Oligonucleotide conjugation to a cell-penetrating (TAT) peptide by Diels–Alder cycloaddition†

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Modifed oligonucleotides are routinely employed as analytical probes for use in diagnostics, *e.g.* in the examination of specific RNA sequences for infectious diseases, however, a major limiting factor in oligonucleotide-based diagnostics is poor cellular uptake of naked oligonucleotides. This problem can be overcome by covalent attachment of a so-called 'cell-penetrating peptide' to form an oligonucleotide peptide conjugate. Stepwise solid phase synthesis of such a conjugate is difficult and expensive due to the conflicting chemistries of oligonucleotides and peptides. A simple approach to overcome this is post-synthetic conjugation. Diels–Alder cycloaddition is an attractive methodology for oligonucleotide peptide conjugation; the reaction is fast, chemoselective and the reaction rate is greatly enhanced in aqueous media – ideal conditions for biological moieties. An oligodeoxyribonucleotide sequence has been derivatised with a series of dienes at the 5′-terminus, using a series of unique dienyl-modified phosphoramidites, and investigation into the effect of diene type on the efficiency of conjugation, using Diels–Alder cycloaddition with a maleimido-derivatised cell-penetrating (TAT) peptide, has been performed. This led to the observation that the optimal diene for conjugation was cyclohexadiene, allowing conjugation of oligodeoxyribonucleotides to a cell-penetrating peptide by Diels–Alder cycloaddition for the first time.

Introduction

Modified oligonucleotides have been used in a variety of applications such as antisense[1,2] and gene-silencing therapies.[3] However, a major limiting factor in oligonucleotide-based therapeutics is poor access of naked oligonucleotides to the intracellular environment, as a result of oligonucleotide size and of the repulsion of the negatively charged phosphate backbone encountered when faced with the anionic charges of the phospholipid cell membrane.[4] The use of modified oligonucleotides as probes for the detection of specific DNA sequences is also well documented,[5–8] but while laboratory-based analysis of single stranded DNA sequences using modified oligonucleotides is well established, little work has been carried out on the detection of nucleic acid sequences in living cells, partly due to the limitation of poor cellular uptake.

Previously, translocation of negatively charged oligonucleotides across cell membranes for delivery into common laboratory cell lines in culture (such as HeLa cells) has been achieved by complexation of the oligonucleotides with cationic lipids;[9] however, there are problems with cytotoxicity and stability associated with this method.[10,11] More recent studies have uncovered the existence of certain proteins, including HIV-1 TAT transactivation protein,[12] *Drosophila* Antennapedia homoprotein[13] and the HSV-1 structural protein, VP22,[14] which have been shown to have the ability to traverse the cell membrane and reach the cell nucleus, whilst maintaining their biological activity. Their ability to do this has been attributed to short 'protein-transduction domains' (PTDs).[15,16] It has since been reported that short peptide sequences derived from these PTDs have been internalised by cells and, when complexed or conjugated to bioactive molecules, have been able to deliver these molecules into the intracellular environment.[17] Such peptide sequences have become known as cell-penetrating peptides (CPPs),[18] and have shown delivery of a wide range of bioactive components into cells, including oligonucleotides.[19]

Conjugation of oligonucleotides to peptides is made difficult due to the conflicting protecting group strategies required to avoid side reactions of the peptide side chains and the oligonucleotide bases. In-line solid phase synthesis of an oligonucleotide peptide conjugate has been achieved, but requires the development of a new series of protecting groups.[20] A simpler method for the synthesis of an oligonucleotide peptide conjugate involves separate preparation and functionalisation of the oligonucleotide and the peptide in a manner that allows their conjugation to be performed post-synthetically.[21–23]

Diels–Alder cycloaddition[24] is an attractive methodology for preparation of an oligonucleotide peptide conjugate in this way. Diels–Alder cycloaddition is selective for a diene and a dienophile, ruling out the need for oligonucleotide and/or peptide protecting groups, and the rate of the reaction has been shown to be greatly enhanced in aqueous systems,[25–27] presenting ideal conditions for bioconjugation. The reaction was first documented by Hill *et al.*[28] as an effective method for oligonucleotide modification. Diels–Alder cycloadditions have since been used for oligonucleotide labelling[29–33] and immobilisation of oligonucleotides onto glass

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slides.[34] Diels–Alder cycloaddition has also more recently been reported to be effective for oligonucleotide peptide conjugation.[35] Cycloadditions, in aqueous media, of 5′-(1,3-hexadienyl)-modified thymine dimers to a short series of maleimido-modified dipeptides, and of 5′-(1,3-hexadienyl)-modified 8mer and 15mer oligonucleotide sequences to simple maleimido-modified 8mer and 20mer peptide sequences, were reported in an investigation into the effect of sequence length on conjugate yield, using Diels–Alder cycloaddition. Composition of the peptide sequence used, as opposed to the oligonucleotide sequence used, has a greater effect on the outcome of cycloadditions and, as such, is the crucial factor in these types of conjugations. The peptide sequences used in this study were not highly charged, as is the case for CPP sequences, which are generally highly cationic and are notoriously difficult peptide sequences to handle.

