Purification was by wet flash column chromatography, eluting with 10 % EtOAc in petroleum ether (30 ^oC - 40 ^oC), to afford the title compound as a clear liquid (4.493 g, 66 %). δ_H (400 MHz, $CDCl_3$) 1.97 - 2.00 (1H, m, C*H*$_2$CH), 2.09 - 2.17 (1H, m, C*H*$_2$CH), 2.24 - 2.26 (1H, m, C≡C*H*), 4.00 (2H, d, *J* 8.0, C*H*$_2$NH), 4.06 (2H, d, *J* 4.0, C*H*$_2$O), 5.31 (1H, s br, N*H*), 5.75 - 5.99 (4H, m, 2 x C*H*=C*H*); δ_C (100.6 MHz, $CDCl_3$) 28.2, 31.0, 33.0, 67.0, 71.8, 80.0, 124.2, 125.6, 125.8, 126.4, 156.3; *m/z* 192.1020 ([M + H $^+$] $C_{11}H_{13}NO_2$ requires 192.0946).

5',3'-Bis-(*O*-*tert*-butyldimethylsilyl)-5-((cyclohexa-2,4-dienylmethyl prop-2-ynylcarbamate)-3-yl)-2'-deoxyuridine (10)



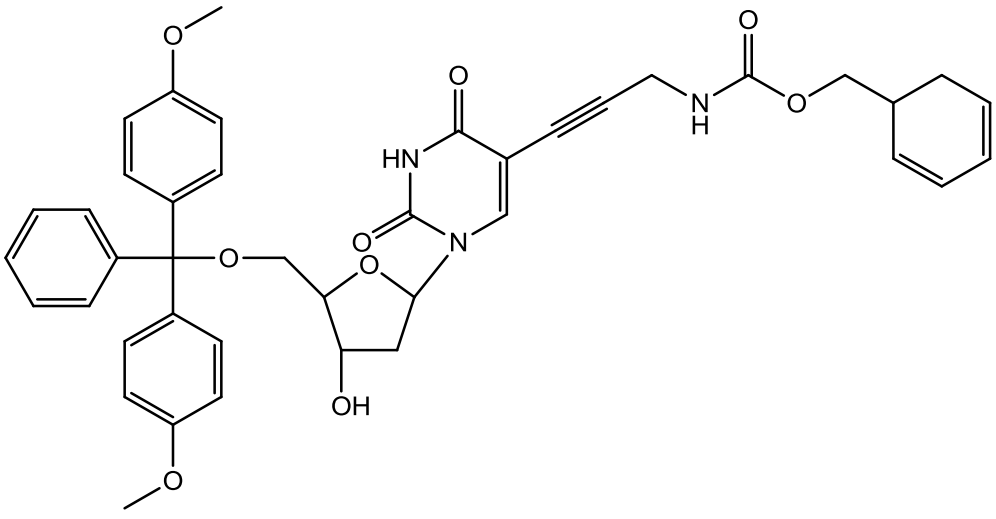
To a solution of 5',3'-bis-(*O-tert*-butyldimethylsilyl)- 2'-deoxyuridine (**4**) (5.00 g, 8.6 mmol) in DMF (20 ml) was added CuI (327 mg, 1.7 mmol) and $PdCl_2(PPh_3)_2$ (602 mg, 0.9 mmol), under dark conditions. The mixture was thoroughly degassed then degassed DIPEA (7.5 ml, 42.9 mmol) was added. The mixture was stirred at room temperature for 1 hr. A thoroughly degassed solution of cyclohexa-2, 4-dien-1-ylmethyl prop-2-yn-1-ylcarbamate (**9**) (2.462 g, 12.9 mmol), in DMF (8 ml), was added, portionwise, over 1 hr. The mixture was stirred at room temperature overnight. The reaction was quenched by addition of water 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. Purification was by wet flash column chromatography, eluting with 30 % EtOAc in petroleum ether (60 ^oC - 80 ^oC), to afford the title compound as a yellow oil (2.901 g, 52 %). δ_H (400 MHz; $CDCl_3$) 0.08 (6H, s, $(CH_3)_2Si$), 0.14 (6H, s, $(CH_3)_2Si$), 0.89 (9H, s, $C(CH_3)_3$), 0.93 (9H, s, $C(CH_3)_3$), 1.98 - 2.00 (1H, m, CH_2CH) 2.21 - 2.27 (2H, m, 2' IdU), 2.21 - 2.28 (1H, m, CH_2CH), 2.54 - 2.67 (1H, m, CH_2CH), 3.75 - 3.91 (2H, m, 5' IdU), 3.98 (2H, d, *J* 4.0, CH_2NH), 4.03 (1H, d, *J* 4.0, 4' IdU), 4.19 (2H, d, *J* 4.0, CH_2O), 4.40 (1H, quintet, *J* 2.0, 3' IdU), 5.63 - 5.98 (4H, m, 2 x CH=CH), 6.28 (1H, t, *J* 6.0, 1' IdU), 8.00 (1H, s, 6 IdU); δ_C (100.6 MHz, d_6-acetone) -5.3, -5.1, -4.7, -4.5, 18.6, 19.1, 26.1, 26.5, 42.0, 63.8, 73.4, 86.1, 88.9, 91.3, 100.0, 124.8, 126.1, 127.5, 143.7, 150.2; *m/z* 668.3163 ($[M + Na^+]$ $C_{32}H_{51}N_3O_7Si_2$ requires 668.3158).

