

# Plant derived cholesterol modifications: Comparative use in oligonucleotide synthesis

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## Introduction

The introduction of hydrophobic residues into oligonucleotides with a view to improving their penetration into cells is an idea that has recently met with some success.<sup>1</sup> Cholesteryl-conjugated oligonucleotides have in particular been the subject of substantial interest in antisense and other studies due to the lipophilicity and good availability of cholesterol. One such study,<sup>2</sup> for example, has shown the use of cholesteryl-modified siRNA in therapeutic gene silencing.

Historically this has been done by post-synthetic conjugation of an amino-modified oligo to cholesterol chloroformate,<sup>3</sup> however direct attachment during synthesis is much more convenient. 5'-Attachment is possible *via* a modified phosphoramidite and various products

have been proposed.<sup>4,5,6</sup> Similarly, 3'-modification can be achieved by an analogous cholesterol-CPG.

Despite the commercialisation of cholesterol products to date, the strict guidelines imposed by regulatory authorities now make it essential to use non-animal based products in pharmaceutical drug and diagnostics development for humans.

After considerable investigation of competing products<sup>7</sup>, we now present three optimised cholesterol products manufactured entirely from plant derived cholesterol: two 5'-cholesterol phosphoramidites (**2170**, **2189**); and a 3'-cholesterol solid support (**2394**).

5'-Cholesterol-CE Phosphoramidite (**2170**) and 5'-Cholesterol-TEG-CE Phosphoramidite (**2189**) have the advantage that they are not susceptible to 1,2-diol elimination and are therefore completely stable during deprotection. There is therefore no need for DMTr protection which can interfere with the purification of oligonucleotides modified with such hydrophobic groups. 3'-Cholesterol SynBase™ CPG 1000 (**2394**) is similarly stable to deprotection and does not undergo 1,2-diol elimination. In fact, the dR backbone allows the cholesterol modification to fit directly into the natural sugar phosphate backbone hence there are no adverse structural effects on the duplex oligonucleotide.

The coupling efficiencies of the cholesterol phosphoramidites have proved to be better than analogous commercially available cholesterol modifications.

1 See for example: **Modification of antisense phosphodiester oligodeoxynucleotides by a 5' cholesteryl moiety increases cellular association and improves efficacy**, A.M. Krieg, K. Tonkinson, S. Matson, Q. Zhao, M. Saxon, L.-M. Zhang, U. Bhanja, L. Yakubov and C.A. Stein, *Proc. Natl. Acad. Sci. USA*, **90**, 1048-1052, 1993; and **Cholesterol conjugated oligonucleotide and LNA: A comparison of cellular and nuclear uptake by Hep2 cells enhanced by Streptolysin-O**, Š. Holasová, M. Mojžišek, M. Bunček, D. Vokurková, H. Radilová, M. Šafařová, M. Červinka and R. Haluza, *Molecular and Cellular Biochem.*, **276**, 61-69, 2005.

2 **Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs**, J. Soutschek, A. Akinc, B. Bramlage, K. Charisse, R. Constien, M. Donoghue, S. Elbashir, A. Geick, P. Hadwiger, J. Harborth, M. John, V. Kesavan, G. Lavine, R.K. Pandey, T. Racie, K.G. Rajeev, I. Röhl, I. Toudjarska, G. Wang, S. Wuschko, D. Bumcrot, V. Kotliansky, S. Limmer, M. Manoharan and H.-P. Vornlocher, *Nature*, **432**, 173-178, 2004.