This study further investigates the use of the Diels–Alder reaction for the conjugation of 5′-dienyl-modified oligodeoxyribonucleotides to peptides, specifically a cell-penetrating peptide, with a view to enhancing cellular uptake of the oligonucleotide for use as a biomolecular probe. A series of three different 5′-dienyl modifications have been investigated and their effect on the efficiency of conjugation to a maleimido-modified TAT peptide derivative, based on the PTD Tat (47–57), has been observed.

Materials and methods

^{1}H NMR and ^{13}C NMR were recorded on a Brüker DPX 400 MHz spectrometer with the appropriate solvent peak as reference. *J* values are quoted in Hertz. Mass spectrometry was carried out either as a service by Swansea EPSRC mass spectrometry centre or on a Shimadzu Axima-CFR system. Analytical and preparative HPLC were performed on a Dionex UVD170U detector fitted with a P680 pump through either a DNA-Pac 100 column (Dionex) or a 1 ml Resource Q column (Amersham Biosciences). Desalting HPLC was performed on a Dionex UVD170U detector fitted with a P680 pump and a CD20 Conductivity Detector, using a HiTrap (5 ml) size exclusion column. Oligonucleotides were synthesised on a MerMade 6 Nucleic Acid Synthesiser. Oligonucleotide synthesis reagents were purchased from Link Technologies. Tube-O-Dialyzers were purchased from G-Biosciences. ZipTip™ C_{18} purification pipette tips were purchased from Millipore Corp.

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

To a solution of sorbic acid (500 mg, 4.5 mmol, 1.0 eq) in MeCN (anhydrous, 10 ml) was added CDI (868 mg, 5.4 mmol, 1.2 eq), under dark conditions. The mixture was stirred at room temperature for 3 h. 6-Aminohexan-1-ol (784 mg, 6.6 mmol, 1.5 eq) was added and the mixture was stirred at room temperature overnight. The solvent was removed under reduced pressure. The product was dissolved in EtOAc (50 ml) and washed with citric acid (10% (m/v), 2 × 30 ml), NaOH (10% (m/v), 2 × 30 ml), water (30 ml) and sat. NaCl (30 ml). The organic layer was dried over Na_2SO_4. The solvent was removed under reduced pressure to afford the amide **1a** as a white solid (284 mg, 30%). δ_H (400 MHz; $CDCl_3$) 1.25–1.61 (8H, m, 4 × CH_2), 1.84 (2H, d, *J* 5.8, CH_2CO), 3.34 (2H, q, *J* 6.7, $NHCH_2$), 3.64 (2H, t, *J* 6.5, CH_2OH), 5.48 (1H, s br, NH), 5.70 (1H, s, CH_2=CH), 5.74 (1H, s, CH_2=CH), 6.04–6.19 (2H, m, CH–CH), 7.16–7.22 (1H, m, CH=CH). δ_C (400 MHz, d_6-acetone) 14.4, 18.5, 26.3, 27.5, 33.7, 39.7, 6.2, 124.0, 131.0, 136.7, 10.2, 166.5. *m/z* 212.1648 ([M + H$^+$] $C_{12}H_{21}NO_2$ requires 212.1651).

3-Furan-2-yl-*N*-(6-hydroxyhexyl)propanamide, 1b

To a solution of 3-(2-furyl)propanoic acid (252 mg, 1.8 mmol, 1.0 eq) in MeCN (5 ml, anhydrous) was added CDI (350 mg, 2.2 mmol, 1.2 eq) and the mixture was stirred at 40 °C for 1 h 30 min. 6-Aminohexan-1-ol (220 mg, 1.9 mmol, 1.1 eq) was added and the mixture was stirred at room temperature for 2 h. The solvent was removed under reduced pressure. The product was dissolved in EtOAc (30 ml) and washed with citric acid (10% (m/v), 2 × 20 ml), NaOH (10% (m/v), 2 × 20 ml), water (20 ml) and sat. NaCl (20 ml). The organic layer was dried over Na_2SO_4. The solvent was removed under reduced pressure to afford the amide **1** as a pale yellow solid (302 mg, 70%). δ_H (400 MHz; $CDCl_3$) 1.26–1.60 (8H, m, 4 × CH_2), 2.49 (2H, t, *J* 7.5, CH_2CO), 2.99 (2H, t, *J* 7.5, furan CH_2), 3.24 (2H, t, *J* 6.5, $NHCH_2$), 3.64 (2H, t, *J* 6.5, CH_2OH), 5.44 (1H, s br, NH), 6.04 (1H, dd, *J* 3.1, 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 (400 MHz, CDCl3) 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).

