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Lipophilic Modification of Oligonucleotides

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Abstract: Methods for attaching lipophilic side-chains to an amino-modified uracil base in oligonucleotides *via* orthogonal amide and disulphide linkages are described. The oligonucleotide conjugates are characterised in terms of their distinctive chromatographic and spectroscopic properties.

The modification of oligonucleotides by attachment of a lipophilic residue can confer increased duplex stability[1] and enhanced nuclease resistance.[2] Oligonucleotides conjugated to lipophilic compounds have been shown to be taken up into the cell more efficiently,[3] exhibit better antiviral activity[4,5,6] and be more efficacious in antisense experiments.[5,7] Modifications reported include attachment of cholesterol,[3,4,6,7,8] vitamin E,[9] cholic acid,[2] and alkyl groups.[1,5,10] These studies generally involve attachment of the lipophilic group to the 3'- or 5'-terminus of the oligonucleotide. Attachment to the 2'-hydroxyl on the ribose ring[10] and modification of the phosphate group have also been reported.[1] In this paper we describe an alternative approach to preparing oligonucleotide conjugates by attaching the lipophilic group to a preformed oligonucleotide incorporating a modified nucleobase.

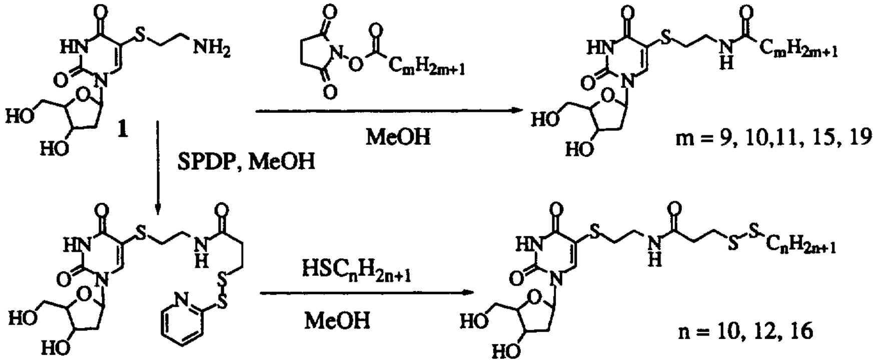
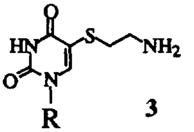


Figure 1. Conjugation of **1** with lipophilic side-chains *via* amide and disulphide linkages

To establish the coupling protocols the modification reactions were initially carried out on the unprotected monomer **1**. This 5-amino-modified 2'-deoxyuridine incorporating a short thioether side-chain was synthesised according to the procedure of Bergstrom *et al.*.[11] It was coupled to hydrophobic side-chains by one of two orthogonal approaches, involving either amide or disulphide bond formation (Figure 1). The amides were made by reacting a series of *N*-hydroxysuccinimide esters (m = 9, 10, 11, 15, 19) directly with the primary amine **1** in methanol. The disulphides were made by initial reaction with the bifunctional linker 3-(2-pyridyldithio)propionic acid *N*-hydroxysuccinimide ester (SPDP),[12] followed by reaction with thiols (n = 10, 12, 16), with concomitant loss of thiopyridone.[13]

