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Attachment of Vitamin E Derivatives to Oligonucleotides during Solid-Phase Synthesis.

David W. Will and Tom Brown*.

Edinburgh Centre for Molecular Recognition, Department of Chemistry, University of Edinburgh, West Mains Rd. Edinburgh,EH9 3JJ, Scotland.

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Abstract: **Compounds have been synthesized which allow the attachment of Vitamin E to the 5'-, and 3'-ends of oligonucleotides, and the attachment of a Vitamin E moiety on an octamethylene spacer to the 5'-end, all during solid-phase synthesis. The effects on cellular uptake, distribution, and antiviral activity of the attachment of the extremely lipophilic Vitamin E moiety to phosphorothioate 'antisense' oligonucleotides are now being investigated.**

The use of antisense oligonucleotides as drugs to inhibit viral replication in cells has attracted great interest in recent years as the potential of this strategy has become apparent[1]. Some of the problems inherent in such a strategy have been solved but many difficulties remain. One of the major problems encountered is the low permeability of cell membranes to large, polyionic oligonucleotides. Attempts to increase the cellular uptake of antisense oligonucleotides by the conjugation of lipophilic molecules are well known. Such lipophilic molecules fall into two main groups:

(i) Those known to interact specifically with cell membranes. *eg.* cholesterol[2-5] and certain lipids[6].

(ii) Those which impart general lipophilicity to the oligonucleotide. *eg.* alkyl chains[7,8].

Vitamin E (α-tocopherol) is an attractive candidate as a lipophilic carrier for the following reasons:

(1) It is inexpensive and essentially non-toxic.

(2) It has only one functional group for derivatization, the rest of the molecule is chemically inert.

(3) It is found mainly in association with membranes of subcellular organelles[9], such as the endoplasmic reticulum and mitochondria, rather than in the plasma membrane. This maximizes the possibility of intracellular transport.

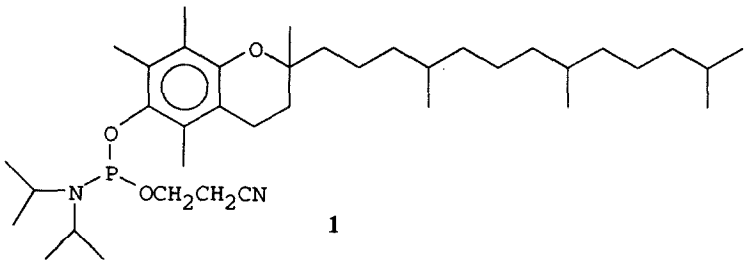
(4) In membranes, the phytyl chain of the Vitamin E is embedded in the membrane, with the phenolic hydroxyl group towards the surface[10,11]. Thus attachment of the oligonucleotide to the phenolic hydroxyl group should have a minimal effect on the interactions of the Vitamin E with membranes.

Here we describe the synthesis of molecules which allow the attachment of Vitamin E to the 5'-, and 3'-ends of oligonucleotides, and the attachment of a Vitamin E moiety *via.* an octamethylene spacer to the 5'-end, during solid-phase synthesis. In all experiments DL-α-tocopherol was used.

(1) Synthesis of Vitamin E derivatives.

(a) Unspaced Vitamin E Phosphoramidite **1** (Scheme 1)

This was prepared by phosphitylation of Vitamin E with 2-cyanoethyl N,N-diisopropylchlorophosphoramidite and diisopropylethylamine in THF.

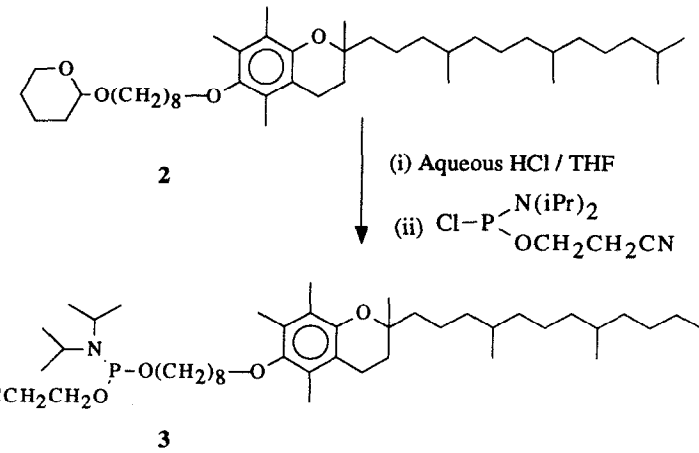


1

Scheme 1

(b) Octamethylene-spaced Vitamin E phosphoramidite **3** (Scheme 2).

Vitamin E was reacted with commercially available 1-Bromo-8-tetrahydropyranyloxyoctane (Lancaster) in DMSO in the presence of powdered KOH [12] to form the asymmetric ether **2**. The THP protecting group was removed by treatment with aqueous HCl in THF, and the resulting alcohol was phosphitylated.