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

A solution of imidazole (2.43 g, 35.7 mmol, 2.0 eq) in DMF (anhydrous, 8 ml) was cooled to 0 °C in an ice bath. *tert*-Butyldimethylsilyl chloride (3.23 g, 21.4 mmol, 1.2 eq) was added and the mixture was stirred at 0 °C for 20 min. 3-Cyclohexene-1-methanol (2.00 g, 17.8 mmol, 1 eq) was added at 0 °C and the mixture was stirred at room temperature for 1 h. Water was added to quench the reaction. The mixture was extracted with diethyl ether (3 × 100 ml). The combined organic layers were washed with water (50 ml) and sat. NaCl (50 ml), and then dried over Na_2SO_4. The solvent was removed under reduced pressure to afford the alkene **2** as a clear liquid (3.80 g, 94%) without further purification. δ_H (400 MHz, $CDCl_3$) 0.05 (6H, s, $Si(CH_3)_2$), 0.91 (9H, s, $C(CH_3)_3$), 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, CH_2O), 3.49 (1H, d, *J* 3.6, CH_2O), 5.64–5.75 (2H, m, CH=CH). δ_C (400 MHz; $CDCl_3$) –5.2, 18.6, 25.6, 25.9, 26.2, 28.4, 36.5, 68.1, 126.4, 127.2.

***trans*-1,2-Dibromo-3-(*tert*-butyldimethylsilyloxymethyl)-cyclohexane, 3**

A solution of crude alkene **2** (3.80 g, 16.8 mmol, 1.0 eq) in DCM (30 ml) was placed in a flask filled with nitrogen, and cooled to 0 °C. Bromine (2.68 g, 16.8mmol, 1.0 eq) in DCM (2 ml) was added slowly. The mixture was stirred at room temperature for 1 h. DCM (40 ml) was added to the resulting red-brown mixture. The mixture was washed with $Na_2S_2O_3$ (10% (m/v), 50 ml) and water (50 ml). The organic layer was dried over Na_2SO_4. The solvent was removed under reduced pressure to afford the dibromide **3** as a brown liquid (5.22 g, 81%) without further purification. δ_H (400 MHz; $CDCl_3$) 0.05 (6H, s, $Si(CH_3)_2$), 0.89 (9H, s, $C(CH_3)_3$), 1.93–1.99 (5H, m, cyclohexyl), 2.40–2.50 (2H, m, cyclohexyl), 3.48 (2H, d, *J* 5.8, CH_2O), 4.66–4.72 (2H, m, 2 × CHBr). δ_C (400 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 dibromide **3** (5.22 g, 13.5 mmol) in THF (anhydrous, 40 ml) was added Aliquat 336® (109 mg, 0.27 mmol, 0.02 eq). The mixture was cooled to 0 °C and potassium *tert*-butoxide (3.34 g, 29.7 mmol, 2.2 eq) was added, all at once. Immediately a yellow precipitate formed. The mixture was stirred at 0 °C for 5 min, then at room temperature for 1 h. Petroleum ether (bp 30–40 °C, 150 ml) was added. The mixture was washed with sat NH_4Cl (2 × 50 ml) and water (2 × 50 ml). The organic layer was dried over Na_2SO_4. The solvent was removed under reduced pressure to afford the diene **4** 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 × CH=CH). δ_C (400 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 diene **4** (3.25 g, 14.3 mmol, 1 eq) in methanol (anhydrous, 25 ml) was added a catalytic amount of acetyl chloride (153 µl, 2.15 mmol, 0.15 eq). The mixture was stirred at room temperature for 10 min. DCM (40 ml) was added to quench the reaction. The solvents were removed under reduced pressure. The product was purified by wet flash column chromatography, eluting with petroleum ether (bp 30–40 °C)–EtOAc (9 : 1) to afford the diene **5** as a pale 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 × CH=CH). δ_C (400 MHz; $CDCl_3$) 25.4, 35.9, 65.5, 124.0, 125.7, 124.9, 127.0.

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

To a solution of diene **5** (274 mg, 2.5 mmol, 1.0 eq) in acetonitrile (anhydrous, 8 ml) was added CDI (484 mg, 3.0 mmol, 1.2 eq) in the dark. The mixture was stirred at room temperature for 1 h. 6-Amino-1-hexanol (350 mg, 3.0 mmol, 1.2 eq) was added and the mixture was stirred at room temperature overnight. The solvent was removed under reduced pressure. The product was dissolved in DCM (30 ml) and washed with water (2 × 15 ml) and sat. NaCl (15 ml). The organic layer was dried over Na_2SO_4. Purification was by trituration of the product, dissolved in the minimum amount of DCM, with petroleum ether (bp 60–80 °C) to afford the carbamate **6** as a pale pink powder (311 mg, 49%). δ_H (400 MHz; d_6-acetone) 1.31–1.55 (8H, m, 4 × CH_2), 2.21–2.25 (2H, m, CH_2CH), 2.56–2.57 (1H, m, CH_2CH), 3.10 (2H, t, *J* 6.4, CH_2NH), 3.52 (2H, dd, *J* 11.7, 6.4 CH_2OH), 3.91 (2H, d, *J* 7.9, CH_2O), 5.64–5.70 (2H, m, CH=CH), 5.87–5.98 (2H, m, CH=CH). δ_C (400 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]hexyl diisopropylamidophosphite, 7a

