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SYNTHESIS AND STUDIES OF 2´-O-ALKYLATED OLIGONUCLEOTIDES WITH ENHANCED STABILITY AND CELLPENETRATING PROPERTIES

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SYNTHESIS AND STUDIES OF 2´-O-ALKYLATED OLIGONUCLEOTIDES WITH ENHANCED STABILITY AND CELLPENETRATING PROPERTIES

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Stefan Milton

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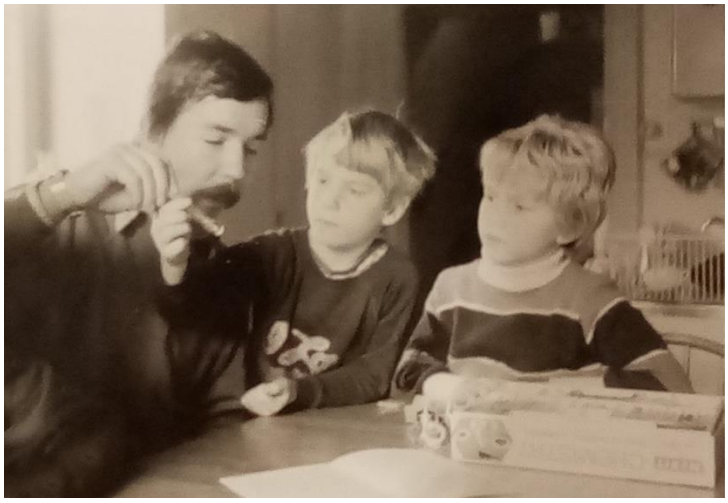
Preface

My great interest in science in general and chemistry in particular started early. I remember the first time I saw a match being lit against a torch, which amazed me greatly. How is that possible? Luckily, I had an uncle Leif Granström who was a geologist and could explain in detail and pedagogically how chemistry works. Already at the age of four, I wanted the experimental box "Little Chemist" which was available in the well-sorted toy store. Two years later I got it! My father, who had extensive knowledge in chemistry, instructed me in various types of experiments. When I started high school, I got a dedicated chemistry teacher who encouraged my great interest in chemistry and let me experiment in the school laboratory. Lots of fireworks of course. In the gymnasium, I chose to continue with my interest by choosing a technical line with a focus on chemistry. After the gymnasium, I went on to the chemical engineering education at KTH. The education was strongly focused on industrial process technology and I later chose to continue my education in chemistry at Stockholm University. There I chose the organic chemistry department that I knew was outstanding. I studied advanced organic chemistry and was lucky enough to meet Susanna Sigurdsson who introduced me to her professor Roger Strömberg. I was very inspired by the exciting field of nucleotide chemistry that Roger was active in. At the end of my studies, I had the opportunity to do my degree project with Roger, who had just moved to Bioorganic Chemistry at MBB at Karolinska Institutet together with Ester Yeheskiely. Then I started my journey as a doctoral student, that you can read about in this thesis.



My Father Ulf ,brother Oskar and I with the "Little Chemist"

“Why, sometimes I've believed as many as six impossible things before breakfast.”

― Lewis Carroll, Alice in Wonderland

ABSTRACT

Basic structures of natural oligonucleotides (ONs) are RNA and DNA are giving rise to conformations and secondary structures inside cells and are the fundamentals for life. Many chemically modified oligonucleotides have been synthesized over the years. Studying the mechanisms on how RNA and DNA interact with different type of modified oligonucleotides will and have given new understandings of their functions. It is also important in the order of pursuing a new type of medicines. Today we can find that these molecules have been realized in certain types of medicines for hereditary diseases and as vaccines, for example.

This thesis describes synthetic strategies to be able to perform 2´-OH alkylations and which protecting groups are required to reach the target compounds. This includes the synthesis of modified nucleotide building blocks intended for oligonucleotide synthesis. The goal is to reach synthesis methods of oligonucleotides with a 2´-O-carbamoylmethyl (CM) functionality including the version of the CM where the amido group is extended with ethylene-amine giving the 2´-O-[N-(aminoethyl)- carbamoyl]methyl (AECM) modification (Figure 1) . Thereafter, to study these molecules with respect to:

- Protecting group strategy for the synthesis of the 2´-O-modified building blocks.
- Chemical stability of the modification during conditions used in oligonucleotide synthesis.
- Stability against degradation by nucleases (enzymes) provided by the CM and AECM modification.
- Binding ability to RNA and DNA.
- Study of how the 2´-O-AECM functionality affects cell uptake.

