Phosphate groups are often crucial to biological activity and interactions of oligonucleotides, but confer poor membrane permeability. In addition, the group’s liability to enzymatic hydrolysis is an obstacle to its use in therapeutics and in biological tools. We present the synthesis of N-oxyamide and squaramide modifications at the 5'-end of oligonucleotides as phosphate replacements and their biological evaluation using the 5'-exonuclease SNM1A. The squaryl diamide modification showed minimal recognition as a 5'-phosphate mimic; however, modest inhibition of SNM1A, postulated to occur through metal coordination at the active site, was observed. Their facile incorporation after solid-phase synthesis and recognition by the exonuclease makes squaryl diamides attractive neutral 5'-phosphate replacements for oligonucleotides. This work is the first example of squaryl diamide modifications at the 5'-terminal position of oligonucleotides and of the potential use of modified oligonucleotides to bind to the metal center of SNM1A.

Introduction

Modified nucleotides are commonly used as antiviral agents and oligonucleotides such as siRNA are becoming increasingly popular as potential therapeutics. Two of the major obstacles in this area are the enzymatic lability of phosphate groups, which serve as key recognition elements, and the poor membrane permeability conferred by them. Phosphate bioisosteres such as phosphonates, phosphorothioates, boronophosphates and squaramides, are commonly used to overcome these problems and have been the subject of several reviews. Mononucleotide analogues with isosteres at the 5'-position are being investigated as antiviral agents, as the introduction of the mimic overcomes the rate limiting phosphorylation of the nucleoside drug. The activity of siRNA depends on 5'-terminal phosphorylation by kinases, which limits chemical modifications of siRNA as extensive modification can compromise recognition by kinases. The introduction of bioisosteres could circumvent this issue. The introduction of 5'- (E)-vinylphosphonate in place of a 5'-phosphate group conferred enhanced activity on single stranded siRNA in cells and animals due to enhanced nuclease resistance.

Substitution of the phosphate group by 5'-methylene phosphonate lowered the activity of single stranded siRNA, but conserved it for double stranded siRNA. A 5'-malonate group retained or enhanced silencing activity and metabolic stability of siRNA compared to its phosphorylated analogue. However, the use of terminal 5'-phosphate replacements on oligonucleotides is challenging, as the mimetics need to withstand solid-phase oligonucleotide synthesis or be amenable to introduction using mild conditions post solid-phase synthesis.

In this regard, squaramides are of interest as they are neutral phosphate bioisosteres formed by the reaction of squarate esters with amines. The reaction is highly chemoselective, facilitating the introduction of amine substituents in aqueous medium and thereby permitting late-stage diversification. Squaramides have a partial negative charge on the carbonyl oxygen atoms and can coordinate to magnesium ions, replicating the behavior of a phosphate group. Oligonucleotides containing an internal squaramide form duplexes despite their bent structure at the squaramide linkage. Mononucleotides with 5'-squaramides co-crystallize in the active site of translation initiation factor eIF4E and nucleotides containing this group bind to the active site of ADP ribosylhydrolase MDO1. Squaramides have also been employed as isosteres for phospholipids with potential applications in liposome-based therapies. Their widespread use makes them promising candidates as 5'-phosphate mimetics for short oligonucleotides.

Furthermore, phosphate groups often coordinate to metal ions in proteins, suggesting metal chelators such as hydroxamic acid, used to target metalloproteases, could also act as phosphate replacements. N-Oxyamides, derivatives of hydroxamic acids, can withstand solid-phase oligonucleotide synthesis and potentially bind metals in a similar manner to the phosphate group. Mononucleotides containing amides as
phosphate bioisosteres successfully bind to MDO1, supporting this hypothesis.\textsuperscript{[13]} The objective of this work is the synthesis of oligonucleotides containing 5'-phosphate replacements, specifically an N-oxyamide and different squaramides, and their evaluation as phosphate isosteres. The DNA repair exonuclease SMN1A, which acts with a 5'-to-3' polarity, was selected since its activity is absolutely dependent upon the presence of a terminal 5'-phosphate group.\textsuperscript{[19]} Moreover, SMN1A possesses an unprecedented capacity to digest substrates containing drastically altered bases.\textsuperscript{[20,21]} We have examined whether the replacements act as a substrate for the exonuclease SMN1A or whether they inhibit enzymatic activity.