Amide **1a** (150 mg, 0.71 mmol, 1.0 eq) was dried overnight over P_2O_5, under vacuum. To a suspension of **1a** in MeCN (anhydrous, 10 ml) was added (2-cyanoethoxy)bis(*N*,*N*-diisopropylamino)phosphine (321 mg, 1.1 mmol, 1.5 eq). 5-Benzylthio-1*H*-tetrazole (0.3 M in MeCN, 3.5 ml, 1.1 mmol, 1.5 eq) was added slowly. The mixture was stirred at room temperature for 1 h. The solvent was removed under reduced pressure. The product was purified by wet flash column chromatography, eluting with EtOAc over a column pre-equilibrated with Et_3N. The product was co evaporated with MeCN (anhydrous, × 3) to afford the phosphoramidite **7a** as a yellow oil (184 mg, 63%). δ_P (400 MHz; d_8-THF) 145.50.

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

Amide **1b** (150 mg, 0.6 mmol, 1.0 eq) was dried overnight over P_2O_5, under vacuum. To a solution of **1b** in MeCN (4 ml, anhydrous) was added (2-cyanoethoxy)bis(*N*,*N*-diisopropylamino)phosphine (189 mg, 0.6 mmol, 1.0 eq). 5-Benzylthio-1*H*-tetrazole (0.3 M in MeCN, 2.5 ml, 0.75 mmol, 1.2 eq) was added slowly and the mixture was stirred at room temperature for 2 h 30 min. The solvent was removed under reduced pressure. The product was purified by wet flash column chromatography, eluting with EtOAc over silica pre-equilibrated with Et_3N. The product was co evaporated with MeCN (anhydrous, × 3) to afford the phosphoramidite **7b** as a yellow oil (204 mg, 74%). δ_P (400 MHz; d_8-THF) 146.20.

(2,4-Cyclohexadienyl-1-yl)methyl-6-{[(2-cyanoethoxy)-(diisopropylamino)phosphino]oxy}hexylcarbamate, 7c

Carbamate **6** (150 mg, 0.6 mmol, 1.0 eq) was dried overnight over P_2O_5, under vacuum. To a solution of **6** in MeCN (anhydrous, 10 ml) was added (2-cyanoethoxy)bis(*N*,*N*-diisopropylamino)phosphine (276 mg, 0.9 mmol, 1.5 eq). 5-Benzylthio-1*H*-tetrazole (0.3 M in MeCN, 2.9 ml, 0.9 mmol, 1.5 eq) was added slowly. The mixture was stirred at room temperature for 1 h. The solvent was removed under reduced pressure. The product was purified by wet flash column chromatography, eluting with EtOAc over silica pre-equilibrated with Et_3N. The product was co evaporated with MeCN (anhydrous, × 3) to afford the phosphoramidite **7c** as a yellow oil (172 mg, 64%). δ_P (400 MHz; d_8-THF) 147.19.

Solid phase synthesis of dienyl-modified oligonucleotides

Phosphoramidites **7a**, **7b** and **7c** were made up to a concentration of 0.1 M in MeCN (anhydrous) and used in the final cycle of oligodeoxyribonucleotide synthesis. The modified oligonucleotide was cleaved from the solid support by treatment with conc. NH_4OH at room temperature for 1 h. Deprotection of the oligonucleotide was achieved by incubation in conc. NH_4OH at 40 °C overnight. NH_4OH was removed under reduced pressure and the oligonucleotide was re-dissolved in H_2O (distilled, 1 ml) then purified by ion-exchange HPLC using a DNA-Pac 100 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. The purified oligonucleotide was concentrated under reduced pressure, re-dissolved in H_2O (distilled, 1 ml) then desalted using a HiTrap (5 ml) size exclusion column [3 ml min^{-1}, water (distilled)]. MALDI-TOF mass spectrometry was performed 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.

Synthesis of oligonucleotide-TAT peptide conjugates

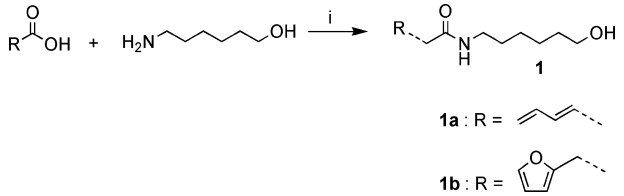
Stock solutions of unpurified dienyl-modified oligodeoxyribonucleotides were made up to concentrations of 1 mM in water (distilled). To 20 µl oligonucleotide stock solution in an Eppendorf tube was added NaOAc buffer (50 µl, 0.1 M, pH 4.8), $Cu(NO_3)_2$ (25 µl, 0.01 M), formamide (60 µl) and maleimido-YGRKKRRQRRR, (purchased from CSS-Albachem, 5 µl, 40 mM in DMF). The reaction was mixture was heated at 40 °C overnight. Purification of the oligonucleotide-TAT conjugates was by ion-exchange HPLC 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 0–100% Buffer B over 22 min. The conjugate product was collected and dialyzed. MALDI-TOF mass spectrometry was performed using a matrix of 40 mg ml^{-1} dihydroxyacetophenone in MeOH and 80 mg ml^{-1} dihydrogen ammonium citrate in water (1 : 1 v/v). ZipTip™ purification was performed prior to MALDI-TOF analysis.