Following protection of the 5'-hydroxyl with a dimethoxytrityl group and activation of the 3'-hydroxyl as the phosphoramidite, the trifluoracetamide of nucleoside **1** was incorporated[14] centrally into the 17mer oligonucleotide **3** of sequence 5'-ACAAAAGCXGGAGCTCC-3'.[15] The commercially available phosphoramidite **2**[16] was similarly used to make the corresponding oligonucleotide **4**.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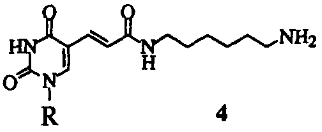
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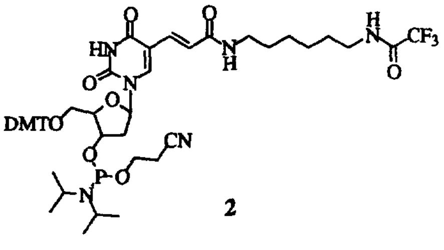
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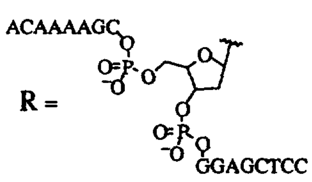
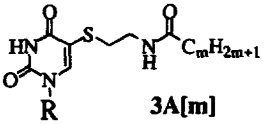
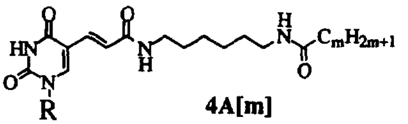
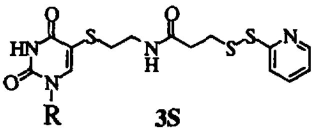
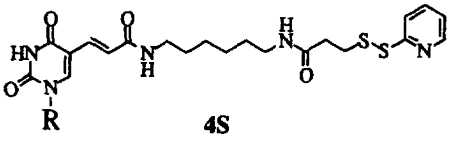


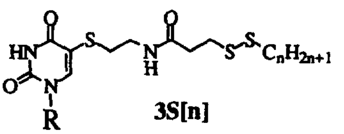
The couplings carried out on the monomer **1** (Figure 1) were adapted[17] for the two amino-modified oligonucleotides **3** and **4** to give the two series of amides **3A[m]** and **4A[m]**, the SPDP coupled products **3S** and **4S** and the derived conjugates **3S[n]** and **4S[n]** (where m and n indicate the number of carbons in the linear alkyl chain)

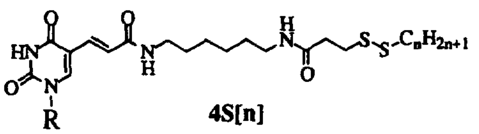

3A[m]


4A[m]


3S


4S


3S[n]


4S[n]

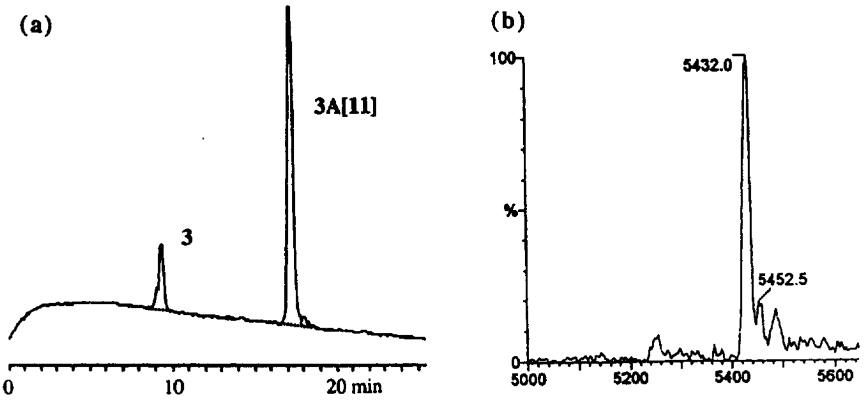
The oligonucleotide coupling products were purified by spun-column chromatography[18] and analysed by reverse phase HPLC.[19] Retention times of the oligonucleotide conjugates on the reverse phase column increased markedly with the length of the attached alkyl chain. Product peaks were collected, evaporated to dryness and analysed by electrospray mass spectrometry (ESMS).[20] It was noted that increasing the length of the alkyl chain in the modified oligonucleotide side-chain resulted in large systematic increases in the time taken for the signal for the oligonucleotide conjugate to be detected in the mass spectrometer. This effect was so pronounced that with alkyl chains longer than 14 carbons, product signals took over 20 min to appear and became too weak to detect. The signal for unmodified oligonucleotides typically took 3–5 min. to appear. Figure 2 shows an example of an HPLC trace with the corresponding ESMS spectrum of the purified product. Table 1 shows the data for all the oligonucleotide conjugates prepared.

Table 1. Characterisation of Oligonucleotide Products by HPLC and ESMS.

