An Acid Free Deprotection of 5′Amino-Modified Oligonucleotides

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Abstract

The installation of reactive chemical handles onto synthetic oligonucleotides is essential for the generation of oligonucleotide conjugates, which are ubiquitous across the healthcare, diagnostic, and research industries. This is typically accomplished by the incorporation of a monomethoxytrityl (MMTr) protected primary amine as it allows for purification via high-throughput reverse phase cartridge purification and can be deprotected with standard laboratory reagents. While the MMTr group provides these advantages over alternative protecting groups, conventional deprotection methods rely on aqueous acids, which are inherently reversible in nature and are known to cause depurination of oligonucleotides. In this study we demonstrate a novel equilibrium driven deprotection strategy for MMTr-protected amino oligonucleotides, which simplifies the purification process while increasing synthetic efficiency and quality of the oligonucleotide. The method utilizes only water and heat to achieve selective deprotection of MMTr groups, eliminating the need for acid treatment. This deprotection approach offers a safe, mild, operationally simple, and environmentally friendly alternative to conventional methods.

Introduction

Nucleic acid conjugates have revolutionized disparate fields ranging from therapeutic intervention to early diagnostic detection and DNA based data storage. The impact of nucleic acid conjugates has been accelerated by the constant evolution of synthetic methods for the incorporation of chemically reactive handles for downstream derivatization. One clear example being the field of nucleic acid therapeutics where the incorporation of ligands has greatly improved both the drug-like properties and cell specific delivery of antisense oligonucleotides (ASOs) and small interfering RNAs (siRNAs).[1–4] Installation of reactive chemical handles during synthesis that enable efficient post-synthesis conjugation is critical for the generation of these

conjugates. Although several handles exist for which robust conjugation reactions are widely employed,[5,6] the amide bond forming reaction between an amine and activated ester is universal due to the cost-effectiveness and relatively simple preparation of starting materials.[7–9]

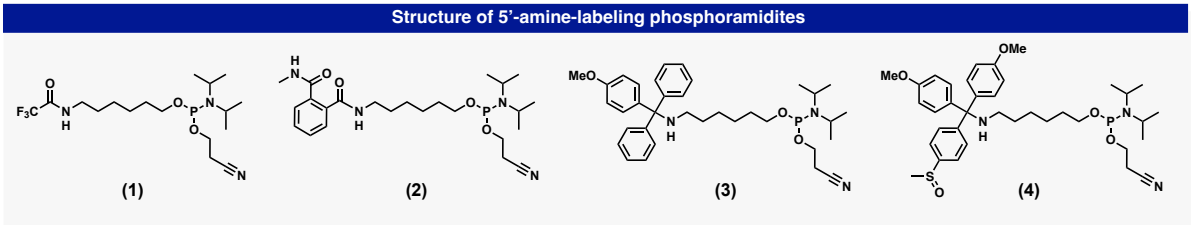

Figure 1: Commercially available 5'-amine-labeling amidites with various protecting groups.

There exist two major classes of phosphoramidite reagents capable of labeling oligonucleotides with 5'-amines that differ in their amino protecting group and therefore, method of deprotection. The first group contains base labile protection including both trifluoroacetyl (TFA) (**1**) and phthalic acid diamide (PDA) (**2**) (Figure 1). In the former case, the TFA group is readily deprotected during standard cleavage and deprotection in concentrated aqueous ammonia, while in the latter case, the PDA group requires a 1:1 mixture of concentrated aqueous ammonia and methylamine (AMA). This approach requires conjugation to occur in the crude synthesis mixture and hinges on the subsequent conjugate being sufficiently distinct chemically, to allow isolation from the truncated oligonucleotide sequences generated during synthesis. This requires the use of HPLC purification, which is expensive, time consuming, and not accessible in every lab.

The trityl-based reagents, MMTr (**3**) and 5'-DMS(O)MT (**4**), are perhaps the most used due to their convenient hydrophobic handle that enables routine cartridge purification. The compatibility with cartridge purification greatly accelerates the ability to generate many high-quality conjugates without the need for laborious purifications. One significant challenge in using these reagents (which is even noted on supplier websites) is that the trityl group shows a propensity to either remain attached during deprotection or unexpectedly detach during routine handling.[10] In addition to these challenges, the requisite acidic deprotection has been shown to cause depurination of the oligonucleotide resulting in abasic sites and cleavage products.[11–13] Previous studies have explored alternative approaches to minimize depurination, such as using less acidic deprotection cocktails for DMTr and MMTr protected oligonucleotides.[14,15] Alternatively protected amino amidites have also received much

attention, however, none have made significant improvements over (**3**), which to date remains the most practical and utilized option.[16]

A practical method for synthesizing 5′-amine labeled oligonucleotides using (**3**) that solves for these challenges would greatly facilitate the synthesis of routine oligonucleotide conjugates and hopefully open avenues for modifications that are not amenable to conventional deprotection strategies. Herein, we report a completely acid-free deprotection method specifically tailored for MMTr-protected amino oligonucleotides. This approach addresses the limitations of previous methods and provides a reliable strategy for achieving efficient amine deprotection without the aforementioned drawbacks, thus adding to the toolbox of methods that oligonucleotide chemists can employ during the preparation of oligonucleotide conjugates in targeted drug delivery, diagnostics, and therapeutic interventions.