Results

The aim of this study was to synthesise oligonucleotide-TAT peptide conjugates, for cell-penetration of oligonucleotides, using the Diels–Alder cycloaddition reaction. This was achieved by reaction of a series of 5′-dienyl-modified oligodeoxyribonucleotide sequences with an *N*-terminus maleimido-modified TAT peptide derivative (47–57, YGRKKRRQRRR). The dienyl moiety was incorporated into the oligonucleotide due to the relative stability of 1,3-dienes towards solid phase synthesis conditions.[36] To date, maleimide is the dominating dienophile reported for use in bioconjugation reactions using Diels–Alder cycloaddition, mainly as a result of the copious number and variety of commercially available compounds containing the maleimido moiety.

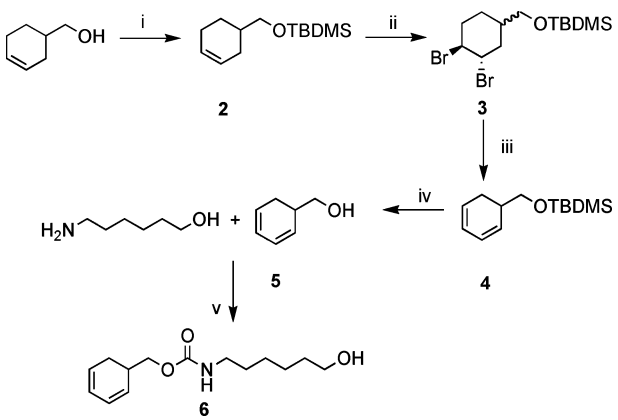
Synthesis of dienyl-modified oligonucleotides

A series of three different dienyl-modified oligodeoxyribonucleotide sequences were produced using dienyl-modified phosphoramidites to modify at the 5′-terminus. The dienyl moieties chosen for this purpose were 1,3-butadiene, furan and cyclohexadiene. For all three dienyl modifications the same 12mer oligodeoxyribonucleotide sequence was generated. In order to synthesise a phosphoramidite for 5′-modification, an alcohol is required and, in the cases of the 1,3-butadienyl and the furanyl modifications, this was achieved by amide formation, using CDI (Scheme 1).



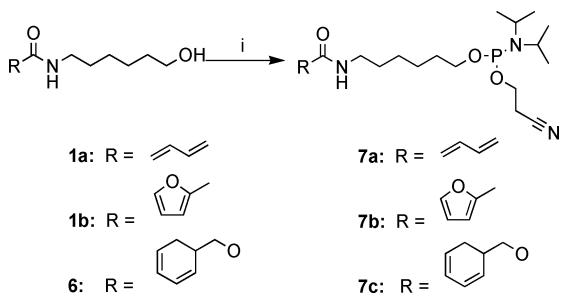
Scheme 1 (i) CDI, rt, MeCN, **1a** 70%, **1b** 30%.

In the case of the cyclohexadienyl modification, synthesis of an alcohol for phosphitylation was more complex. Preparation of alcohol **6** was achieved using a previously described method (Scheme 2).[28] The hydroxyl functionality of 3-cyclohexene-1-methanol was first protected by silylation. Treatment of **2** with molecular bromine formed a diastereomeric mixture of dibromides, **3**. A double elimination reaction, using potassium *tert*-butoxide, was then performed, generating cyclic 1,3-diene **4**. Deprotection of **4**, using a catalytic amount of acetyl chloride in anhydrous methanol,[37] afforded the cyclic 1,3-dienyl alcohol **5**. Addition of a C-6 linker to form alcohol **6** for phosphitylation was achieved by carbamate synthesis, using 6-amino-1-hexanol and CDI, as described previously for the butadienyl and furanyl modifications.



Scheme 2 (i) TBDMSCl, imidazole, DMF, 0 °C, 94%, (ii) Br_2, DCM, 0 °C, 81%, (iii) K^{t}BuO, Aliquat 336, THF, 0 °C, 45%, (iv) AcCl, MeOH, rt, 81%, (v) CDI, MeCN, r.t, 49%.

Phosphitylation of alcohols **1a**, **1b** and **6** was achieved by reaction of each of the alcohols with tetraisopropyl phosphitylating agent in the presence of a tetrazole catalyst (Scheme 3).



Scheme 3 (i) $[(^iPr)_2N]_2CN(CH_2)_2OP$, 5-benzylthio-1*H*-tetrazole, MeCN, rt, **7a** 63%, **7b** 74%, **7c** 64%.

Oligodeoxyribonucleotides 5′-X CGC ATT CAG GAT, where X is the butadienyl, furanyl or cyclohexadienyl modification, were synthesised using standard phosphoramidite methodology.[38] Deprotection of the modified oligonucleotides was achieved using conc. aq. ammonia at 40 °C, overnight. Ion-exchange HPLC analysis and purification was followed by characterisation of the modified oligonucleotides by MALDI-TOF mass spectrometry analysis (Table 1).

