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Thioctic acid modification of oligonucleotides using an H-phosphonate

Jennifer A. Dougan, Andrew. K. Reid, Duncan Graham *

Centre for Molecular Nanometrology, Department of Pure and Applied Chemistry, University of Strathclyde, Glasgow G1 1XL, Scotland, United Kingdom

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ABSTRACT

The H-phosphonate of a derivative of thioctic acid (TA) was synthesised and used to introduce a disulfide moiety at the 5′-end of oligonucleotides. This method overcomes the difficulties experienced with the phosphoramidite approach when employing a cyclic disulfide in the starting alcohol. The disulfide-modified oligonucleotides are subsequently used in metallic nanoparticle (Au and Ag) and surface functionalisation for sensitive, sequence specific analytical detection strategies.

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The simple modification of oligonucleotide sequences is required for a variety of applications.[1] One such application is the sensitive and specific detection of oligonucleotide sequences of interest, which is of pivotal importance for the understanding and management of genetic disease states.[2,3] At the intersection of bio- and nano-technology the conjugation of oligonucleotide probes to metallic nanoparticles has been proven as an effective method for the sensitive and selective detection of target sequences via strong plasmon-derived absorption,[4] scattering,[5] surface-enhanced resonance Raman scattering (SERRS)[6] and/or fluorescence (via quantum dots)[7] properties. Enhanced conjugate stability is a key requirement as these detection strategies are employed in increasingly complex matrices, the presence of common biological buffer additives, in serum or in vivo, for example. As ligands for oligonucleotide-nanoparticle attachment, multi-dentate thiol groups have been shown to confer improved stability with respect to monothiol standards.[8–10] Tethering oligonucleotides to surfaces via thioctic acid has been shown to provide probe conjugates of enhanced stability with both Au and Ag nanoparticles[10] and allow for an improved detection reproducibility when employed as a surface attachment group for oligonucleotide detection on a gold nanostructured chip via SERRS.[11]

To date, oligonucleotides have been modified by thioctic acid (TA) at the 3′-terminus by the reaction of a pre-formed *N*HS-ester intermediate with amino-modified controlled pore glass (CPG) solid support for subsequent oligonucleotide synthesis.[10] 5′-Modification has been achieved by both solution and solid phase post-synthetic coupling of amino-modified oligonucleotides using the pre-formed *N*HS-ester of thioctic acid (unpublished results). It would, however, be more convenient for 5′- or mid-sequence modification of oligonucleotides to proceed via a solid phase synthesis approach with the potential for automation.

The most obvious synthetic method for the modification of the 5′-termini of oligonucleotides uses the phosphoramidite chemistry developed by Caruthers et al. in the early 1980s.[12–14] In order to employ the phosphoramidite method, the modifying group must first possess an alcohol which reacts with the P^{III} phosphitylating reagent, **2.** The P^{III} phosphoramidite product, **3**, can then be introduced on to the oligonucleotide chain on the solid support in the presence of an activator.

Thioctic acid, TA, can be readily derivatised,[15] in this case, via an amide formation with aminohexanol to yield amide **1**. However, attempts at phosphitylating the resulting TA-alcohol, **1**, did not provide the desired phosphoramidite (Scheme 1). The phosphitylating reagent, 2-cyanoethoxy-bis(*N,N*-diisopropylamino) phosphine (**2a**) was used under standard conditions.[16] Later attempts were conducted using the chloro-analogue, 2-cyanoethoxy-*N,N*-diisopropylaminochloro-phosphine (**2b**) a less stable and inherently more reactive species. However, this also failed to yield the desired product.

It was suspected that the highly reactive nature of the P^{III} intermediate, **3**, had resulted in side-reactions with the cyclic disulfide of TA rendering the product unsuitable for addition to the oligonucleotide chain. Certainly, ^{31}P NMR analysis showed a variety of phosphorus species that suggested a mixture had, indeed, been formed. It should be noted that the phosphoramidite of a cyclic disulfide, dithiothreitol (DTT), has previously been reported and, indeed, allows for both terminal and internal modifications.[17] However, it is tentatively suggested that, in contrast to the six-membered ring of DTT, the TA-phosphoramidite suffers from self-reactivity and instability as a result of steric strain imparted by the two sulfurs in a five-membered ring system.