Oligonucleotide	HPLC retention time (min.)	% Recovery[a]	% Yield[b]	Expected Mass[c]	Observed Mass (± std. devn.)
3	9			5246	5249±2
3A[9]	14	72	85	5400	5401±2
3A[11]	17	63	95	5428	5432±2
3A[13]	22	56	78	5456	5460±1
3A[15]	26	53	47	5484	-
3A[17]	29	94	5	5512	-
3A[19]	-	73	0	5540	-
3S	11	80	47	5443	5441±5
3S[10]	22	42	60	5506	5505±6
3S[12]	25	57	53	5534	5531±8
3S[16]	32	37	18	5590	-
4	10			5339	5339±3
4A[9]	17	62	97	5493	5493±4
4A[11]	22	77	98	5521	5520±7
4A[13]	26	67	97	5549	-
4A[15]	29	93	94	5577	-
4A[17]	32	75	79	5505	-
4A[19]	35	70	28	5633	-
4S	12	100	44	5536	5530±12
4S[10]	25.4 / 25.6[d]	72	57	5599	5601±9
4S[12]	29.0 / 29.6[d]	62	67	5627	-
4S[16]	35.5 / 36.2[d]	65	42	5683	-

Notes (a) Percentage oligonucleotide recovered after spun-column chromatography based on amount of 3 or 4 used. (b) Product as a percentage of total oligonucleotide recovered. (c) Calculated for the monoanion. (d) These reactions gave two unresolvable peaks in the HPLC. These were treated as a single entity for ESMS analysis.

Figure 2 (a) HPLC trace for purification of 3A[11]. (b) ESMS of 3A[11] after HPLC.



The use of two orthogonal approaches allows two different lipids to be attached to a doubly modified oligonucleotide. The methodology described in this paper is now being generalised to the synthesis of oligonucleotide conjugates with carbohydrates and proteins.

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13. Products were characterised by ^{1}H NMR, infrared and ultraviolet spectroscopy, high resolution mass spectrometry, and melting point.
14. Oligonucleotides **3** and **4** were synthesised by Oswel DNA Service based at the University of Southampton, on an Applied Biosystems ABI 394 DNA synthesiser, using phosphoramidite chemistry.
15. This sequence was chosen to hybridise to one end of the polylinker sequence of pBluescript II SK$^-$, however for this study it should just be considered a typical oligonucleotide.
16. Amino-Modifier C6 dT available from Glen Research.
17. a) The couplings to the *N*-hydroxysuccinimide esters were performed by heating 20 μg of oligonucleotide **3** or **4** in 20 μl HEPES buffer (0.1 M, pH 8.2) with 100 μg ester (dissolved in 20 μl acetonitrile) at 50 °C for 16 h.

    b) The couplings to SPDP were carried out by reacting 40 μg of oligonucleotide **3** or **4** in 20 μl HEPES buffer (0.1 M, pH 8.2) with 100 μg of SPDP (dissolved in 20 μl acetonitrile) at room temperature for 16 h. The product was purified by spun column chromatography and then diluted with 60 μl HEPES buffer (0.1 M, pH 8.2). An aliquot (20 μl) of this solution was reacted with each thiol (100 μg dissolved in 20 μl acetonitrile) at room temperature for 96 h.
18. The samples were loaded onto 1 ml Sephadex G-25 columns with milli Q water as eluant, following the method of Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning A Laboratory Manual;* Cold Spring Harbor Laboratory Press, 1989; pp. E.37-E.38.
19. A Tosahaas TSKgel OligoDNA RP column (4.6 mm x 15 cm) was used on a Gilson HPLC system with the following gradient of acetonitrile in 0.1 M ammonium acetate pH 7.0: 0-2 min., 5%; 2-32 min., 5-50%; 32-43 min., 50%; 43-44 min., 50-5%; 44-45 min., 5%. The flow rate was 1 ml/min.
20. ESMS data were acquired on a VG BioQ quadrupole mass spectrometer used in negative ion mode. Samples were run in 100:100:1 water:acetonitrile:880 ammonia.

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