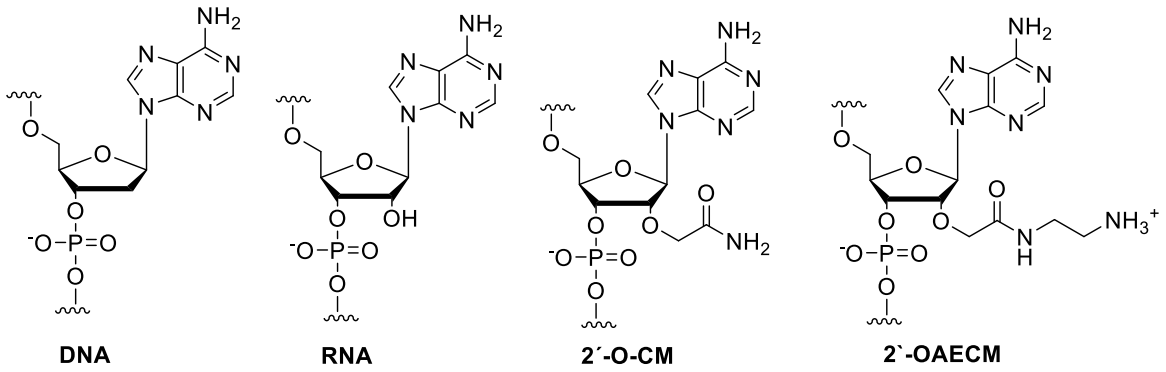


Figure 1: Parts of oligonucleotides showing their structural difference.

Paper I present the synthesis of a 2 ′-O-carbamoylmethyl (CM) containing H-phosphonate building block as well as synthesis of model dinucleotide. Paper II studies the stability of the 2´-*O*-carbamoylmethyl (CM) group under ammonolysis conditions used during oligonucleotide synthesis as well as enzymatic stability of the phosphate diester bond vicinal to the 2´-O-carbamoyl group. Paper III present two different classes of protection for the uridine lactam function, intended for use in synthesis of 2'-O-alkyl-uridines. These are benzoyl protections and different acetal functions. Paper IV includes strategies for the synthesis of a 2`-

O-[N-(aminoethyl)-carbamoyl]methyl (AECM) modified H-Phosphonate building block and the synthesis of a model dinucleotide. In addition, studies on the chemical and enzymatic stability of this dinucleotide is reported. Paper V present the synthesis of a 2`-O-AECM modified phosphoramidite building block and the synthesis of AECM-modified oligonucleotides. These AECM-oligonucleotides are studied with respect to resistance towards enzymatic degradation and cellular uptake.

LIST OF SCIENTIFIC PAPERS

I. “Synthesis of A 2′- O -(Carbomoylmethyl)Ribonucleoside H-Phosphonate Building Block and A Model Dinucleotide”
Stefan Milton, Raunak, Esther Yeheskiely & Roger Strömberg.
Nucleosides, Nucleotides & Nucleic Acids,Volume 26, 2007 - Issue 10-12

II. “Stability of a 2′-O-(Carbamoylmethyl)adenosine-Containing Dinucleotide”
Stefan Milton, Charlotte Ander, Esther Yeheskiely, Roger Strömberg.
EurJOC, 2012, 3, 539-543

III. “Evaluation of lactam protection for synthesis of 2'-O-alkylated uridines”
Stefan Milton, Roger Strömberg.
Nucleosides Nucleotides Nucleic Acids, 2007;26(10-12):1491-3

IV. “Synthesis and Stability of a 2′-O-[N-(Aminoethyl)carbamoyl]methyladenosine-Containing Dinucleotide”
Stefan Milton, Charlotte Ander, Dmytro Honcharenko, Małgorzata Honcharenko, Esther Yeheskiely, Roger Strömberg.
EurJOC, 2013,31, 7184-7192.

V. “Nuclease resistant oligonucleotides with cell penetrating properties” Stefan Milton, Dmytro Honcharenko, Cristina S. J. Rocha, Pedro M. D. Moreno, C. I. Edvard Smith and Roger Strömberg. ChemCom, 2015,51(19), 4044-4047.

Scientific papers not included in the Thesis:

Honcharenko D, Rocha CSJ, Lundin KE, Maity J, Milton S, Tedebark U, Murtola M, Honcharenko M, Slaitas A, Smith CIE, Zain R, Strömberg R.
2′-O-Aminoethylcarbamoylmethyl (AECM) modification allows for lower phosphorothioate content in splice-switching oligonucleotides with retained activity. *Nucl Acid Ther*, 2021, accepted with minor revision.