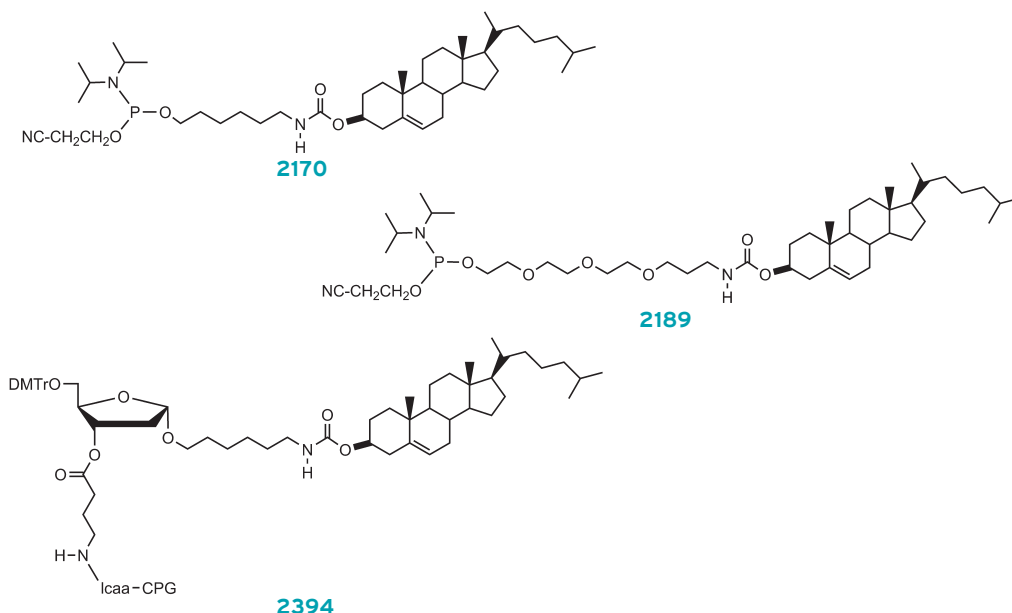
3 See for example: (a) **Cholesteryl-conjugated oligonucleotides: synthesis, properties, and activity as inhibitors of replication of human immunodeficiency virus in cell culture**, R.L. Letsinger, G. Zhang, D.K. Sun, T. Ikeuchi and P.S. Sarin, *Proc. Natl. Acad. Sci.*, **86**, 6553-6556, 1989; and (b) **A simplified synthesis of acridine and/or lipid containing oligodeoxynucleotides**, C.J. Marasco, Jr., N.J. Angelino, B. Paul and B.J. Dolnick, *Tetrahedron Lett.*, **35**, 3029-3032, 1994. Other methods, such as conjugation *via* a disulphide bond to terminal phosphate groups, have also been used. See for example: **Antisense effects of cholesteryl-oligonucleotide conjugates associated with poly(alkylcyanoacrylate) nanoparticles**, G. Godard, A.S. Boutorine, E. Saison-Behmoaras and C. Héline, *Eur. J. Biochem.*, **232**, 404-410, 1995.

4 **Assembling liposomes by means of an oligonucleotide tagged with a lipophilic unit**, N. Maru, K. Shohda and T. Sugawara, *Nucleic Acids Symposium Series No. 48*, 95-96, 2004.

5 **Synthesis and physical properties of anti-HIV antisense oligonucleotides bearing terminal lipophilic groups**, C. MacKellar, D. Graham, D.W. Will, S. Burgess and T. Brown, *Nucleic Acids Research*, **20**, 3411-3417, 1992.

6 **Mode of action of 5'-linked cholesteryl phosphorothioate oligodeoxynucleotides in inhibiting syncytia formation and infection by HIV-1 and HIV-2 in vitro**, C. A. Stein, Ranajit Pal, A. L. DeVico, G. Hoke, S. Mumbauer, O. Kinstler, M. G. Sarngadharan and R. L. Letsinger, *Biochemistry*, **30**, 2439 - 2444, 1991.

7 **Plant derived cholesterol modifications: A comparison of commercially available cholesterol phosphoramidites and solid supports for use in oligonucleotide synthesis of DNA and RNA (TC and TBDMS chemistries)**, S. Aitken, U. Ixkes, C. McKeen and D. Picken, originally presented as a poster at TIDES 2010, Boston. Copies are available at [www.linktech.co.uk](http://www.linktech.co.uk) or by request.



These products are compatible with automated standard and modified DNA and RNA synthesis and although **2170** is soluble in dichloromethane rather than acetonitrile this in fact proved to have the best coupling efficiency out of all commercially available cholesterol amidites. For all our cholesterol amidites there is no requirement for the use of mixed solvents since the TEG-based **2189** is readily soluble in acetonitrile.

Finally, these products can be used without IP restrictions.