Results and Discussion

Synthesis of modified oligonucleotides

The synthetic targets were designed based on known isosteres and metal-chelating groups, with the aim of achieving similar molecular recognition by SMN1A through hydrogen bonding or metal coordination compared to the natural 5'-phosphate group or phosphodiester bond.\textsuperscript{[22]}

N-Oxyamide-containing oligonucleotides 1a-b, based on the known metal-binder hydroxamic acid, and three squaramide derivatives 2a-4b, based on their use as phosphate backbone replacements in DNA,\textsuperscript{[13]} were synthesized (Figure 1).

![Figure 1. Structure of modified oligonucleotides 1–4 containing phosphate replacements at the 5'-end, positive control 5a with 5'-phosphate group and negative controls 6a-b with 5'-OH group.](image)

The introduction of an acetyl protected 5'-hydroxamic acid group, designed to undergo deprotection after solid-phase synthesis, was attempted but in our hands no suitable phosphitylating conditions could be identified. The synthesis of the 5'-oxyamide phosphoramidite 11 was carried out in six steps, giving good to excellent yields (Scheme 1). Oxyamine 9 was prepared from thymidine (7) in three steps as reported previously.\textsuperscript{[23]} Briefly, a Mitsunobu reaction was used to introduce the oxyamine functionality, followed by protecting group manipulations to afford oxyamine 9. Amide coupling between the oxyamine 9 and acetic acid, followed by TBDMS deprotection, gave alcohol 10. Subsequent phosphitylation yielded phosphoramidite 11 for incorporation into oligonucleotides. Modified oligonucleotides bearing a 3'-Cy3 fluorophore 1a or a free 3'-OH group 1b were synthesized to allow for visualization where necessary. As the coupling of the modified monomer was not quantitative and separation of the unmodified 20mer from the oxyamide-containing 21mer proved challenging, the oligonucleotides were used as a 2:3 mixture of 20mer to 21mer 1a and a 2:3 mixture of 20mer to 21mer 1b. Consequently, observed activity will be underestimated due to the presence of inactive 5'-OH oligonucleotide.

The 5'-squaryl monoamide phosphoramidite 15 was synthesized in five steps, giving moderate to excellent yields, using modified literature procedures (Scheme 2a).\textsuperscript{[24–26]} Intermediate iodothymidine 12 was prepared from thymidine (7) using an Appel reaction\textsuperscript{[24]} before reaction with sodium azide\textsuperscript{[25]} and Staudinger reduction to give amine 13. This monomer was reacted with diethyl squarate to give squaryl monoamide 14,\textsuperscript{[26]} which was successfully phosphitylated. However, incorporation of phosphoramidite 15 into oligonucleotides in solid-phase synthesis was unsuccessful. The squaramide modification was therefore introduced by reaction of 5'-amino oligonucleotides 16a-b in aqueous solution with dimethyl squarate to give

![Scheme 1. Synthesis of 5'-oxyamide modified oligonucleotides 1a-b.](image)
The fluorescent modified oligonucleotides 1a-4a and the positive control 5a were incubated with SNM1A and their hydrolysis products were analyzed by Urea PAGE. If a substituent in the 5′-position binds similarly to the natural substrate, hydrolysis of the oligonucleotide backbone is anticipated (i.e. the modified oligonucleotide acts as a substrate) (Figure 2a). 5′-Phosphorylated oligonucleotide 5a is a substrate for SNM1A and is hydrolyzed to eleven nucleotides in size (Figure 2b, lane 2), consistent with Allerston et al.22 No hydrolysis was observed for the squaryl monoamide modified oligonucleotide 2a or the N-oxyamide modified oligonucleotide 1a (Figure 2b, lanes 4 and 10) while minimal digestion was observed for the squaryl diamide modified oligonucleotides 4a and 3a relative to the control 5a (Figure 2b, lanes 6, 8 and 2). These results suggest that the squaryl diamides were binding in a similar manner to the 5′-phosphate group present in the natural substrate to a small degree, while oxyamides and squaryl monoamides do not show any recognition in this context. Further attempts to optimize the hydrolysis with regards to concentration of the modified oligonucleotide 4a resulted in no observable cleavage when deviating from the conditions shown.