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LIST OF ABBREVIATIONS

A	Adenosine
AECM	2`-O-[N-(aminoethyl)- carbamoyl]methyl
CM	Carbamoylmethyl
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DCA	Dichloroacetic acid
DCM	Dichloromethane
DiPEA	N,N-Diisopropylethylamine
DMAP	4-dimethylaminopyridine
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
DMT	Dimethoxytrityl
EMA	European medicines agency
EXON	Encoding part of a gene
EtOH	Ethanol
FDA	Food and Drug Administration
HMBC	Heteronuclear Multiple Bond Correlation
HMDS	Hexamethyldisilazane
HPLC	High-performance liquid chromatography
LAH	Lithium aluminium hydride ($LiAlH_4$)
MeCN	Acetonitrile
MeOH	Methanol
MPDS	methylene-bis-diisopropylsilyl
NaH	Sodium hydride
NMR	Nuclear magnetic resonance
ON	Oligonucleotide
OXP	Bis(2-oxo-3-oxazolidinyl) phosphinic chloride
PDE	Phosphodiesterase
RISC	RNA-induced silencing complex

SEM	2-(Trimethylsilyl)ethoxymethyl
TBDMS	Tert-butyldimethylsilyl
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TIPS	Tetraisopropyldisiloxane
TLC	Thin layer chromatography
TMS-Cl	Trimethylsilyl chloride
TOM	Triisopropylsiloxymethyl
MMT	Monomethoxytrityl

1 INTRODUCTION

There is great interest in developing the chemistry around oligonucleotides (ONs). The effect of many types of nucleotide modifications incorporated into ONs has been studied. Most modifications of ONs aim to target RNA and DNA oligomers. Synthesis methods for building the crucial internucleosidic phophodiester linkage has been known since the early 1950:s (1, 2) and ON synthesis was pioneered during the coming decades to lead to the first chemical synthesis of a Gene (3), but they continue to be developed and refined (12). One type of many ON modifications that have been of great interest is 2´-O-Alkyl modifications, which this thesis will focus on. The field of ON applications is constantly growing.

Basic structures of RNA and DNA giving rise to conformations and secondary structures:

There are essentially two different major conformations of what is called the sugar pucker in nucleic acids. The stereoelectronic effects of DNA and RNA differ, due to the electronegative 2´-OH group, which is present in RNA but which DNA lacks. The stereoelectronic gauche effect contributes to that RNA normally assumes a so-called North (C3´-endo) conformation which preference in an RNA/RNA double helix results in an A-form conformation. While DNA having a hydrogen in the 2´ position assumes a South sugar pucker (C2´ endo) conformation and a DNA/DNA double helix forms a B-conformation (4-6). The A-form and B-form conformations differ significantly with respect to the distance between the phosphate groups (7, 8). DNA duplexes rich in poly dA-dT (A-tract) do not adopt the A-form but DNA duplexes rich in poly dG-dC (G-tract) can adopt the A-form. DNA/RNA hybrid duplexes usually adopts the A-form, just like many 2´-O-Alkyl/DNA hybrids do (Figure 2) (9).