Results and Discussion

Innovation: Conception of an acid-free deprotection method

Oligonucleotide (**5**) (Figure 2a) was treated with the industry standard protocol for the deprotection of trityl protected 5′-amine-modified oligonucleotides, which requires incubation in 20% acetic acid in water for 60 minutes at room temperature. In our hands, this resulted in incomplete deprotection (Figure 2b) as well as the detection of depurinated species (see Supporting Information, Figure S1). Increased percentages of acetic acid, incubation time, and reaction temperature all resulted in minimal changes in overall conversion or levels of observed depurination (see Supporting Information, Figure S2).

Routine analysis of our purified MMTr-ON oligonucleotide before deprotection, indicated *ca*. 5% MMTr-OFF material was present after concentrating *in vacuo*. Investigations were then aimed at understanding this intriguing observation. To determine if the small amount of deprotected product was generated by the conditions present in the drying step, namely heating an aqueous solution of the oligonucleotide, (**5**) was heated at 60 °C in non-buffered water and curiously, full deprotection was observed after one hour (Figure 2c).

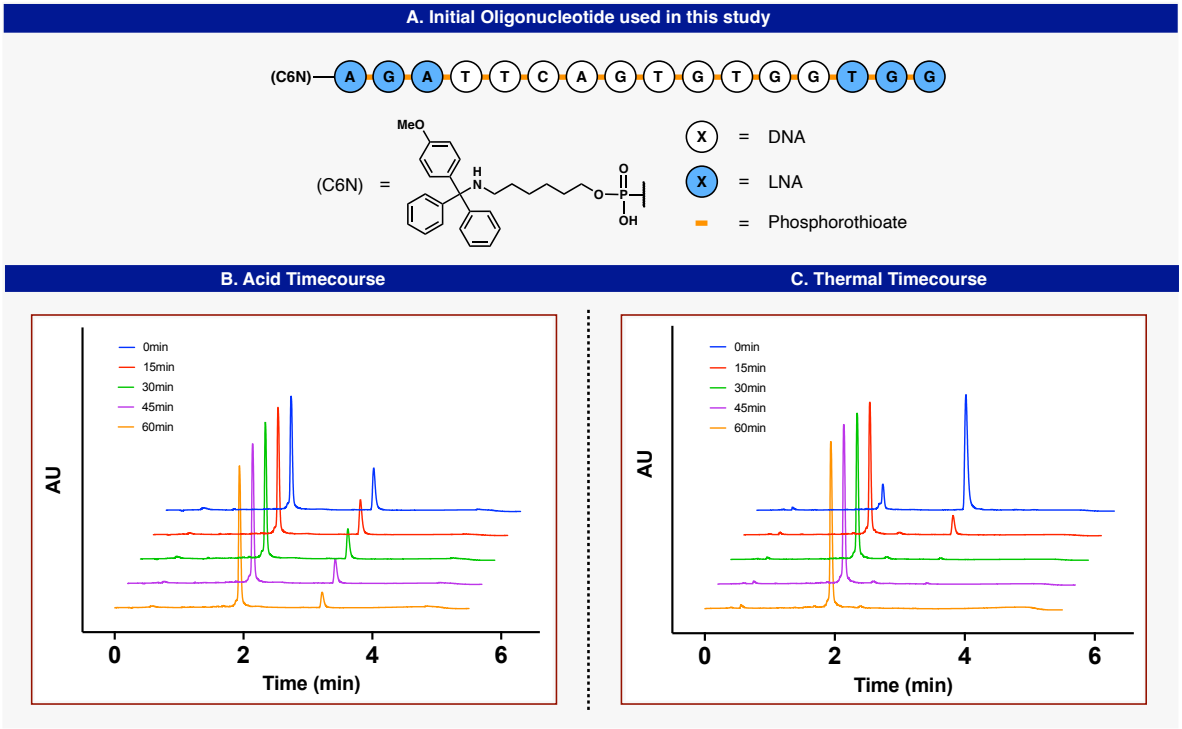

Figure 2. Oligonucleotide and timecourses. (A) Initial oligonucleotide used in this study. (C6N) = incorporated (**3**). (B, C) MMTr deprotection at 0, 15-, 30-, 45-, and 60-minute timepoints using current 20% aqueous acetic acid and water heated to 60 °C, respectively. Traces obtained with IPRP-LCMS.

