

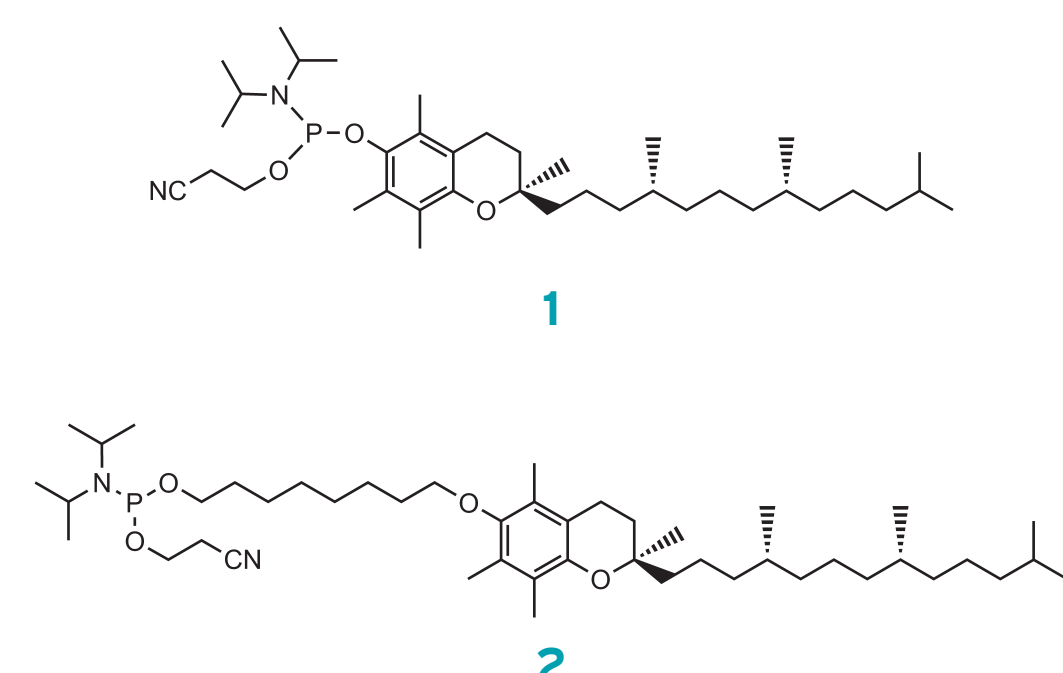
# Tocopherol (Vitamin E) Modified Oligonucleotides

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## Introduction

As with cholesterol modifications, tocopherol has been shown to have potential use in the delivery of oligonucleotides into cells. Vitamins such as tocopherol are not produced by the target cells, but are used by the latter and therefore vitamins are recognised. They are thought to be internalised by cells only after interaction with a binding protein and therefore have the potential for specific targeting of a cell type.<sup>1,2,3</sup> We have looked at the use of tocopherol CEP (**1**) and octyltocopherol CEP (**2**) in oligonucleotide synthesis with the aim to define the optimum deprotection conditions. The synthesis conditions were not optimised since these are reported elsewhere.<sup>4</sup>



The hydrophobic nature of tocopherol has also been utilised as a means of improving the purification of ribozymes.<sup>5</sup> We have also investigated the use of tocopherol as a means of allowing an initial purification of thiol-modified oligos with a view to improving the efficiency of a second ion-exchange purification. Thiol-modified oligonucleotides are often used as a means of incorporating labels such as dyes by reaction with maleimides or acetamides. There is often a need to carry out multiple purifications in order to ensure the purity of the thiol-modified oligonucleotide prior to conjugation and a simple, efficient method of removing DMT-containing N-1 failures would be advantageous.

## Experimental

### Synthesis Conditions

Synthesis was carried out on an ABI 394 DNA/RNA automated synthesiser using the following reagent specifications: standard CEPs and spacers (Bz-dA, dmF-dG, Ac-dC, dT, SP18, SP9, SPC12 and SPC3) - 0.1M in acetonitrile, coupling time = 30s; tocopherol CEPs (**1** and **2**) - 0.1M anhydrous, alcohol-free, dichloromethane, coupling time = 900s; solid support - dT 1000Å SynBase™ 1µmol column; activator - 0.25M ETT<sup>6</sup>; cap A - THF/pyridine/acetic anhydride (8:1:1); cap B - 10% methylimidazole in THF; oxidiser - 0.02M iodine in THF/pyridine/water (7:2:1); deblock - 3% TCA/DCM.