A comparison of deprotection conditions of oligonucleotides functionalised with these modifications has been undertaken. In particular, degradation during deprotection was investigated where the cholesterol modification can potentially be lost *via* cleavage at the carbamate bond (**all**), or the anomeric position (**2394**). The optimum deprotection conditions for each of these modifications were then determined.

## Experimental

### Synthesis Conditions

Syntheses were carried out on either an Expedite 8909 or an ABI 394 DNA/RNA automated synthesiser using the following reagent specifications. All amidites were used at 0.1M concentrations. Standard amidites [Bz-dA (**2003**), dmf-dG (**2030**), Ac-dC (**2034**), and dT (**2001**)], TBDMS-RNA amidites [Bz-A (**2036**), dmf-G (**2033**), Ac-C (**2038**), and U (**2040**)] and 5'-Cholesterol TEG phosphoramidite (**2189**) were dissolved in anhydrous acetonitrile (**4050**). 5'-Cholesterol phosphoramidite (**2170**) was dissolved in anhydrous, alcohol-free dichloromethane, and TC-RNA amidites [Bz-A (**2179**), iBu-G (**2178**), Ac-C (**2180**), and U (**2177**)] were dissolved in anhydrous toluene (**4055**). Solid supports used throughout were 1 $\mu$ mol 1000Å SynBase columns derivatised with either dT (**2271**) or 3'-Cholesterol (**2394**).

For all oligonucleotides, the following ancillary reagents were used: 0.25M and 0.5M ETT (for DNA and RNA respectively) (activator, **3140** and **3145**), THF/pyridine/acetic anhydride (8:1:1) (Cap A, **4110**), 10% Methylimidazole in THF (Cap B, **4120**), 0.02M iodine in THF/pyridine/water (89.6:0.4:10) (oxidiser, **4132**) and 3% TCA/DCM (deblock, **4140**)

Standard coupling times were used for DNA amidite couplings, 15min for all cholesterol amidite couplings and TBDMS-RNA amidite couplings, and 3min for all TC-RNA amidite couplings.

The same sequence was used for all oligonucleotides:

TTC GGC TTG TCC GTG GAA TCT CAC AGC TTA T (DNA)  
UUC GGC UUG UCC GUG GAA UCU CAC AGC UUA T (RNA)

with the cholesterol modification at either the 3' or 5' end as appropriate. Unmodified controls were also synthesised and used in HPLC analysis to identify the retention time of the unlabelled oligonucleotide.

### Deprotection Conditions

The deprotection conditions used in the study are shown in Table 1. To avoid any complications in terms of RNA degradation, RNA deprotections were initially carried out on the corresponding DNA oligonucleotide and the results confirmed with deprotection on the RNA oligonucleotide.

Table 1. Deprotection conditions

ID	Conditions	Temp /°C	Time /min
A	AMA	65	10
B	AMA	RT	120
C	Ammonia	55	240
D	Et <sub>3</sub> N/Et <sub>3</sub> N:3HF/NMP (1.5:2:3)	65	150
E	EDA/Toluene (1:1)	RT	120

## Analysis Conditions

All oligonucleotides were analysed in their crude form and for each modification HPLC and MS data are shown for oligonucleotides deprotected with AMA, 65°C, 10min. LCMS data shown corresponds to RNA oligonucleotides synthesised *via* TC-RNA chemistry and deprotection conditions of EDA/toluene 1:1, RT, 2h.

RP-HPLC was carried out using an Agilent Eclipse XBD-C18 5µm, 4.6x150mm column using buffer A: 0.1M TEAA and buffer B: 100% MeCN over a gradient of 0-100% B over 27.5min at a flow rate of 1ml/min.

MALDI-TOF mass spectrometry was carried out on a Bruker Ultraflex. Eurogentec S.A (Liege, Belgium) provided this service.

LCMS data was supplied by Zoltan Timar of Agilent Technologies, Inc. (Boulder, CO, US).

## Results & Discussion

HPLC analysis on the crude oligonucleotides showed the presence of one, or a combination of the following adducts which were confirmed by mass spectrometry (MS).