In order to circumvent the problem of disulfide reaction at the P^{III} centre, an H-phosphonate approach was exploited. H-Phosphonate chemistry originated in the 1950s in the laboratory of Lord Todd and co-workers.[18] The H-phosphonate method was found to be suitable for solid phase synthesis,[19–21] particularly when using pivaloyl chloride as an activator.[18,19] Nevertheless, it was largely superceded by the phosphoramidite approach. The intermedi-

* Corresponding author. Tel.: +44 0141 548 4701; fax: +44 0141 552 0876.
E-mail address: duncan.graham@strath.ac.uk (D. Graham).

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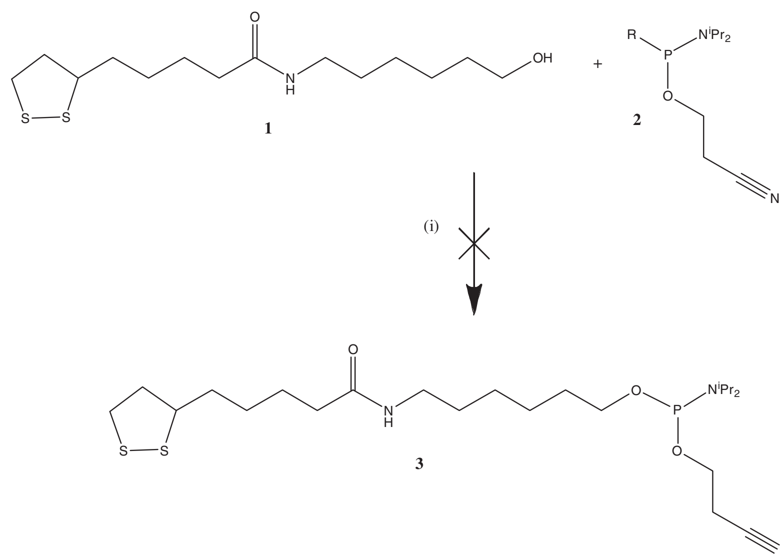


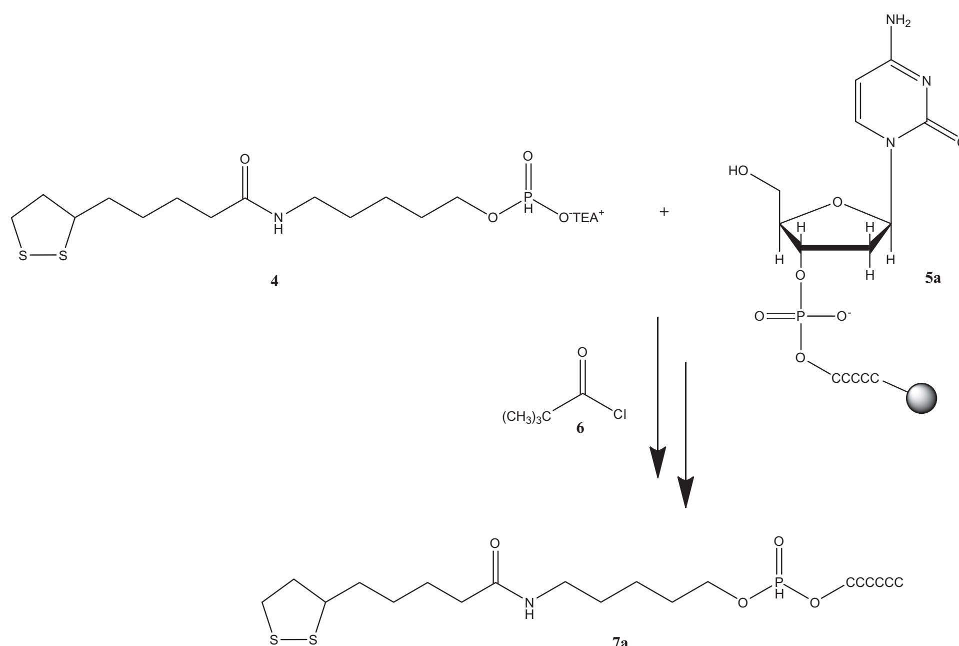
Scheme 1. Reagents and conditions: (i) BTT (0.25 M, 4.2 ml), CH_2Cl_2/THF (anhydrous), N_2, 1–4 h. Unsuccessful phosphitylation reactions, where the phosphitylating reagent, **2a**, R = diisopropylamino- and **2b**, R = chloro–.