### Deprotection Conditions

The deprotection conditions tested are shown in Table 1. Thereafter, all deprotections were carried out using AMA at room temperature for 2h.

Table 1. Deprotection conditions used on toc-modified poly-T oligos.

Method	Solution	Time	Temp.
A	AMA	10min	65°C
B	AMA	2h	65°C
C	Ammonium hydroxide	16h	55°C
D	i. AMA	i. 10min	i. 65°C
		ii. 2.5h	ii. 65°C
E	EDA/toluene	4h	RT

### Analysis Conditions

RP-HPLC on all oligos was carried out using a Waters X-Bridge OST C18, 2.5µm, 4.6x50mm column, Buffer A: 0.1M TEAA, Buffer B: MeCN, with a gradient of 0-50% B over 15min (X-Bridge oligos gradient) at a flow rate of 1ml/min.

MALDI-TOF was carried out on a Bruker Ultraflex. This data was provided by EGT-SA. LCMS was carried out on an Agilent 6220 Accurate Mass TOF LCMS with 1260 pumps and DAD, using a Zorbax C18, 3.5µm, 2.1x30mm column, Buffer A: 190mM HFIP, 7mM TEAA, 5% MeOH in water, Buffer B: MeOH, with a gradient of 0-100% B over 12min. All samples were at a concentration of 20D/ml.

**1 Delivery of Oligonucleotides and Analogues: The Oligonucleotide Conjugate-Based Approach.** F. Marlin, P. Simon, T. Saison-Behmoaras and C. Giovannangeli, *ChemBioChem*, **11**, 1493-1500, 2010.

**2 Efficient in vivo delivery of siRNA to the liver by conjugation to alpha-tocopherol.** K. Nishina, T. Umno, Y. Uno, T. Kubodera, T. Kanouchi, H. Mizusawa and T. Yokota, *Mol. Ther.*, **16**, 734-740, 2008.

**3 Resolution of liver cirrhosis using vitamin-A coupled liposomes to deliver siRNA against a collagen-specific chaperone.** Y. Sato, K. Murase, J. Kato, M. Kobune, T. Sato, Y. Kawano, R. Takimoto, K. Takada, K. Miyaniishi, T. Matsunaga, T. Takayama and Y. Niitsu, *Nat. Biotechnol.*, **26**, 431-442, 2008.

**4 Attachment of Vitamin E Derivatives to Oligonucleotides during Solid-Phase Synthesis.** D. Will and T. Brown, *Tet. Letts.*, **33**, 2729-2732, 1992.

**5 Fast and simple purification of chemically modified hammerhead ribozymes using a lipophilic capture tag.** B.S. Sproat, T. Rupp, N. Menhardt, D. Keane, and B. Beijer, *Nucleic Acids Res.*, **27**, 1950-1955, 1999.

**6** While this was the only activator used for this work, 0.5M ETT, 0.3M BTT and 0.25M DCI have all been used to successfully couple both tocopherol derivatives **1** & **2** to oligonucleotides.

Table 4. Summary of MS data of tocopherol-thiol-modified oligonucleotides before and after TOPS purification.

Oligo before TOPS	Calculated MW	Observed MW	Oligo after TOPS	Calculated MW	Observed MW
Toc-O-C6-SS-C6-oligo	10281.8914	10314	HS-C6-O-oligo	9667	9661
Toc-O-C6-SS-C6-oligo	10281.8914	10314	HS-C6-O-oligo	9667	9657
DMT-O-C6-SS-C6-oligo	9791.6614	9799.56	HS-C6-O-oligo	9667	9665

## Results & Discussion

### Deprotection Optimisation

A set of tocopherol (**1**) modified T6 oligonucleotides were synthesised using the conditions described above. A poly T sequence was chosen to prevent any misleading results arising from incomplete deprotection of amino nucleobases. After synthesis, the resin was emptied from the column into a sample tube and the appropriate deprotection solution added. Cleavage and deprotection were carried out simultaneously. The oligonucleotides were then deprotected as shown in Table 1.