Table 1 Mass spectroscopic characterisation data of dienyl-modified oligonucleotides, 5′-X CGC ATT CAG GAT

5′-Modification (X)	Calcd *M*	MALDI-TOF MS Found [M – H]$^-$
	3918.6	3917.9
	3946.6	3946.7
	3960.6	3959.8

Diels–Alder cycloadditions

Diels–Alder cycloadditions for the conjugation of the dienyl-modified oligodeoxyribonucleotides to a maleimido-modified TAT peptide derivative (47–57, YGRKKRRQRRR) were carried out according to a method described previously,[39] but with the addition of copper(II) nitrate and formamide to the reaction mixture. Copper(II) nitrate was shown in work by Grondin *et al.*[40] to improve the yields of Diels–Alder cycloadditions involving benzotriazole maleimide.

This was tested using crude 5′-furanyl-modified oligonucleotide and fluorescein maleimide. It was found that the yield of oligonucleotide fluorescein conjugate was greatly improved when the cycloaddition was performed in the presence of copper(II) nitrate (see ESI), with complete conversion to the conjugate being observed. Turner *et al.* recommend the use of formamide as a denaturing agent in conjugations of oligonucleotides to highly cationic peptides.[41] It was found that formamide was a necessary component in the cycloadditions involving TAT peptide; in the absence of the denaturing agent the reaction did not proceed at all. Solutions of crude 5′-dienyl-modified oligodeoxyribonucleotides were used; purification was performed after conjugation to the TAT peptide derivative. Optimal conditions for the cycloadditions were found to be heating at 40 °C overnight, and the use of 10 equivalents of the maleimido-modified TAT peptide derivative. HPLC analysis and purification for characterisation was performed using ion-exchange chromatography (Resource Q column), and under highly denaturing conditions (50% formamide),[41] followed by dialysis. Characterisation of the oligonucleotide TAT peptide conjugates was achieved by MALDI-TOF mass spectrometry analysis (Table 2). It was found that solutions of the conjugates for analysis by MALDI-TOF MS had first to be purified using ZipTip™C_{18} purification pipette tips and that the best conditions for analysis were in positive mode.

Table 2 Mass spectroscopic characterisation data of maleimido TAT oligonucleotide peptide conjugates, TAT 5′-X CGC ATT CAG GAT

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

Diels–Alder cycloaddition for the conjugation of sequence 5′-X CGC ATT CAG GAT to the maleimido-modified TAT peptide derivative was first performed was using the 1,3-butadienyl modification. HPLC analysis (Fig. 1(B)) of the conjugation mixture, after heating at 40 °C overnight, showed a new peak with shorter retention time than that of the unconjugated oligonucleotide (Fig. 1(A)), indicating formation of the oligonucleotide TAT peptide conjugate; conjugation of the cationic peptide to the oligonucleotide should neutralise some of the anionic charges carried on the phosphate backbone, thereby reducing the overall charge of the conjugate when compared to the free oligonucleotide. However, the yield of the cycloaddition was low, with only 23% (based on the ratio of peak areas) conversion to the conjugate produced. Formation of the oligonucleotide TAT conjugate was confirmed by MALDI mass spectrometry analysis (Table 2).

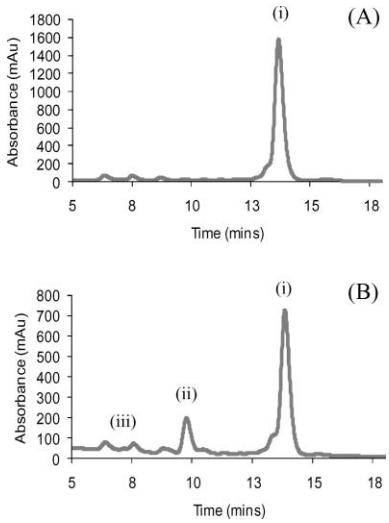


Fig. 1 Ion-exchange HPLC traces of 5′-butadienyl-modified oligonucleotide and oligonucleotide TAT peptide conjugate at 260 nm. (A) Starting material; (i) 5′-X CGC ATT CAG GAT, (B) Diels–Alder reaction mixture; (i) starting material 5′-X CGC ATT CAG GAT, (ii) TAT 5′-X CGC ATT CAG GAT conjugate, (iii) failure sequences.

On repeating the conjugation reaction under identical conditions, but using the furanyl modification, by HPLC analysis (Fig. 2(B)), the yield of the cycloaddition was visibly improved, with conversion to the conjugate increasing to 33% (based on the ratio of peak areas). It was thought that a contributory factor in the increased yield of oligonucleotide TAT conjugate using furanyl-modified oligonucleotide could be the fixed *cis*-conformation of the heterocyclic diene.