- ~8.5min: Unlabelled DMT-OFF RNA oligonucleotide and/or the DMT-OFF RNA oligonucleotide where cleavage at the carbamate bond has taken place. A in Tables 2 to 4 below.
- ~9.5min: Unlabelled DMT-OFF DNA oligonucleotide and/or the DMT-OFF DNA oligonucleotide where cleavage at the carbamate bond has taken place. A in Tables 2 to 4 below.
- ~12.5min: Unlabelled DMT-ON oligonucleotide and/or the DMT-ON oligonucleotide where cleavage at the carbamate bond has taken place. B in Tables 2 to 4 below.
- ~19min: Cholesterol labelled DNA or RNA oligonucleotide (DMT-OFF). C in Tables 2 to 4 below.

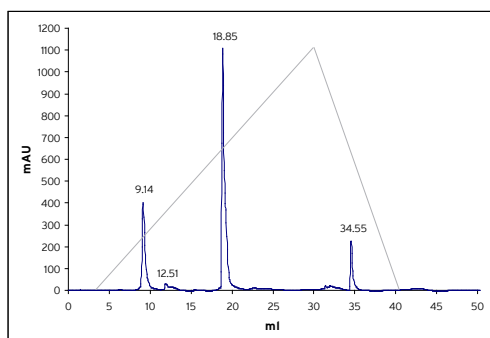


Figure 1. HPLC trace of DNA-oligonucleotide modified with 5'-Cholesterol-CE Phosphoramidite (**2170**), deprotected in AMA, 65°C, 10min.

- ~22min: Cholesterol labelled DNA or RNA oligonucleotide (DMT-ON). C in Tables 2 to 4 below.

In all cases there was a peak around 34min that appears to be product sticking to the HPLC column and being eluted off after 100% acetonitrile had been passed through the column, this is not included in the data shown below.

Where a modification has no DMT protection, this is indicated as 'NA' in the 'DMT' column. Note that since the matrix used in the MALDI-TOF is acidic, and the DMT group therefore removed, the calculated and observed peaks refer to the DMT-OFF product even when the oligonucleotide was synthesised DMT-ON. Where LCMS (ES) data is given the DMT-ON and DMT-OFF values are shown.

## 5'-Cholesterol-CE Phosphoramidite (**2170**)

Since this is based on the standard 5'-C6 linker, the only possible degradation product is cleavage across the carbamate bond.

The data showed that **2170** is compatible with all conditions shown in Table 1 and is therefore compatible with both DNA and RNA chemistries. The optimum deprotection conditions were shown to be AMA, RT, 2h (DNA) and EDA/toluene 1:1, RT, 2h (RNA-TC). The unlabelled oligonucleotide was a result of either failures in synthesising the oligonucleotide itself or incomplete coupling during the addition of the modification. In all cases MS indicated that there was negligible cleavage across the carbamate bond.

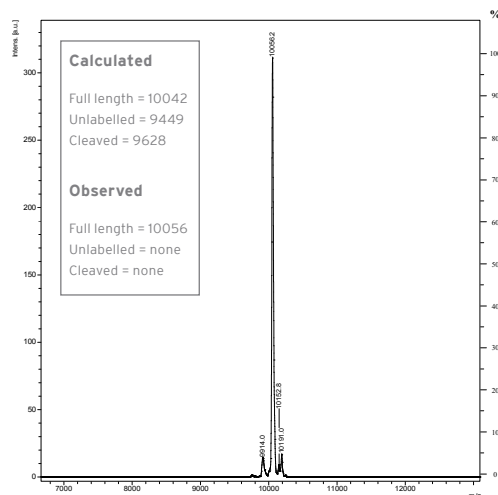


Figure 2. MALDI-TOF MS of DNA-oligonucleotide modified with 5'-Cholesterol-CE Phosphoramidite (**2170**), deprotected in AMA, 65°C, 10min.