Analysis by MALDI MS is shown in Table 2. All cases showed that the full-length product was present (one example is shown in Figure 1). In all but method D this was the main product. In this case the main product was observed as M+158 (Figure 2). This is thought to be due to incomplete removal of the TBDMS deprotection solution prior to analysis since after further desalting only the full-length product was observed. The results show **1** is compatible with all deprotection methods A-E. The deprotection tests were repeated with comparable results using the following sequence TTC GGC TTG TCC GTG GAA TCT CAC AGC TTA T, where the 5'-end was modified by either **1** or **2**.

Table 2. Summary of MS data for toc-modified poly-T oligos.

Method	Calculated MW	Observed MW
A	2248.6072	2255
B	2248.6072	2252
C	2248.6072	2251
D	2248.6072	2414, 2256
E	2248.6072	2255

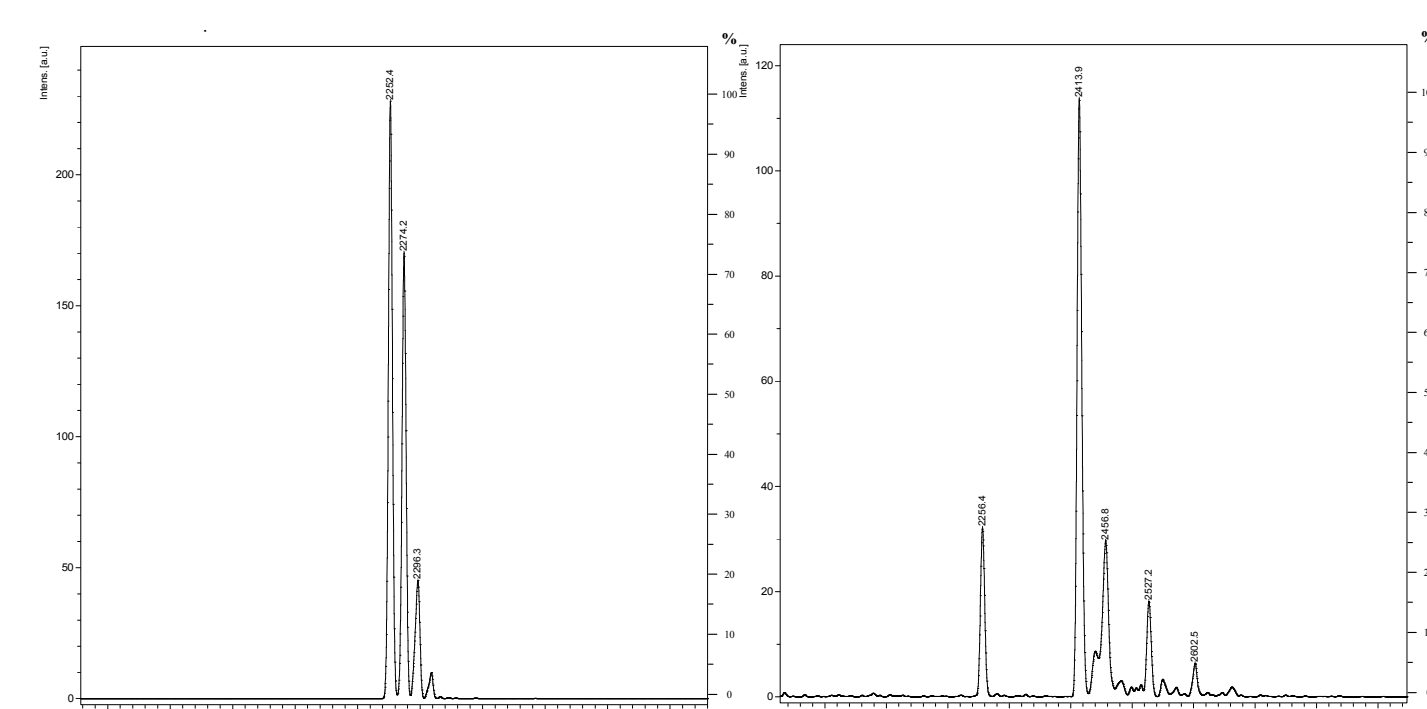


Figure 1. MALDI-MS of tocopherol modified T6 oligonucleotide with method B. Figure 2. MALDI-MS of tocopherol modified T6 oligonucleotide with method D.