5-((Cyclohexa-2,4-dienylmethyl prop-2-ynylcarbamate)-3-yl)-2'-deoxy-uridine (11)



To a solution of 5', 3'-bis-(*O-tert*-butyldimethylsilyl)-5-((cyclohexa-2, 4-dienyl-methyl prop-2-ynylcarbamate)-3-yl)-2'-deoxyuridine (**10**) (2.900 g, 4.5 mmol) in anhydrous MeOH (30 ml) was added a catalytic amount of acetyl chloride (144 µl, 2.0 mmol). The reaction mixture was stirred at room temperature for 6 hrs. DCM (60 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 0 - 10 % MeOH in DCM, to afford the title compound as a pale yellow foam (840 mg, 45 %). δ_H (400 MHz; d_6-acetone) 2.02 - 2.05 (1H, m, C*H*$_2$CH), 2.06 - 2.10 (1H, m, C*H*$_2$CH), 2.25 - 2.34 (2H, m, 2' IdU), 2.48 - 2.57 (1H, m, CH$_2$C*H*), 3.80 - 3.84 (2H, m, 5' IdU), 3.98 (2H, d, *J* 4.0, C*H*$_2$NH), 4.02 - 4.05 (1H, m, 4' IdU), 4.10 (2H, d, *J* 8.0, C*H*$_2$O), 4.51 (1H, quintet, *J* 4.0, 3' IdU), 5.63 - 5.95 (4H, m 2 x C*H*=C*H*), 6.28 (1H, t, *J* 4.0, 1' IdU), 8.26 (1H, s, 6 IdU); δ_C (100.6 MHz, d_6-acetone) 31.4, 32.2, 33.4, 41.2, 62.2, 66.3, 68.6, 71.4, 86.0, 88.5, 89.7, 99.2, 124.3, 125.7, 125.8, 127.1, 144.3, 150.0, 156.7, 161.8; *m/z* 418.1614 ([M + H^+]) $C_{20}H_{23}N_3O_7$ requires 418.1536).