**Evaluation of modified oligonucleotides as inhibitors of SNM1A**

As an alternative mechanism of action, coordination of the phosphate replacement to the zinc ions in the active site of SNM1A was examined by incubating non-fluorescent modified squaryl monoamide oligonucleotides 2a-b with quantitative conversion. Diethylamine or methylamine derivatives were then generated by reaction of 2a-b with the desired amine to give squaryl diamides 3a-b and 4a-b (Scheme 2b). Finally, oligonucleotides containing a 5′-OH group were synthesized as negative controls (6a-b), with 6a phosphorylated enzymatically using T4 polynucleotide kinase to give 5′-phosphate containing oligonucleotide 5a as a positive control for enzymatic activity (Figure 1).

**Scheme 2.** Synthesis of 5′-modified oligonucleotides. a) Synthesis of 5′-squaramide modified phosphoramidite 15. b) Synthesis of squaramide-modified oligonucleotides 2a-b, 3a-b and 4a-b after solid-phase synthesis.

**Figure 2.** Evaluation of modified oligonucleotides as substrates for SNM1A. a) Modified 3′-Cy3 labelled oligonucleotides are hydrolyzed if binding to the active site in a similar manner to oligonucleotides containing a 5′-phosphate group. b) Digestion of oligonucleotides 1a-5a (0.8 pmol, 80 nM) by SNM1A (25 fmol, 2.5 nM) after 60 min. nt = nucleotides.
oligonucleotides 1b-4b with SNM1A in the presence of fluorescent 5'-phosphorylated oligonucleotide 5a. In cases where the phosphate replacements bind stably to the enzyme active site, they act as inhibitors and hydrolysis of the natural substrate is less efficient (Figure 3a). In this assay, as expected, the 5'-OH negative control 6b did not affect the hydrolysis rate of the fluorescent 5'-phosphorylated oligonucleotide 5a when added in equimolar concentrations compared to the substrate (Figure 3b, lanes 1–2), indicating that any observed inhibitory effect is due to the presence of the 5'-modification rather than simply due to the presence of additional oligonucleotides. One equivalent of modified oligonucleotide compared to the substrate led to decreased enzyme activity for the squaryl diamides 3b and 4b (Figure 3b, lanes 5–6), but not for oxyamide 1b and squaryl monoamide 2b (Figure 3b, lanes 3–4). This suggests that squaryl diamides 3b and 4b form stable complexes with the metal center, blocking the enzyme active site, analogous to the binding of the cephalosporin ceftriaxone.\textsuperscript{[27]} This inhibitor was shown to bind to the metal center in SNM1A through two carbonyl groups when co-crystallized with SNM1A.\textsuperscript{[28]} Next, a concentration-dependence study was carried out with oligonucleotide 4b (Figure 3c) to explore this finding. Firstly, for the negative control 6b, a decrease in the hydrolysis rate was observed when present in concentrations above one equivalent compared to the phosphorylated oligonucleotide (Figure 3c, lanes 3 and 5). However, this effect was less than with the modified oligonucleotide 4b (Figure 3c, lanes 4 and 6). The most pronounced difference after 60 min was observed at 3.3 equivalents of oligonucleotide 4b or 6b (2.7 pmol, 264 nM) relative to 5a (0.8 pmol, 80 nM) after varying incubation times with 5 min preincubation (Figure 3d, lanes 5–19). The addition of either oligonucleotide leads to a decrease in hydrolysis rate, visible from 10 minutes onwards (Figure 3d, lanes 5–19). However, the modified oligonucleotide 4b has a greater impact on the rate than the 5'-OH negative control 6b between 20 and 60 minutes incubation (Figure 3d, lanes 8–16), with the

![Figure 3](image_url)