### Versatility of Tocopherol (**1**)

While **2** already encompasses a C8 spacer, **1** has the versatility to be used with either a hydrophobic spacer such as spacer C12 or a hydrophilic spacer such as spacer 18 (HEG). To show this a series of tocopherol (**1**) oligonucleotides were synthesised with a spacer between the oligonucleotide and tocopherol. In all cases the sequence used was TTC GGC TTG TCC GTG GAA TCT CAC AGC TTA T. The MS data is shown in Table 3.

Table 3. Summary of MS data for toc-spacer-modified oligos.

Spacer	Calculated MW	Observed MW
None	9954.8966	9965
18	10298.0117	10309
C12	10218.0372	10229
C3	10091.8968	10103
9	10165.9334	10177

In all cases the desired product was obtained indicating that **1** gives the option of incorporating either a hydrophilic or hydrophobic spacer between the tocopherol and the oligonucleotide.

### Use of Tocopherol Modifications to Aid Purification

The possibility of using hydrophobic modifications such as tocopherol or cholesterol derivatives was investigated using **1**. The same 31mer mixed DNA sequence was used as before where the thiol-modifier (**3**) was used to incorporate O-C6-S-S-C6-O between the oligonucleotide and **1** (Figure 3). As a control the same oligonucleotide sequence was modified with **3** where the terminal DMT group was retained. A 25s coupling time for the thiol modification was used, rather than the recommended 300s, to enhance failures in the crude.

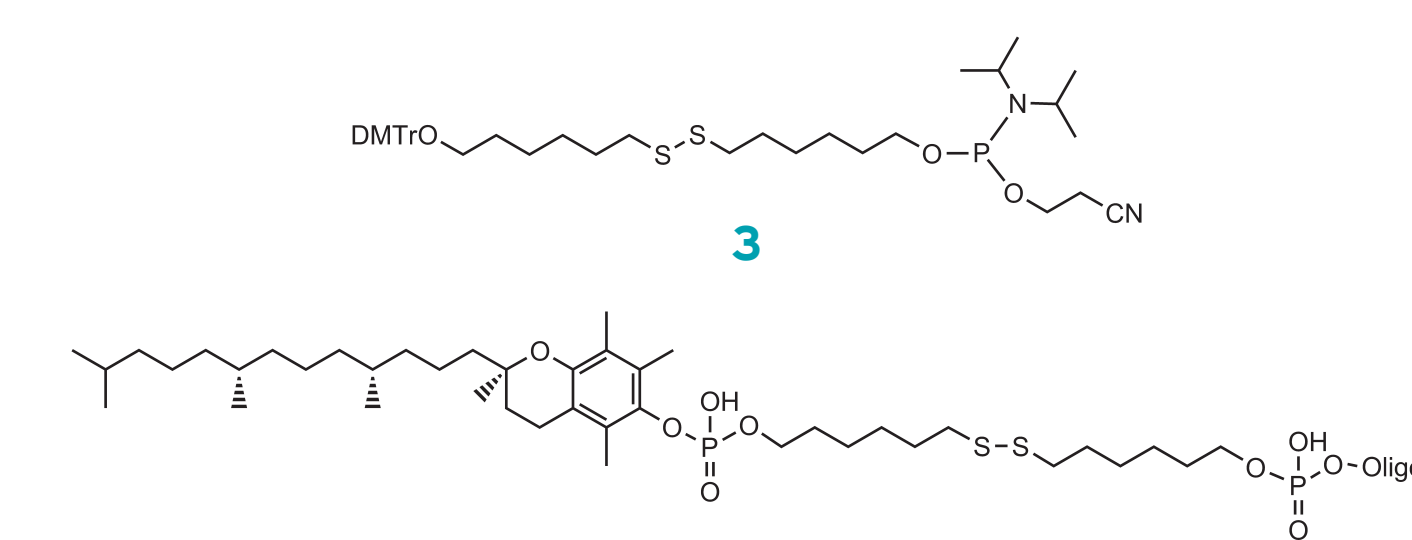


Figure 3. Tocopherol-thiol-modified oligonucleotide.