Conjugation of sequence 5′-X CGC ATT CAG GAT to the maleimido-modified TAT peptide derivative was repeated once more, using the cyclohexadienyl modification. The cyclic nature of this third modification led again to a fixed *cis*-conformation of the diene, but this time the modification was not in possession of any aromatic character. Once more, the cycloaddition reaction was performed with heating at 40 °C overnight. The following HPLC analysis (Fig. 3(B)) of the conjugation mixture showed a significant increase in yield of the oligonucleotide TAT conjugate, when compared with the butadienyl and furanyl oligonucleotide modifications, with 72% (based on the ratio of peak areas) conversion of the oligonucleotide to the conjugate being achieved.

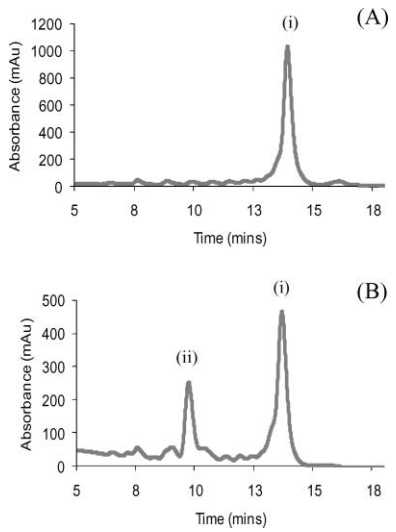


Fig. 2 Ion-exchange HPLC traces of 5′-furanyl-modified oligonucleotide and oligonucleotide TAT peptide conjugate at 260 nm. (A) Starting material; (i) 5′-X CGC ATT CAG GAT, (B) Diels–Alder reaction mixture; (i) starting material 5′-X CGC ATT CAG GAT, (ii) TAT 5′-X CGC ATT CAG GAT conjugate.

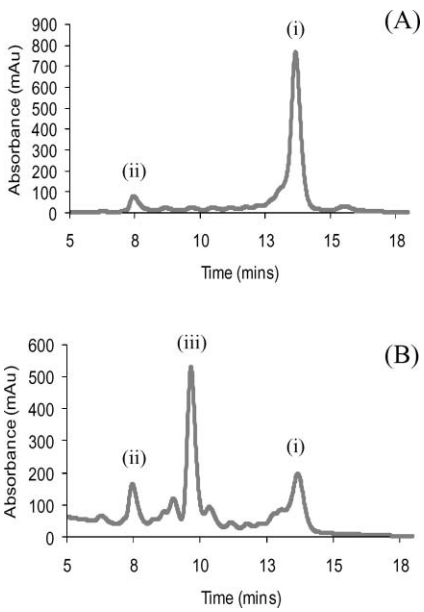


Fig. 3 Ion-exchange HPLC traces of 5′-cyclohexadienyl-modified oligonucleotide and oligonucleotide TAT peptide conjugate at 260 nm. (A) Starting material; (i) 5′-X CGC ATT CAG GAT, (ii) failure sequences, (B) Diels–Alder reaction mixture; (i) starting material 5′-X CGC ATT CAG GAT, (ii) failure sequences, (iii) TAT 5′-X CGC ATT CAG GAT conjugate.

A sample of TAT 5′-X CGC ATT CAG GAT, where X = cyclohexadiene, was heated over a period of 5 hours at 37 °C and monitored by HPLC to see if it was possible that a retro-Diels–Alder reaction could occur, resulting in cleavage of the oligonucleotide from the peptide. No evidence of this was observed.

Conclusions

A series of 5′-dienyl-modified oligonucleotides have been generated by synthesis of different dienyl-modified phosphoramidites. The data shows that the best results for conjugation of these oligonucleotides to a maleimido-modified TAT peptide derivative, using the Diels–Alder reaction, were achieved when using 5′-cyclohexadienyl-modified oligonucleotide. This result has important implications for the synthesis of oligonucleotide peptide conjugates; the Diels–Alder reaction is fast, chemoselective and is effective in aqueous conditions, suitable to the biological natures of both oligonucleotides and peptides; it is also a significant development in the use of the Diels–Alder reaction for conjugation of oligonucleotides to meaningful peptides, such as cell-penetrating peptides.