To further investigate the factors driving this deprotection, several variables were evaluated including temperature, buffer system and solvent, with a fixed timepoint of one hour (Table 1). While evaluating temperature, it was found that below 60°C, full conversion was not achieved (Table 1, entries 1-4), therefore, all other experiments were caried out at this temperature. Various solutions commonly used in oligonucleotide purification and handling were evaluated. Complete deprotection was observed in phosphate buffered saline (PBS, pH 7.4) and TE buffer (pH 8.0) indicating that pH need not be acidic to drive the reaction to completion (Table 1, entries 7-8). However, concentrated ammonium hydroxide (NH_4OH) led to only trace product formation (Table 1, entries 6).

The pH 7.0 and buffered samples (pH 7.4, and pH 8) all contained white precipitate after incubation, determined to be MMTr-OH (see Supporting Information, Figure S3). It was postulated that solubility characteristics of this byproduct drives the system to deprotection, by minimizing the known reversibility of MMTr deprotection[17] through sequestration of the

byproduct as an insoluble species. For further evidence supporting this equilibrium reaction, incubation of (**5**) in a 50:50 mix of MeCN/H_2O caused incomplete deprotection likely due to the increased solubility of MMTr-OH, resulting in an equilibrium shift (Table 1, entries 5).

Table 1. Evaluation of reaction parameters.

Optmization Table: Thermal Deprotection					
Entry	**Temp (°C)**	**Time (min)**	**Solvent/pH**	**Conversion**	**Depurination Observed?**
State of The Art	RT	60	20% Acetic Acid / Water	92%	Yes
This Work	60	60	Water	**100%**	No
Temperature					
1	RT	60	Water	15%	No
2	35	60	Water	45%	No
3	45	60	Water	94%	No
4	80	60	Water	**100%**	No
Solvent					
5	60	60	50% Acetonitrile / Water	55%	No
6	60	60	28% NH_4OH	1%	No
7	60	60	PBS (pH 7.4)	**100%**	No
8	60	60	TE Buffer (pH 8.0)	**100%**	No

To rule out the possibility of this thermal deprotection being biased by a particular oligonucleotide sequence, a panel of oligonucleotides with all possible 5'-neighboring bases as well as adjacent bases was synthesized (Figure 3, see Supporting Information for full list of sequences evaluated, Table S1). Two other therapeutically relevant antisense oligonucleotides (**12** and **13**) were also synthesized and evaluated with both the acid and thermal treatments. Complete deprotection was achieved in all thermally deprotected samples, showcasing the sequence independence of these methods.