After synthesis each of the oligonucleotides were cleaved and deprotected using AMA at room temperature for 2h then immediately loaded onto a Varian DNA TOPS column. The standard protocol for purifying DMT-ON DNA oligonucleotides was carried out. For the tocopherol oligonucleotides an extra step of addition of 100mM TCEP in water to each of the columns (leaving for 5min before being removed) was carried out. This step was repeated

and the resulting thiol-modified oligonucleotide was eluted from the column with MeCN/water (1:1). In all cases the desired thiol-modified oligonucleotide was obtained. Examples of the HPLC and MS data of the tocopherol-O-C6-S-S-C6-O-oligonucleotide before cartridge purification and the HS-C6-O-oligonucleotide after purification are shown in Figures 4-7. HPLC of the latter shows two peaks since the dimerised oligonucleotide (oligo-S-S-oligo) is observed. The MS data is summarised in Table 4.

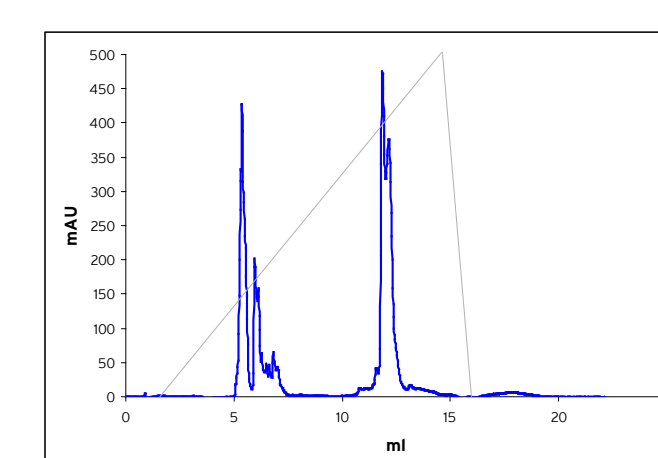


Figure 4. HPLC data of tocopherol-thiol-modified oligonucleotide.

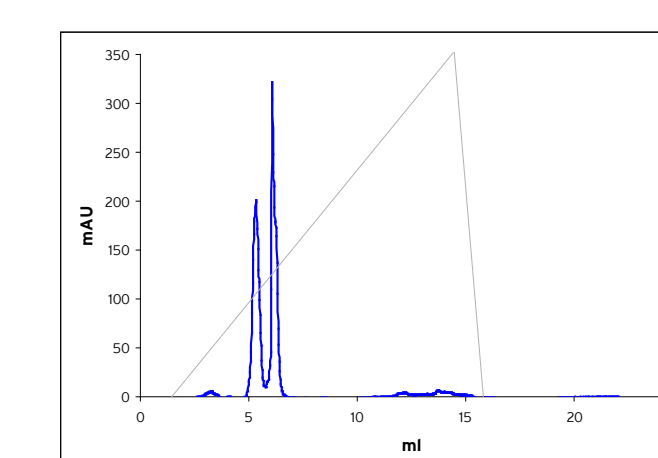


Figure 5. HPLC data of thiol-modified oligonucleotide.