References

1 N. Dias and C. A. Stein, *Mol. Cancer Ther.*, 2002, **1**, 347.
2 D. A. Braasch and D. R. Corey, *Biochemistry*, 2002, **41**, 4503.
3 X. Chen, N. Dudgeon, L. Shen and J. H. Wang, *Drug Discovery Today*, 2005, **10**, 587.
4 F. Debart, A. Saïd, G. Deglane, H. M. Moulton, P. Clair, M. J. Gait, J.-J. Vasseur and B. Lebleu, *Curr. Top. Med. Chem.*, 2007, **7**, 727.
5 U. Englisch and D. H. Gauss, *Angew. Chem., Int. Ed. Engl.*, 1991, **30**, 613.
6 Z. Zhu, J. Chao, H. Yu and A. S. Waggoner, *Nucleic Acids Res.*, 1994, **22**, 3418.
7 A. Castro and J. G. K. Williams, *Anal. Chem.*, 1997, **69**, 3915.
8 C. Wojczewski, K. Stolze and J. W. Engels, *Synlett*, 1999, **10**, 1667.
9 C. F. Bennett, M.-Y. Chiang, H. Chan and J. E. E. Shoemaker, *Mol. Pharmacol.*, 1992, **41**, 1023.
10 J. J. Turner, G. D. Ivanova, B. Verbeure, D. Williams, A. A. Arzumanov, S. Abes, B. Lebleu and M. J. Gait, *Nucleic Acids Res.*, 2005, **33**, 6837.
11 S. Resina, S. Abes, J. J. Turner, P. Prevot, A. Travo, P. Clair, M. J. Gait, A. R. Thierry and B. Lebleu, *Int. J. Pharm.*, 2007, **344**, 96.
12 M. Green and P. M. Lowenstein, *Cell*, 1988, **55**, 1179–1188.
13 A. Joliot, C. Pernelle, H. Deagostini-Bazin and A. Prochiantz, *Proc. Natl. Acad. Sci. U. S. A.*, 1991, **88**, 1864.
14 G. Elliot and P. O'Hare, *Cell*, 1997, **88**, 223.
15 E. Vivès, P. Brodin and B. Lebleu, *J. Biol. Chem.*, 1997, **272**, 16010.
16 D. Derossi, A. H. Jolito, G. Chassaing and A. Prochiantz, *J. Biol. Chem.*, 1994, **269**, 10444.
17 G. P. H. Dietz and M. Bähr, *Mol. Cell. Neurosci.*, 2004, **27**, 85.
18 K. M. Stewart, K. L. Horton and S. O. Kelley, *Org. Biomol. Chem.*, 2008, **6**, 2242.
19 D. Lochmann, E. Jauk and A. Zimmer, *Eur. J. Biopharm.*, 2004, **58**, 237.
20 B. G. de la Torre, A. M. Avino, G. Tarrason, M. Royo, F. Albericio and R. Eritja, *Tetrahedron Lett.*, 1994, **35**, 2733.
21 E. Zubin, E. A. Romanova and T. S Oretskaya, *Russ. Chem. Rev.*, 2002, **71**, 239.
22 C.-H. Tung and S. Stein, *Bioconjugate Chem.*, 2000, **11**, 605.
23 N. Venkatsen and B. H. Kim, *Chem. Rev.*, 2006, **106**, 3712.
24 O. Diels and K. Alder, *Liebigs Ann. Chem.*, 1929, **470**, 62.
25 R. Breslow and D. C. Rideout, *J. Am. Chem. Soc.*, 1980, **102**, 7816.
26 C.-J Li and L. Chen, *Chem. Soc. Rev.*, 2006, **35**, 68.
27 J. B. F. N. Egberts, *Pure Appl. Chem.*, 1995, **67**, 823.
28 K. W. Hill, J. Taunton-Rigby, J. D. Carter, E. Kropp, K. Vagle, W. Pieken, D. P. C. McGee, G. M. Husar, M. Leuck, D. J. Anzanio and D. P. Sebesta, *J. Org. Chem.*, 2001, **66**, 5352.
29 L. Fruk, A. Grondin, W. E. Smith and D. Graham, *Chem. Commun.*, 2002, **18**, 2100.
30 D. Graham, A. Grondin, C. MacHugh, L. Fruk and W. E. Smith, *Tetrahedron Lett.*, 2002, **43**, 4785.
31 D. Graham, L. Fruk and W. E. Smith, *Analyst*, 2003, **128**, 692.
32 R. Tona and R. Häner, *Bioconjugate Chem.*, 2005, **16**, 837.
33 E. Anderson and D. Picken, *Nucleosides, Nucleotides, Nucleic Acids*, 2005, **24**, 761.

34 H. A. Lathan-timmons, A. Wolter, J. S. Roach, R. Giare and M. Leuck, *Nucleosides, Nucleotides, Nucleic Acids*, 2003, **22**, 1495.
35 V. Marchán, S. Ortega, D. Pulido, E. Pedroso and A Grandas, *Nucleic Acids Res.*, 2006, **34**, e24.
36 A. Enright and D. Graham, *Curr. Org. Synth.*, 2006, **3**, 175.
37 T. A. Khan and E. Mondal, *Synlett*, 2003, **5**, 694.
38 M. H. Caruthers, S. L. Beaucage, C. Becker, W. Efcavitch, E. F. Fischer, G. Gallupi, R. Goldman, P. deHaseth, F. Martin, M. Matteucci and Y. Stabinsky, in *Genetic Engineering: Principles and Methods*, ed. J. K. Setlow and A. Hollaender, Plenum, New York, 1982, vol. 4, pp. 1–17.
39 M. Leuck and A. Wolter, in *Current Protocols in Nucleic Acid Chemistry*, ed. S. L. Beaucage, D. E. Bergstrom, G. D. Glick and R. A. Jones, John Wiley and Sons, Inc., vol. 2, pp. 4.18.11–4.18.14.
40 A. Grondin, D. C. Robson, W. E. Smith and D. Graham, *J. Chem. Soc., Perkin Trans. 2*, 2001, 2136.
41 J. J. Turner, A. A. Arzumanov and M. J. Gait, *Nucleic Acids Res.*, 2005, **33**, 27.