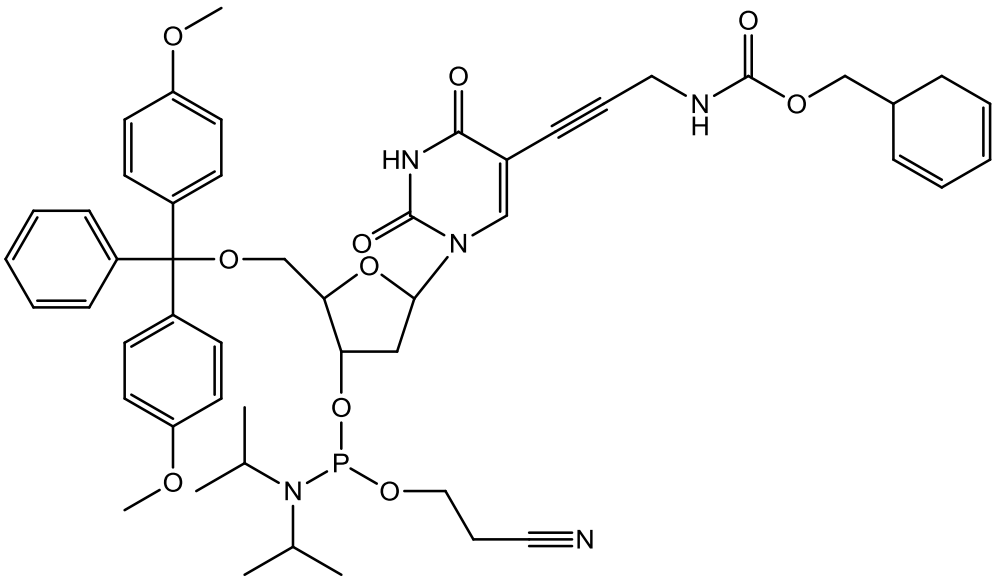
5'-*O*-(4,4'-Dimethoxytrityl)-5-((cyclohexa-2,4-dienylmethyl prop-2-ynylcarbamate)-3-yl)-2'-deoxyuridine (12)



5-((Cyclohexa-2,4-dienylmethyl prop-2-ynylcarbamate)-3-yl)-2'-deoxyuridine (**10**) (835 mg, 2.0 mmol) was co evaporated (x 3) in anhydrous pyridine, then dissolved in anhydrous pyridine (10 ml). To this solution was added DMAP (50 mg, 0.4 mmol) and DIPEA (525 µl, 3.0 mmol). 4, 4'-dimethoxytrityl chloride (813 mg, 2.4 mmol)

was added, portionwise, over 1 hr. The reaction mixture was stirred at room temperature overnight. The reaction was quenched by addition of methanol. All solvents were removed under reduced pressure. The residue was dissolved in DCM, then washed with saturated KCl solution and saturated NaCl solution. The combined organic phases were dried over Na_2SO_4. Purification was by wet flash column chromatography, eluting with 0 - 2 % MeOH in DCM to afford the title compound as an off-white foam (727 mg, 51 %). (Found: C, 65.8; H, 6.7; N, 6.4. $C_{41}H_{41}N_3O_9$ requires C, 68.4; H, 5.7; N, 5.8 %); δ_H (400 MHz, d_6-acetone) 2.08 - 2.09 (1H, m, C*H*$_2$CH), 2.20 - 2.24 (1H, m, C*H*$_2$CH), 2.21 - 2.27 (2H, m, 2' IdU), 2.53 - 2.61 (1H, m, C*H*$_2$CH), 3.25 - 3.46 (2H, m, 5' IdU), 3.80 (6H, s, 2 x C*H*$_3$O), 3.91 (2H, d, *J* 5.5, C*H*$_2$NH), 3.96 (2H, d, *J* 6.9, C*H*$_2$O), 4.09 - 4.11 (1H, m, 4' IdU), 4.61 - 4.62 (1H, m, 3' IdU), 5.65 - 5.97 (4H, m, 2 x C*H*=C*H*), 6.27 (1H, t, *J* 6.7, 1' IdU), 6.90 - 6.94 (4H, m, Ar*H*), 7.38 - 7.55 (9H, m, Ar*H*), 8.02 (1H, s, 6 IdU); δ_C (100.6 MHz, d_6-acetone) 32.6, 33.5, 34.7, 42.5, 56.4, 65.6, 67.5, 73.1, 76.0, 87.1, 88.3, 100.7, 108.3, 114,8, 114.9, 125.6, 126.9, 127.0, 127.3, 128.3, 129.6, 131.8, 137.4, 137.7, 144.6, 146.9, 150.9, 151.2, 160.5, 162.9; *m/z* 720.2921 ([M + H^+] $C_{41}H_{41}N_3O_9$ requires 720.2843).