Table 2. Synthesis results for 5'-Cholesterol-CE Phosphoramidite (**2170**)

Chemistry	Dep <sup>n</sup> Method ID	DMT	% A	% B	% C	Calc. MW	Obs. MW (major peak)
DNA	A	NA	26.29	1.00	72.71	10042	10056
DNA	B	NA	13.67	1.29	85.03	10042	10073
DNA	i. B	NA	13.67	1.29	85.03	10042	10073
	ii. D		19.35	3.33	77.32		10055
DNA	E	NA	19.80	0.00	80.20	10042	10161
DNA	i. A	NA	11.16	0.00	88.84	10042	10056
	ii. D		11.75	1.41	86.84		10045
RNA-TC	E	NA	32.62	0.50	66.89	10392	10406
RNA-TBDMS	i. A	NA	70.73	0.90	28.67	10392	10408
	ii. D						

**3'-Cholesterol SynBase™ CPG 1000/110 (2394)**

This modification is based on the deoxyribose backbone and the only possible degradation products are cleavage across the carbamate bond or at the anomeric position.

Prior to starting the synthesis cycle, the 3'-cholesterol columns underwent an additional detritylation step to ensure that the resin was fully detritylated prior to monomer addition.

The oligonucleotides were synthesised both DMT-OFF and DMT-ON although since the modification occurs at the 3'-end no difference in the results was expected. This was not the case and a reproducible pattern was observed in that the DMT-ON oligonucleotides result in a purer full-length product. However the difference in the percentage of full-length product is only

around 5% therefore the benefits of synthesising the oligonucleotide DMT-OFF (i.e. solubility, purification, no post-synthetic detritylation) outweigh the slight increase in the percentage of full-length product obtained.

The results indicate that **2394** is stable to all the deprotection conditions used and is compatible with both TC and TBDMS-RNA chemistries. The optimum deprotection conditions were shown to be AMA, RT, 2h (DNA) and EDA/toluene 1:1, RT, 2h (RNA-TC). In all cases there was no evidence of any cleavage at the anomeric position and negligible cleavage across the carbamate bond with the exception of the use of EDA/toluene 1:1, RT, 2h with the DNA oligonucleotide where 14% was observed while the predominant component was the desired product.

Table 3. Synthesis results for 3'-Cholesterol SynBase™ CPG 1000/110 (**2394**)

Chemistry	Dep <sup>n</sup> Method ID	DMT	% A	% B	% C	Calc. MW	Obs. MW (major peak)
DNA	A	ON	0.61	4.92	94.46	10157	10175
DNA	A	OFF	9.21	0.67	90.11	10157	10181
DNA	B	OFF	1.71	7.28	91.02	10157	10172
DNA	C	OFF	8.36	0.00	91.64	10157	10178
DNA	i. C	ON	11.23	2.56	86.21	10157	10170
	ii. D		17.51	0.00	82.49		10175
DNA	i. C	OFF	8.36	0.00	91.60	10157	10178
	ii. A		2.76	7.03	90.21		10175
DNA	i. A	OFF	9.21	0.67	90.11	10157	10181
	ii. D		15.78	0.98	83.27		10175
DNA	i. A	OFF	0.61	4.92	94.46	10157	10175
	ii. E		0.00	9.15	90.85		10159
DNA	i. B	OFF	1.70	7.28	91.02	10157	10172
	ii. D		6.04	4.79	89.17		10159
DNA	E	ON	0.00	14.20	85.80	10157	10159
RNA-TC	E	ON	2.97	2.42	94.61	10511 (off)	10515 <sub>(MALDI)</sub>
							10511 <sub>(LCMS)</sub>
						10813 (on)	10813 <sub>(LCMS)</sub>
RNA-TBDMS	i. A	OFF	37.69	0.00	62.31	10201	10216
	ii. D						

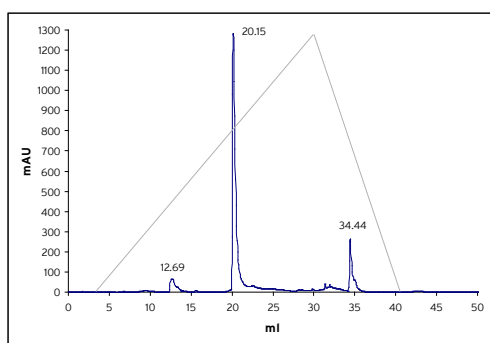


Figure 3. HPLC trace of DNA-oligonucleotide modified with 3'-Cholesterol SynBase™ CPG 1000/110 (**2394**), deprotected in AMA, 65°C, 10min.