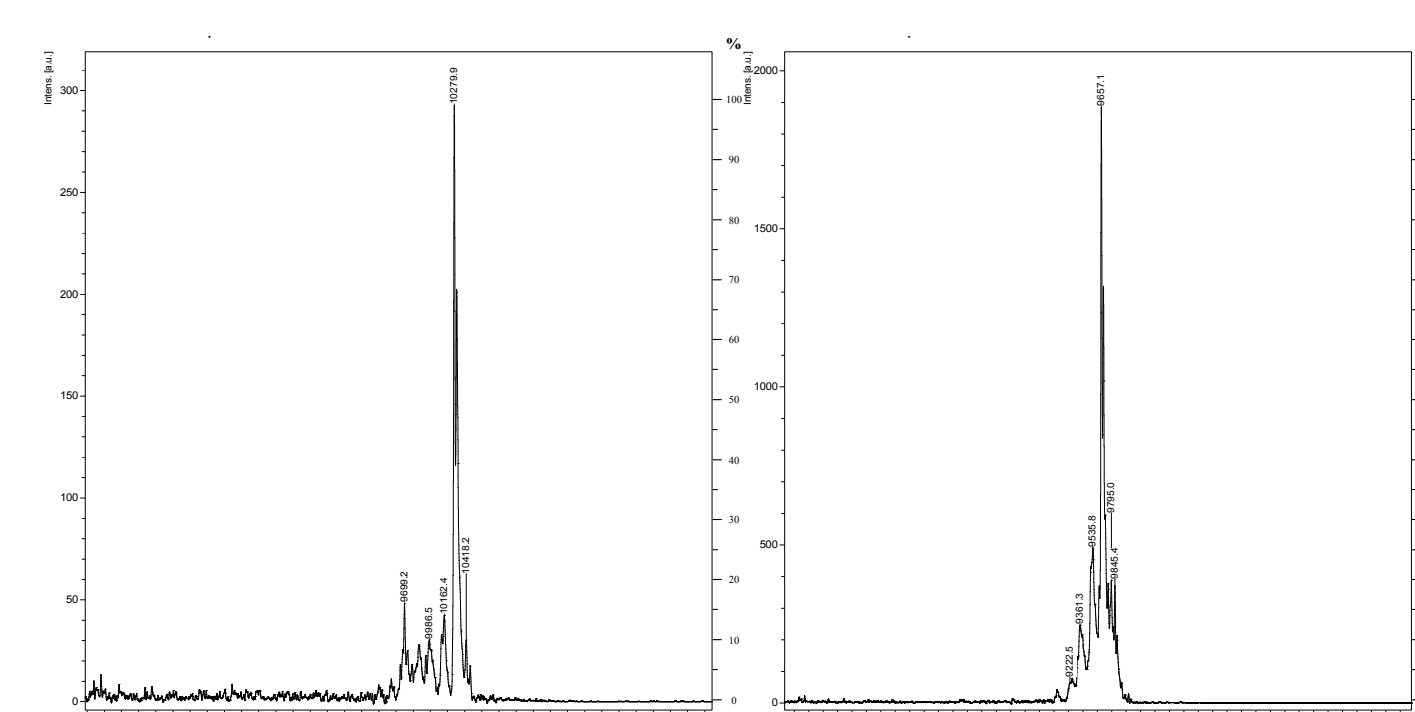


Figure 6. MS data of tocopherol-thiol-modified oligonucleotide. Figure 7. MS data of thiol-modified oligonucleotide.

Initially, the elution step was only carried out at the end of the purification after the reduction step, but this has since been repeated using an elution step to remove any failures which retained a DMT group at the end of the synthesis prior to carrying out the disulphide reduction. Also, to show that all the DMT-containing oligo sequences are efficiently removed, a sample of a tocopherol-thiol-oligonucleotide (Figure 3) mixed with a sample of DMT-O-C6-S-S-O-oligonucleotide (Figure 8) has been purified using the same method. The results are pending. This purification technique will also be carried out with octyltocopherol and other hydrophobic groups.

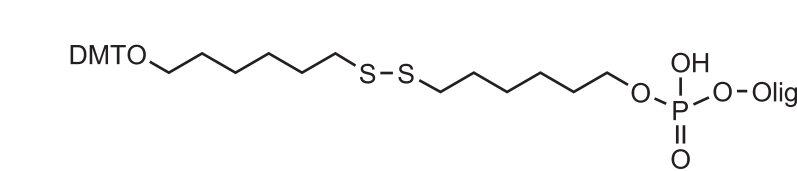


Figure 8. Thiol-modified oligonucleotide.

## Conclusions

Both tocopherol (**1**) and octyltocopherol (**2**) are compatible with all deprotection methods A-E as shown in Table 1.

Tocopherol (**1**) has the versatility to be used in conjunction with any number of hydrophobic or hydrophilic spacers.

Hydrophobic groups such as tocopherol can be used to modify oligonucleotides with a view to a simple and efficient means of removing DMT-containing N-1 failures from thiol modified oligonucleotides.

## Further Information

Any queries regarding this work should be directed to Dr Catherine McKeen, Product Manager, Link Technologies Ltd.

[www.linktech.co.uk](http://www.linktech.co.uk)