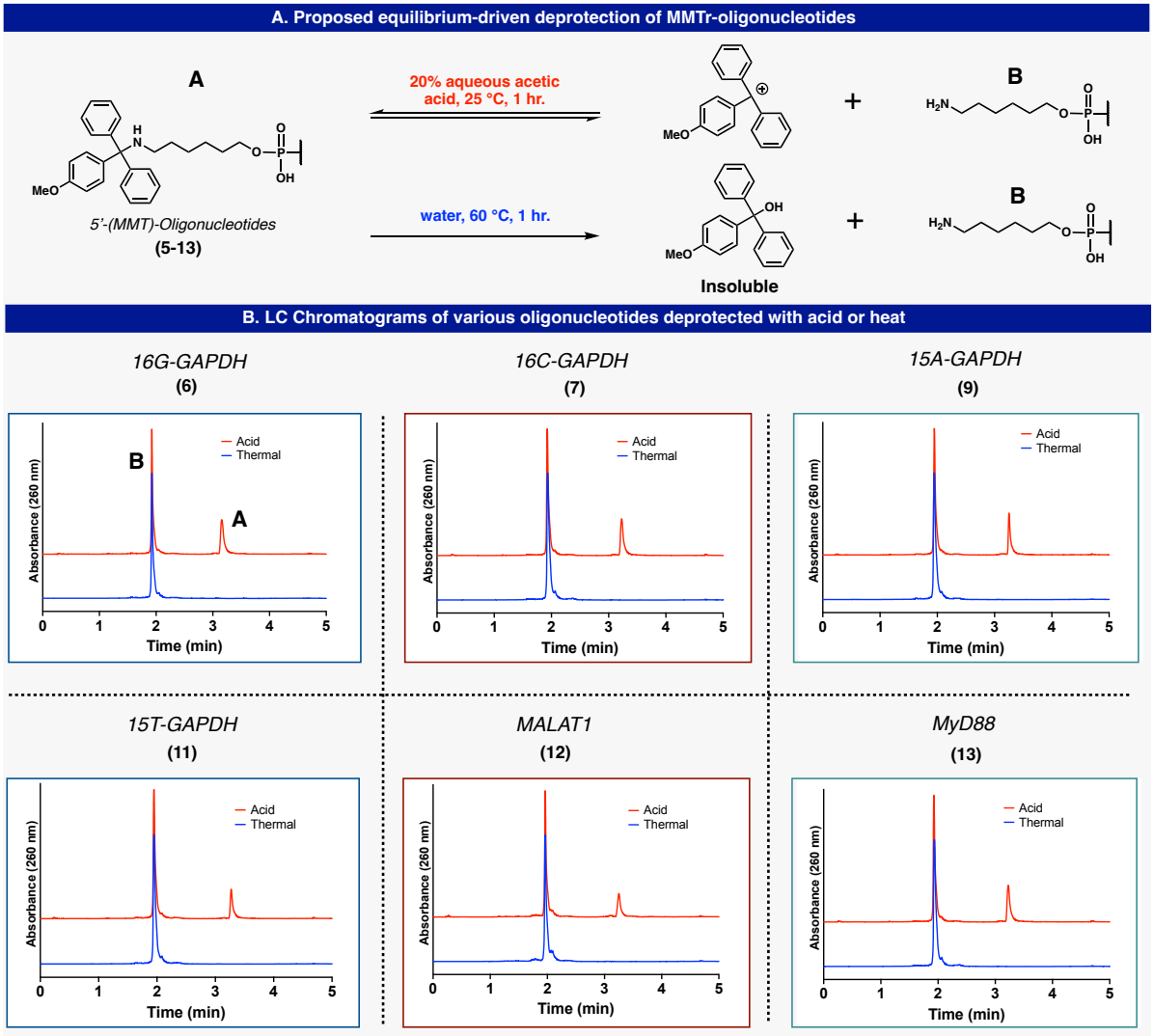

Figure 3. (A) Contrast of uncontrolled vs. solubility driven equilibriums between acid and thermal methods. (B) Acid (red) and thermal (blue) deprotection chromatograms of various oligonucleotides.

To determine whether this thermal deprotection method could also be extended to standard 4,4'-dimethoxytrityl (DMTr) protected oligonucleotides we attempted to deprotect a 5'-DMTr-protected oligonucleotide using similar thermally driven deprotection. Under slightly more aggressive heating, full DMT-deprotection was observed by 1.5 hours at 95°C (Figure 4).

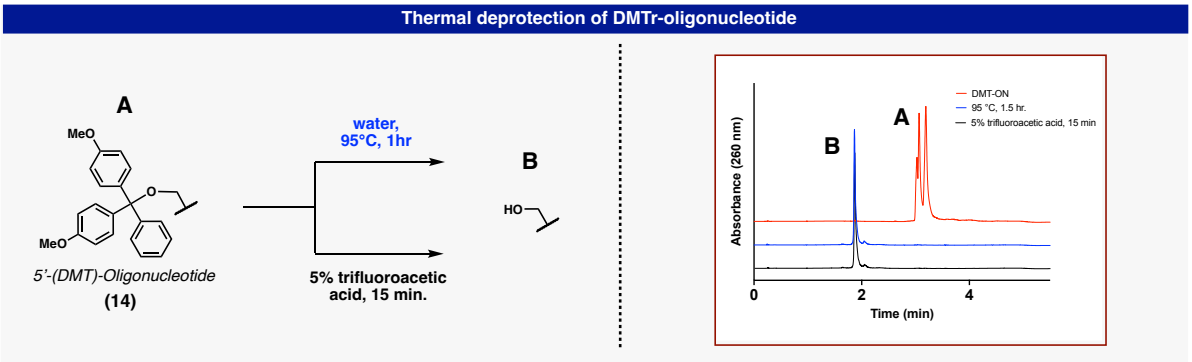

Figure 4. Thermal deprotection of DMTr-protected (**14** see SI for table of sequences).

The simplicity of this equilibrium driven deprotection method and the compatibility with standard oligonucleotide purification techniques has been demonstrated. Following elution of MMTr-ON product from OPC, the resulting protected oligonucleotide can be deprotected in elution buffer once acetonitrile is removed, by simple heating. The insoluble MMTr-OH byproduct can be removed by either extraction with ethyl acetate or by a desalting method of choice. The streamlined purification process reduces the overall complexity and time required for amino-modified oligonucleotide purification while eliminating the use of corrosive acids and the corresponding waste streams. We envision this method to be of utility to the field, increasing access to oligonucleotide conjugate materials and expanding the use case of these species in the improvement of human health and beyond.

Conflict of Interest

All authors are stockholders of Elsie Biotechnologies Inc.

Data Availability Statement

A list of all oligonucleotide sequences used in this study along with corresponding data that support these findings are available in the Supporting Information of this article.

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