**Figure 3.** Evaluation of modified oligonucleotides as inhibitors of SNM1A. a) Modified oligonucleotides act as inhibitors if binding to the metal center in the active site, preventing the hydrolysis of 3'-Cy3-labelled 5'-phosphorylated oligonucleotide 5a. b) Digestion of oligonucleotide 5a (0.8 pmol, 80 nM) by SNM1A (25 fmol, 2.5 nM) in the presence of oligonucleotides 1b-4b or 6b (0.8 pmol, 80 nM) after 60 min. c) Digestion of oligonucleotide 5a by SNM1A (25 fmol, 2.5 nM) in the presence of different amounts of oligonucleotide 4b or 6b relative to 5a (0.8 pmol, 80 nM) after 60 min with 5 min preincubation. d) Digestion of oligonucleotide 5a by SNM1A (25 fmol, 2.5 nM) in the presence of 3.3 equivalents of oligonucleotide 4b or 6b (2.7 pmol, 264 nM) relative to 5a (0.8 pmol, 80 nM) after varying incubation times with 5 min preincubation. nt = nucleotides.
biggest contrast after 60 minutes (Figure 3d, lanes 14–16). The same effects were observed for squaryl diamide 3b (Figures S1-S2 in Supporting Information), suggesting that there is no significant difference between the binding of the secondary squaryl diamide 4b and the tertiary squaryl diamide 3b.

For quantification of the effect of the squaramide-modification, the hydrolysis of 5’-phosphorylated oligonucleotide 5a in the presence and absence of 5’-OH or 5’-modified oligonucleotides 6b and 4b after 10 minutes was analyzed by Urea PAGE (Figure 4a). At this time point the reaction is still in the linear range. The efficiency of hydrolysis was determined by measuring the amount of fluorescent substrate 5a remaining after 10 minutes incubation with SNM1A relative to a control reaction without SNM1A. Nine independent experiments were carried out in triplicate (Figure 4a and S3). Our results show that in the absence of any additional oligonucleotide, 33% of substrate 5a remained after the reaction. While the addition of 5’-OH oligonucleotide 6b increased this to 37%, the addition of 5’-modified oligonucleotide 4b showed a slightly stronger effect, with 41% of oligonucleotide 5a present after the reaction (Figure 4b).

The difference in inhibition between the squaramide-modified oligonucleotide 4b and 5’-OH oligonucleotide 6b illustrates a modest inhibitory effect of the squaramide moiety. This is postulated to occur through the squaramide binding to the metal center of SNM1A. The observed inhibition and proposed binding is consistent with the previously reported metal binding of squaramides[13] and the binding mode of ceftriaxone to the metal center in SNM1A through two carbonyl groups[24].

Conclusions

In conclusion, we report the synthesis of four different 5’-modifications of deoxyoligonucleotides. An N-oxamidemodification was introduced at the 5’-position of a nucleoside and incorporated using solid-phase oligonucleotide synthesis, while three squaramide modifications were introduced chemoselectively after solid-phase synthesis to circumvent difficulties arising during solid-phase oligonucleotide synthesis. This strategy permits access to a wide range of substituted squaramides that can be introduced post-solid-phase, allowing for late stage diversification and circumventing the requirement for compatibility with solid-phase synthesis. The recognition and binding mode of the phosphate replacements was examined using the exonuclease SNM1A, showing minimal recognition through hydrogen bonding for both squaryl diamide modifications. Inhibition was observed for the same squaryl diamides, postulated to be enhanced through metal coordination of the squaramide moiety. Our results demonstrate a novel synthetic methodology for the synthesis of 5’-squaramide modified oligonucleotides and illustrates their potential use as metal-binding phosphate substitutes. The facile introduction of the group as well as of different substituents makes squaramides attractive 5’-modifications for the wider scientific community.

Supporting Information Summary

The supporting information contains procedures for the synthesis of modified nucleosides, NMR spectra, procedures for oligonucleotide synthesis, mass spectra of oligonucleotides, procedures for enzymatic assays and additional figures.

Acknowledgements

This work was supported by the Wellcome Trust ISSF (JM & WD), Trinity College Dublin with an Ussher Postgraduate fellowship (EMD), the RSC with a Researcher Mobility Grant (EMD) and UK BBSRC grants BB/J001694/2 (extending the boundaries of nucleic acid chemistry, TB). We thank O. Gileadi (SGC) for providing us with recombinant SNM1A enzyme.

Conflict of Interest

The authors declare no conflict of interest.

Keywords: Exonuclease · metalloenzyme · 5’-modification · oligonucleotide · squaramide


Submitted: October 26, 2018
Accepted: November 19, 2018