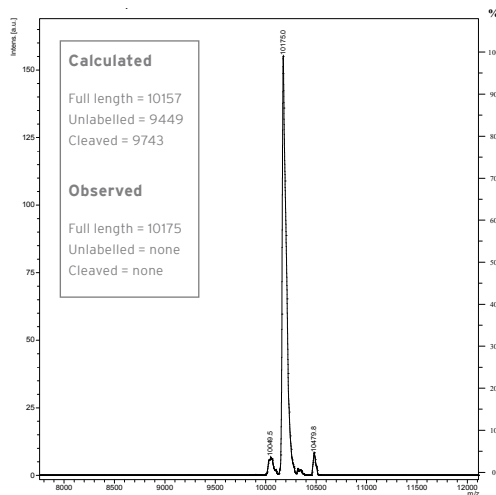


Figure 4. MALDI-TOF MS of DNA-oligonucleotide modified with 3'-Cholesterol SynBase™ CPG 1000/110 (**2394**), deprotected in AMA, 65°C, 10min.

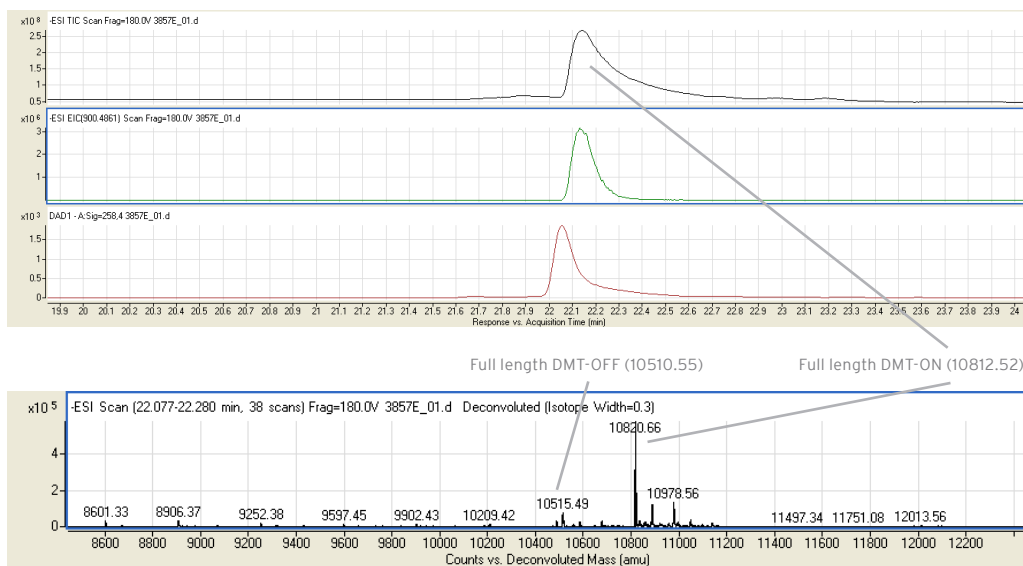


Figure 5. LCMS of DMT-ON RNA-oligonucleotide (TC chemistry) modified with 3'-Cholesterol (**2394**), deprotected in EDA/toluene 1:1, RT, 2hr. Expected mass: DMT-ON 10812.85; DMT-OFF 10510.72. Found 10812.52 and 10510.55.