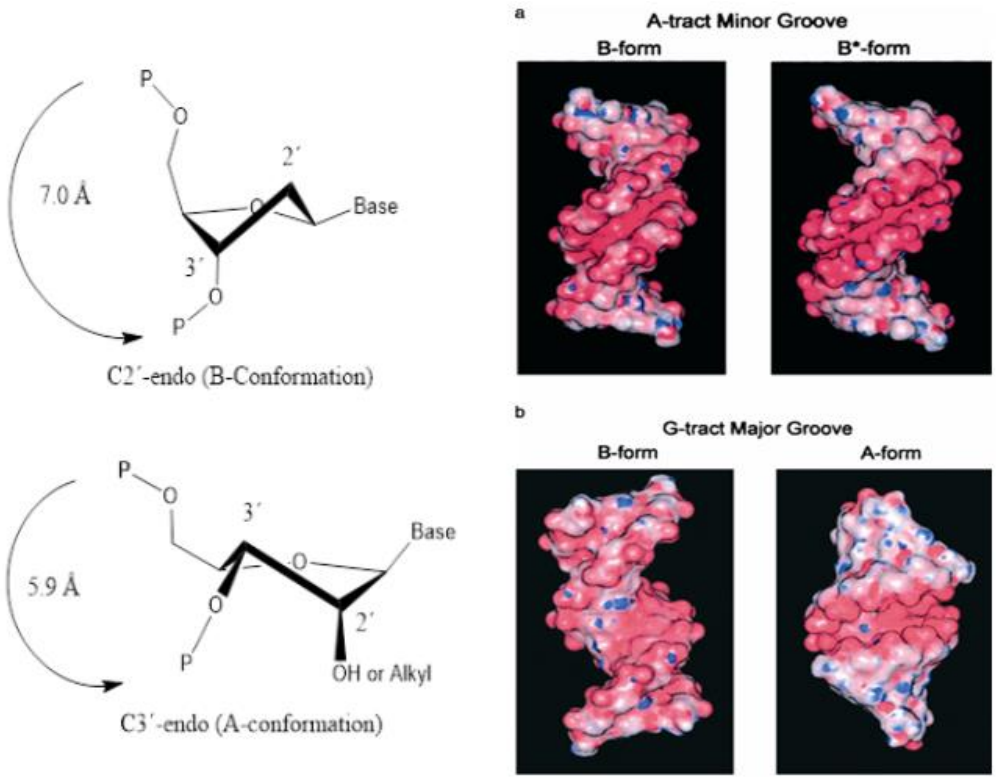

Figure 2. The stereoelectronic effect of the 2´-moiety gives rise to different conformations of the sugar pucker, that results in A and B-form conformations of ON-duplexes.

Oligonucleotide therapy:

Oligonucleotide (ON) therapy is a rapidly growing approach for treatment of disease with more than 10 FDA/EMA approved drugs and many more are currently in clinical trials (10-13). There are a number of different approaches by which therapeutic function can be achieved (Figure 2). These include classical antisense action of a single stranded ON that targets an RNA molecule (most commonly an mRNA). The idea of Antisense comes from the 1960s(14, 15), where the Antisense ONs acts as a steric blocker of the m-RNA, resulting in translation arrest of m-RNA into peptides. The efficiency is usually empowered by catalytic cleavage of the target RNA by the hosts own RNase H enzyme. That normally recognizes double-stranded RNA:DNA hybrids and cleaves the RNA to give a 3´-OH and a 5´-phosphate (16). Another approach is the use of double stranded RNA, short interfering RNA (siRNA) that achieves the same action as an antisense ON aided by enzymatic cleavage of the target by the RNA-induced silencing complex (RISC). Another approach is targeting of pre-mRNA in order to change the maturation, splicing, that creates the mature mRNA. A corrected mRNA can then be produced, by use of a so called splice switching ON, leading to either the native protein or a protein that has partial functionality (Figure 3).

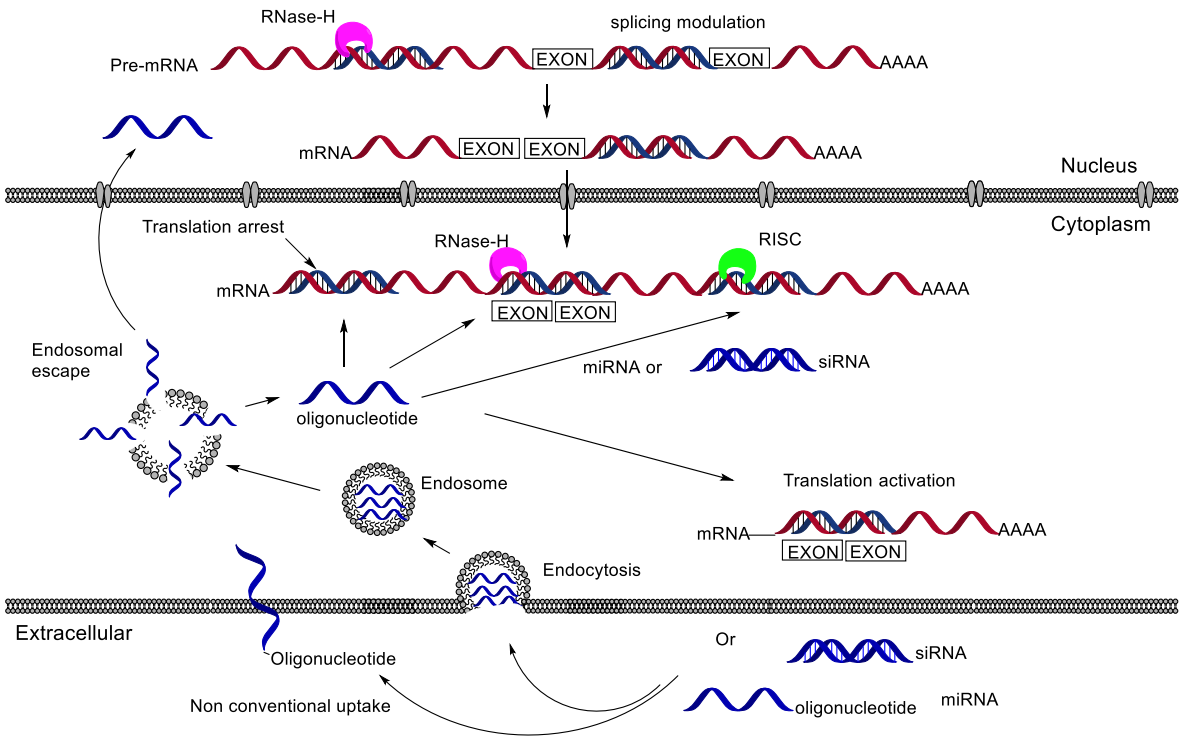

Figure 2. Different modes of action in oligonucleotide therapies.