3'-*O*-(5'-*O*-(4,4'-Dimethoxytrityl)-5-((cyclohexa-2,4-dienylmethyl prop-2-ynylcarbamate)-3-yl)-2'-deoxyuridine)-yl 2-cyanoethyl diisopropyl phosphoramidite (13)



5'-*O*-(4,4'-Dimethoxytrityl)-5-((cyclohexa-2,4-dienylmethylprop-2ynylcarbamate)-3-yl)-2'-deoxyuridine (**12**) (200 mg, 0.3 mmol) was under vacuum, over P_2O_5, overnight. To a solution of (**12**) in anhydrous MeCN (10 ml) was added (2-cyanoethoxy)bis(*N*,*N*-diisopropylamino)phosphine (99 µl, 0.3 mmol). 5-Benzylthio-1*H*-tetrazole (0.3 M in MeCN, 1.4 ml, 0.4 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 Büchi. Purification was by wet flash column chromatography, eluting with 5 % MeOH in DCM 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 (127 mg, 50 %). δ_P (162.0 MHz, d_8- THF) 148.7.

4. 4. 3 Oligonucleotide Synthesis

4.4.3.1 Solid phase synthesis of mid-sequence dienyl-modified oligonucleotides

The following sequence was synthesised on an automated DNA synthesiser:

Oligonucleotide sequence 1: 5'- X CGC ATU* CAG GAT -3'

Where U* is the pre-synthesised dienyl modified phosphoramidite (**7**) or (**13**), made up to a concentration of 0.1 M, in anhydrous MeCN. Oligonucleotide synthesis, purification and MALDI-TOF analysis were as described in 3.4.3.1 - *Synthesis of unmodified oligonucleotides*.

X = MALDI-TOF: $C_{10}H_{10}NO_2$ CGC ATU CAG GAT requires 3795.4; found 3796.2.

X = MALDI-TOF: $C_{11}H_{12}NO_2$ CGC ATU CAG GAT requires 3822.6; found 3820.6.

3.4.3.1 Solid phase synthesis of labelled mid-sequence cyclohexadienyl modified oligonucleotide

The following sequence was synthesised on an automated DNA synthesiser:

Oligonucleotide sequence 2: 5'- CGC ATU* CAG GAT - FAM -3'

Where U* is the pre-synthesised cyclohexadienyl modified phosphoramidite (**13**), made up to a concentration of 0.1 M, in anhydrous MeCN. Synthesis, cleavage and deprotection of 3'- FAM labelled sequences was performed as described for oligonucleotide sequence (**1**), but using a column containing (6-FAM) modified CPG solid support. The labelled sequence was used unpurified in Diels-Alder cycloadditions.

Purification for MALDI-TOF analysis and MALDI-TOF analysis conditions were as described for oligonucleotide sequence (**1**), using HPLC method (2).

Oligonucleotide sequence (**2**): MALDI-TOF: $C_{38}H_{36}NO_{11}$ CGC ATT CAG GAT requires 4390.4; found 4388.8.

3. 4. 4 Diels-Alder Cycloadditions

Stock solutions of unpurified mid-sequence cyclohexadienyl modified oligonucleotide sequences (**1**) and (**2**) were made up to a concentration of 1 mM in distilled water. A stock solution of maleimido modified Tat peptide 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 and purification of the reaction mixture was by ion-exchange chromatography, using HPLC method (3) for unlabelled conjugates and HPLC method (4) for labelled conjugates. Buffer salts and formamide were removed by

dialysis. Synthesis of the conjugate of oligonucleotide sequence (**1**) was confirmed by MALDI-TOF mass spectrometry, using a matrix of 40 mg ml^{-1} dihydroxy-acetophenone 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.

MALDI-TOF: Tat $C_{11}H_{12}NO_2$ CGC ATT CAG GAT requires 5581.6; found 5581.2.

MALDI-TOF: Tat $C_{11}H_{12}NO_2$ CGC ATT CAG GAT - FAM requires 6149.4; found 6149.5.

3. 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 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 (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, 280 and 492 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.

4.5 References

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