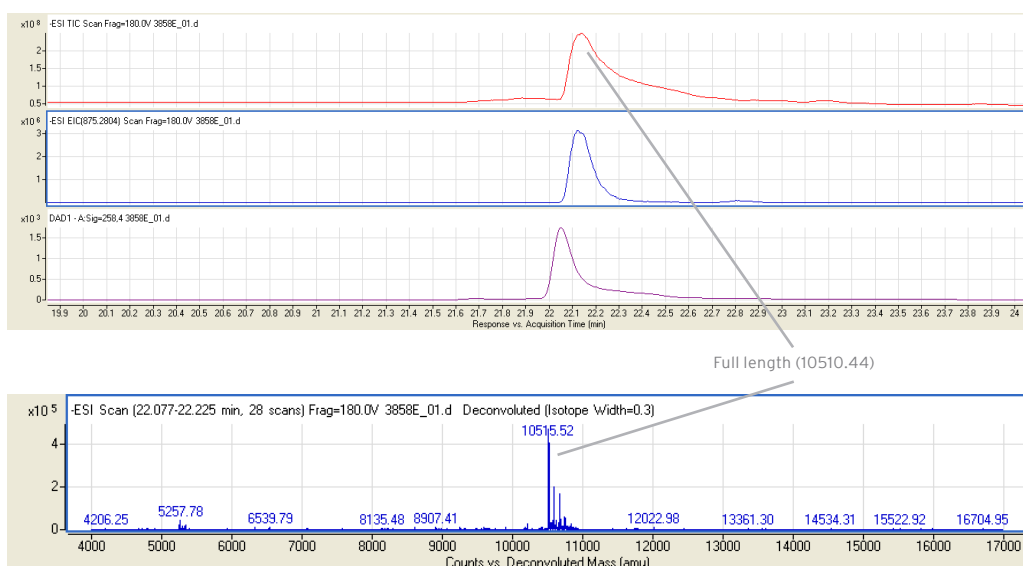


Figure 6. LCMS of DMT-OFF RNA-oligonucleotide (TC chemistry) modified with 3'-Cholesterol (**2394**), deprotected in EDA/toluene 1:1, RT, 2hr. Expected mass: 10510.72. Found 10510.44.

### 5'-Cholesterol-TEG-CE Phosphoramidite (**2189**)

This modification is based on a TEG linker where the only expected degradation product is cleavage across the carbamate bond. For this modification a comparison was made of plant derived 5'-cholesterol TEG and animal derived 5'-cholesterol TEG. In terms of oligonucleotide synthesis, cleavage and deprotection, no

difference in performance was observed.

This is compatible with both TC and TBDMS-RNA chemistries.

The optimum deprotection conditions were shown to be AMA, RT, 2hr (DNA) and EDA/toluene 1:1, RT, 2h (RNA-TC).

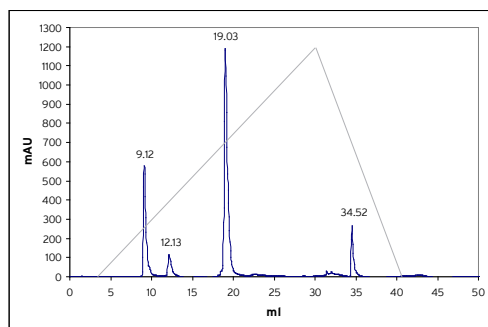


Figure 7. HPLC trace of DNA-oligonucleotide modified with 5'-Cholesterol-TEG-CE Phosphoramidite (**2189**), deprotected in AMA, 65°C, 10min.

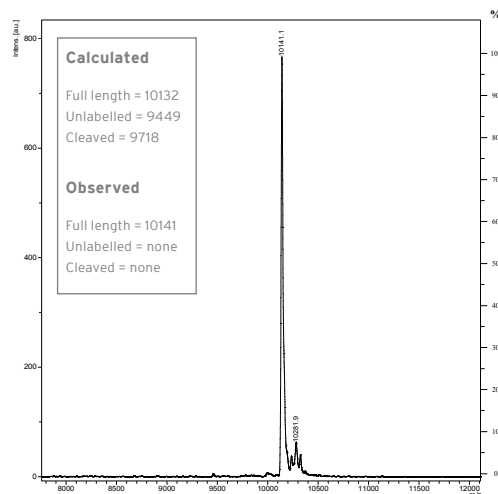


Figure 8. MALDI-TOF MS of DNA-oligonucleotide modified with 5'-Cholesterol-TEG-CE Phosphoramidite (**2189**), deprotected in AMA, 65°C, 10min.