ates of the H-phosphonate method are tetra-coordinated P^{III} species and, as such, are not susceptible to further reaction. The H-phosphonate method is still used in situations where the oxidation step is best avoided.[22] This allows oxidation of the full sequence to be carried out at the end of the oligonucleotide synthesis. Whilst, in the case of TA-modification, oxidation was not a problem, using a tetra-coordinated P^{III} species was an attractive option as it was believed that this might prevent the side-reactions which appeared to be hindering the phosphoramidite method.

The H-phosphonate method also requires a suitable alcohol for functionalisation. In this case, TA was coupled with aminopentanol to introduce the required alcohol moiety. The H-phosphonate of the TA-derivative was then prepared in the standard fashion by dissolving, **4**, in 1 M phosphorus acid in anhydrous pyridine and adding pivaloyl chloride dropwise. The reaction was monitored by thin layer chromatography, and upon completion, was treated with a triethylamine bicarbonate quench. The reaction mixture was worked-up and the product was extracted into dichloromethane. Flash column chromatography yielded the triethylamine salt as a yellow oil in 47% yield.

Oligonucleotide sequences were synthesised on a MerMade 6 synthesiser using standard phosphoramidite techniques. Each 6-mer sequence was prepared on a standard CPG column at 1 μmol loading. Each synthesis was ended with a trityl deprotection step to ensure that the 5′-hydroxy of the terminal nucleoside would be available for reaction with the H-phosphonate, **4**.

The coupling (Scheme 2) was carried out with the H-phosphonate, **4**, dissolved in 1:1 MeCN/pyridine and added to both a cyti-



Scheme 2. H-Phosphonate, **4**, coupling onto cytidine-6-mer, **5a**, using pivaloyl chloride, **6**, yields TA-modified oligonucleotide, **7a**. where **5b** and **7b** are the thymidine analogues.

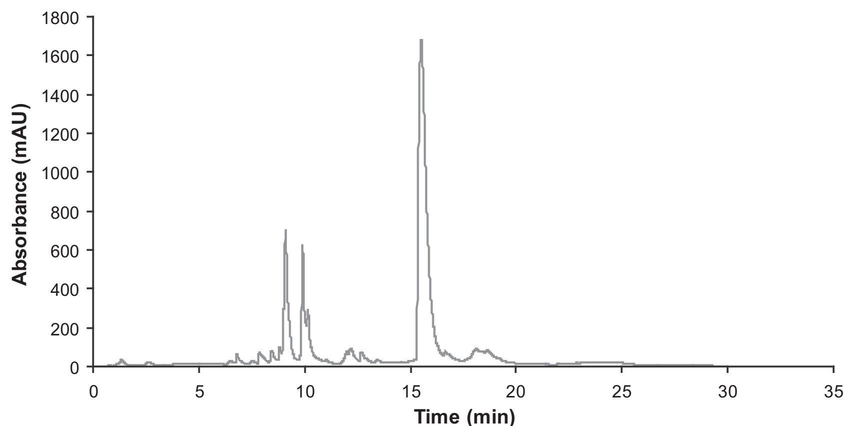


Figure 1. RP-HPLC of TA H-phosphonate with cytidine 6-mer (5a) on solid support (note the cytidine 6-mer was not purified prior to reaction). *t*R = 15.5 min for product **7a**.