2

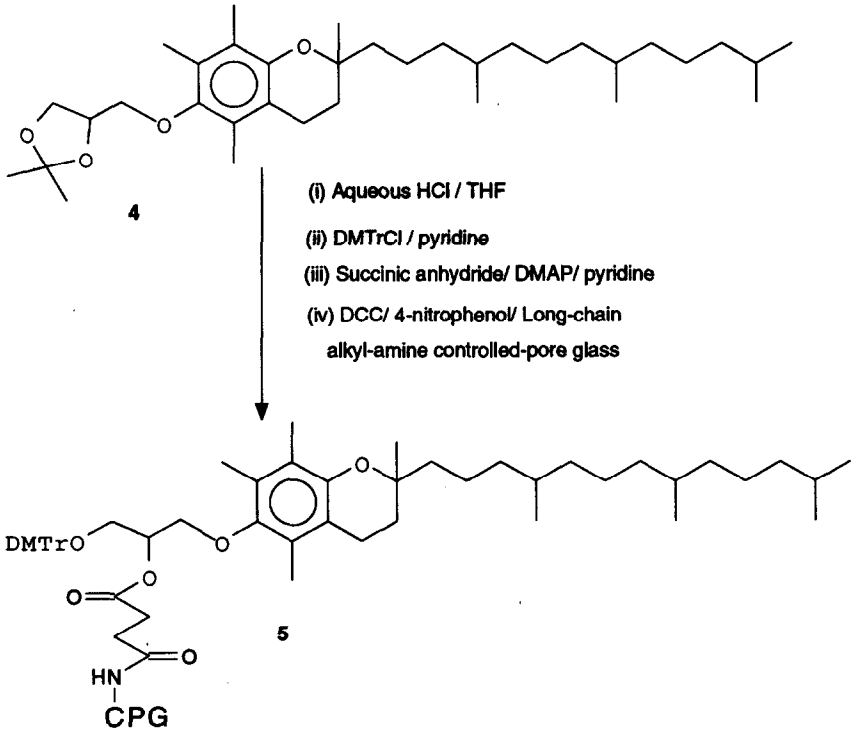
3

Scheme 2

(c) Vitamin E-derivatized Controlled PoreGlass (CPG), **5** (Scheme 3)

Solketal was reacted with 4-toluene sulphonyl chloride in pyridine. The resulting tosyl solketal was reacted with Vitamin E in DMSO in the presence of powdered KOH [12] to form the asymmetric ether **4**. The

acetonide protecting group was removed by treatment with aqueous HCl in THF, and the resulting alcohol was reacted with dimethoxytrityl chloride in pyridine. The remaining secondary alcohol was then reacted with succinic anhydride/ DMAP in pyridine. The resulting tritylated-succinylated Vitamin E solketal derivative was coupled to LCAA-CPG to give **5** with a loading of 24 $\mu mol.g^{-1}$.



Scheme 3

(2) Oligo ucleotide Synthesis.

Normal and phosphorothioate[13] Vitamin E derivatised oligonucleotides were synthesized on an Applied Biosystems 380B DNA synthesizer.

For 3'-Vitamin E oligonucleotides:

- standard DNA and phosphorothioate synthesis cycles were used.

For 5'-Vitamin E oligonucleotides:

-Phosphoramidites **1** and **3** were insoluble in acetonitrile and were used as 0.1*M* solutions in anhydrous dichloromethane,

-Minor alterations to the synthesis cycles were required (the synthesis columns were washed with anhydrous dichloromethane before and after the coupling reaction to prevent precipitation).

-Coupling efficiencies were >95% estimated by HPLC.

Vitamin E oligonucleotides were stable in conc. aqueous ammonia at 55°C for >24h (HPLC analysis).

(3) Oligonucleotide Purification.

The extreme lipophilicity of Vitamin E oligonucleotides greatly facilitates reversed-phase HPLC (RP-HPLC) purification, the products eluting approx. 15min later than all failure sequences in a gradient of acetonitrile in aqueous ammonium acetate. As the Vitamin E moiety is introduced during solid-phase synthesis with high efficiency the product is obtained in high yield. Vitamin E oligonucleotides were characterized by 600MHz NMR spectroscopy.

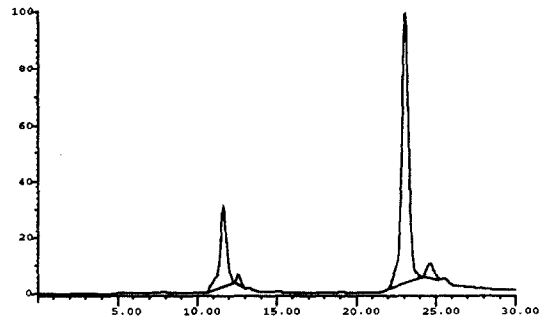


Figure 1: **RP-HPLC trace of a crude 28mer phophorothioate with a 5'-octamethylene spaced Vitamin E attached.**

Multi-milligram quantities of derivatives of a phosphorothioate 28mer antisense to a region near the start codon of the *rev* gene of HIV[14] have been synthesized with all three types of Vitamin E modification. These sequences are now being evaluated for anti-HIV activity in cell culture.

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