Table 4. Synthesis results for 5'-Cholesterol-TEG-CE Phosphoramidite (**2189**)

Chemistry	Dep <sup>n</sup> Method ID	DMT	% A	% B	% C	Calc. MW	Obs. MW (major peak)
DNA	A	NA	30.33	6.16	63.51	10132	10159
DNA	B	NA	18.33	2.37	79.30	10132	10202
DNA	C	NA	28.63	0.00	71.37	10132	10146
DNA	E	NA	0.00	3.24	96.76	10132	10144
DNA	i. A	NA	25.04	4.38	70.59	10132	10134
	ii. D		20.45	0.00	79.55		
DNA	i. C	NA	28.63	0.00	71.37	10132	10146
	ii. D		22.16	0.00	77.83		
DNA	i. C	NA	28.63	0.00	71.37	10132	10146
	ii. A		25.42	3.09	71.48		
DNA	i. B	NA	18.33	2.37	79.30	10132	10202
	ii. D		29.40	1.43	69.18		
RNA-TC	E	NA	41.36	29.69	28.95	10482	10476

### Conclusions

All the cholesterol modifications tested were compatible with all deprotection conditions. A

summary of the optimum and compatibility with RNA chemistries per modification are shown in Table 5.

Table 5. Summary of results

Modification	Optimum Deprotection Conditions (DNA)	Compatibility with RNA Chemistry		Plant Derived
		TC	TBDMS	
5'-Cholesterol ( <b>2170</b> )	AMA, RT, 2h	Yes	Yes	Yes
3'-Cholesterol ( <b>2394</b> )	AMA, 65°C, 10min	Yes	Yes	Yes
5'-Cholesterol TEG ( <b>2189</b> )	AMA, RT, 2h	Yes	Yes	Yes

## Recommended Protocol

### Dissolution

**2170** is dissolved in alcohol-free dichloromethane, **2189** in anhydrous acetonitrile, both to 0.1M according to Table 6. Typical Expedite (0.067M) dilutions are also given should you wish to use these.

Cholesterol phosphoramidites, like other oil products, are dissolved in the appropriate diluent, left for at least 10min, then vortexed prior to attachment of the bottle to the oligo synthesiser. Do not use the "autodilute" function.

### Coupling

A 15min coupling time is recommended for all products.

### Cleavage & Deprotection

Optimum conditions are as described in Table 5.

### Purification

RP-HPLC on a C<sub>18</sub> Semiprep column is recommended using the following conditions: Buffer A (0.1M TEAA (aq)/5% MeCN) and Buffer B (95% MeCN/H<sub>2</sub>O) with the following gradient with a flow rate of 2.5ml/min.

Time/min	%B
0	5
2	5
28	100
31	100
33	5
35	5

Table 6. Phosphoramidite dilutions

Item No.	Mol. Wt.	Unit Wt.	Dilution (0.1M)/ml			Dilution (0.067M)/ml		
			250mg	500mg	1g	250mg	500mg	1g
<b>2170</b>	730.07	592.82	3.42	6.85	13.70	5.11	10.22	20.44
<b>2189</b>	820.15	682.90	3.05	6.10	12.19	4.55	9.10	18.20

## Ordering Cholesterol Modifiers

Product	Pack Size	Cat. No.	Product	Pack Size	Cat. No.
5'-Cholesterol-CE Phosphoramidite	100µmol 250mg	2170-F100 2170-B250	3'-Cholesterol SynBase™ CPG 1000/110	100mg 1g	2394-B100 2394-C001
5'-Cholesterol-TEG-CE Phosphoramidite	100µmol 250mg	2189-F100 2189-B250	ALL-FIT Columns	4 x 0.2µmol 10 x 0.2µmol 4 x 1µmol 10 x 1µmol	2394-P001 2394-P002 2394-P010 2394-P008
			MerMade Columns	4 x 0.2µmol 10 x 0.2µmol 4 x 1µmol 10 x 1µmol	2394-P015 2394-P016 2394-P026 2394-P022

## Storage & Stability

Products are stored dry in a freezer at -10 to -30°C. Phosphoramidite stability in solution is 2-3 days on the synthesiser.

## Further Information

For up to date ordering and protocol information please see [www.linktech.co.uk](http://www.linktech.co.uk), e-mail us at [sales@linktech.co.uk](mailto:sales@linktech.co.uk) or call +44(0)1698 849911.

You may like to know that we also offer a 3'-Palmitate SynBase™ CPG 1000/110 (**2393**) for introduction of a hydrophobic palmitate group at the 3'-end. This has applications similar to cholesterol. For further details please see the web site.

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