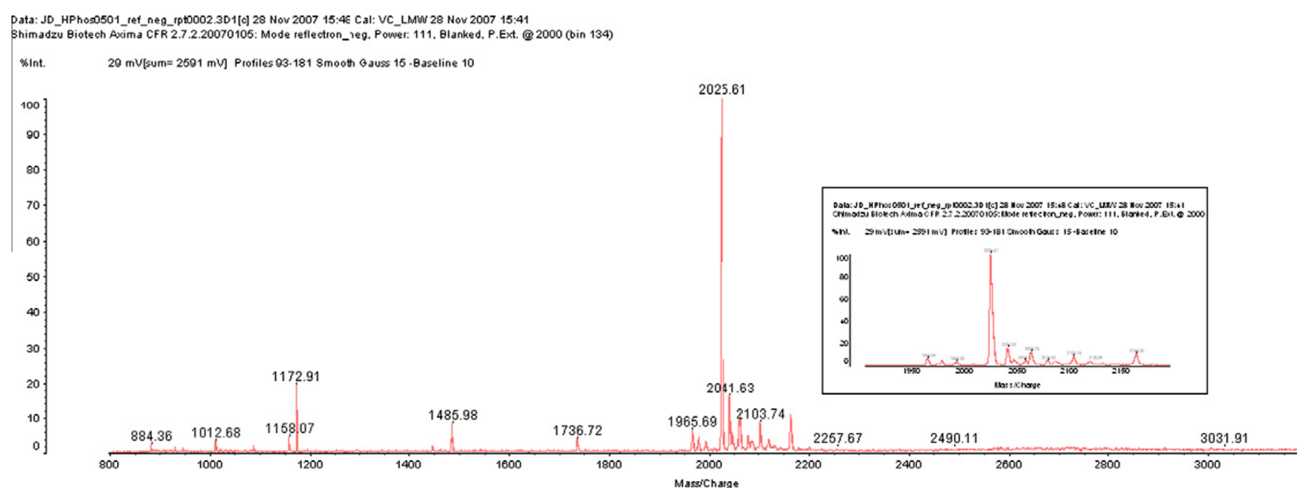


Figure 2. MALDI-TOF mass spectrometry (neg., ref. mode) of **7a** after RP-HPLC purification and ZipTip™ preparation. The desired peak is observed at 2025.6 amu. Other peaks correspond to internal calibration points.

dine-6-mer, **5a**, and thymidine-6-mer, **5b**, on a solid support using pivaloyl chloride, **6**, as activator. The reaction was carried out in a silanised glass vial which was sealed and agitated for five minutes with a mechanical shaker before the reaction mixture was removed and the CPG was washed (3 × MeCN). Oxidation was achieved with standard DNA synthesis-oxidising solution [0.2 M iodine in THF/pyridine/water (7:2:1)]. Following reaction, the CPG was treated with conc. ammonium hydroxide to cleave and deprotect the sequences. This reaction could be carried out via an automated synthesiser. However, care would be required for the timing of reagent delivery to avoid pre-mixing which could lead to self-reaction and the generation of an unreactive pyrophosphate.

Reversed-phase HPLC was used to purify the reaction mixtures an example of which is shown in Figure 1, corresponding to the reaction between TA H-phosphonate, **4**, and the cytidine-6-mer on solid support, **5a**, with pivaloyl chloride. Note that the 6C sequence was not purified prior to reaction with the TA H-phosphonate, **4**. The peak at t_R = 15.5 min corresponds to the thioctic acid-modified sequence, **7a**, as confirmed by MALDI-TOF mass spectrometry (Fig. 2). A mass of 2025.4 was required in negative ionisation mode and modification was confirmed with the observation of a peak at 2025.6. Other peaks relate to an internal calibration standard. Note that, for brevity, HPLC and MALDI-TOF data are not shown for the thymidine-6-mer analogue.

Reported herein is a facile route to 5′-cyclic-disulfide-modified oligonucleotides. The H-phosphonate method should be investigated in cases where the phosphoramidite approach is not viable. Using the H-phosphonate method a route to otherwise troublesome modifications has been shown to be successful. Indeed, it is suggested that this approach could be useful for a variety of modifications with groups that would be reactive to a trivalent, and nucleophilic, P^{III} intermediate.

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Supplementary data

Supplementary data (general experimental methods, reaction conditions and characterisation) associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2010.08.107.

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