All approaches for ON therapy require modifications in order to be efficient since unmodified RNA and DNA ONs are rapidly degraded in biological fluids. Numerous modifications with different properties and traits of ONs has been discovered. A combination of modifications is

often advantageous in order to achieve desirable properties of the ON(13). But some of the traits are contradictory to other desirable properties. Some desirable properties of ONs for "classical" antisense therapeutic applications are:

- A strong binding to the complementary target RNA (typically mRNA) sequence. Rapid binding to the RNA to then detach from the target fragments after cleavage by ribonuclease H.
- High stability against nucleases. Which means that the antisense molecule can function for a longer period of time.
- High target specificity. So that only the target sequences that are meant to be targeted, become the sequences that are affected. This can be adversely affected by too strong binding to target RNA.
- Increased cellular uptake of the ON. This can be done by chemically altering the ON. Some modifications that increase cellular uptake are known, including ON conjugates (17-24).
- Antisense ONs linked to a target-seeking molecule, creating a ON conjugate. That allow the Antisense ON to find the cells that are to be treated. Often the selectivity is looked for in extracellular proteins and receptors, e. g, receptors for steroids, sugars, peptides etc.
- An ON molecule can also be bound to a catalytic molecule, often a metal chelate, that acts as an artificial nuclease. The artificial nuclease cleaves the target m-RNA specifically, often in a double helix with a bulge(25).
- That the Antisense molecule is not toxic or forms toxic fragments and by-products.
- The ON should not induce immune response.

Oligonucleotide synthesis:

There are several different ways to synthesize ONs. All synthetic methods require protecting group strategies. The first synthesis methods were developed for the synthesis of DNA and RNA but are also suitable for many nucleoside modifications. The historically more well-known synthesis methods are the H-Phosphonate (26), Phosphodiester (27), Phosphotriester (28), and phosphite triester methods (28). The most widely used method today is the phosphite triester approach in the form of the phosphoramidite method (29-31). Most commonly used synthesis methods extend the ON from the 3´-end with a subsequent deprotection of the 5´-OH which is then connected to the next 3´-O-Phosphorus derivative, and so on (32). In addition to DNA and RNA building blocks, there are also many modified phosphoramidites available on the market. A phosphoramidite nucleoside building block is usually composed of an acid labile trityl group at the 5`-oxygen (33-35) and a 3´-O-[O-(2-*cyanoethyl*)-N,N-*diisopropyl*]-*phosphoramidite* at the 3´-oxygen. The exocyclic nucleobase-NH_2 groups are often protected as amides (36). Several other protecting groups may be needed, depending on which nucleoside that is used (33, 37). For RNA synthesis the 2´-OH group, several different protecting groups can be used, TBDMS(29), TOM(30, 38) and many more. There are several reasons why the phosphoramidite method gained a lot of ground commercially. Some of the reasons are the high-speed synthesis with fast coupling rates, great product purity and high yields. The

phosphoramidite method usually starts with a solid support from which the oligonucleotide is then built(39). The reason for using a solid support is that you can easily wash off reagents after each reaction step. Otherwise, there would be an increased risk of side reactions during ON synthesis, which would have a negative effect on the yield. For each new building block to be connected, a sequence of several reaction steps takes place (Figure 4). The most commonly used steps are:

- Acidic deprotection of the 5´-O-trityl group and then washing. Often with di-or-tri-chloroacetic acid in dichloromethane.
- Coupling/condensation of the next phosphoramidite to the deprotected 5´-OH hydroxyl. 1H-tetrazole (or derivatives thereof) is often used in acetonitrile. The coupling reaction forms a phosphite triester.
- Capping reaction with acetic anhydride and usually 1-methylimidazole as base. The capping is mainly performed to prevent the unprotected 5´-OH groups that did not react in the previous coupling reaction, from continuing to build on in later couplings. This would otherwise result in a larger number of long oligonucleotides with the wrong sequence.
- Oxidation of the formed phosphite triester to a phosphate triester. The oxidation is usually performed with Iodine / H_2O and Pyridine as solvent and base.

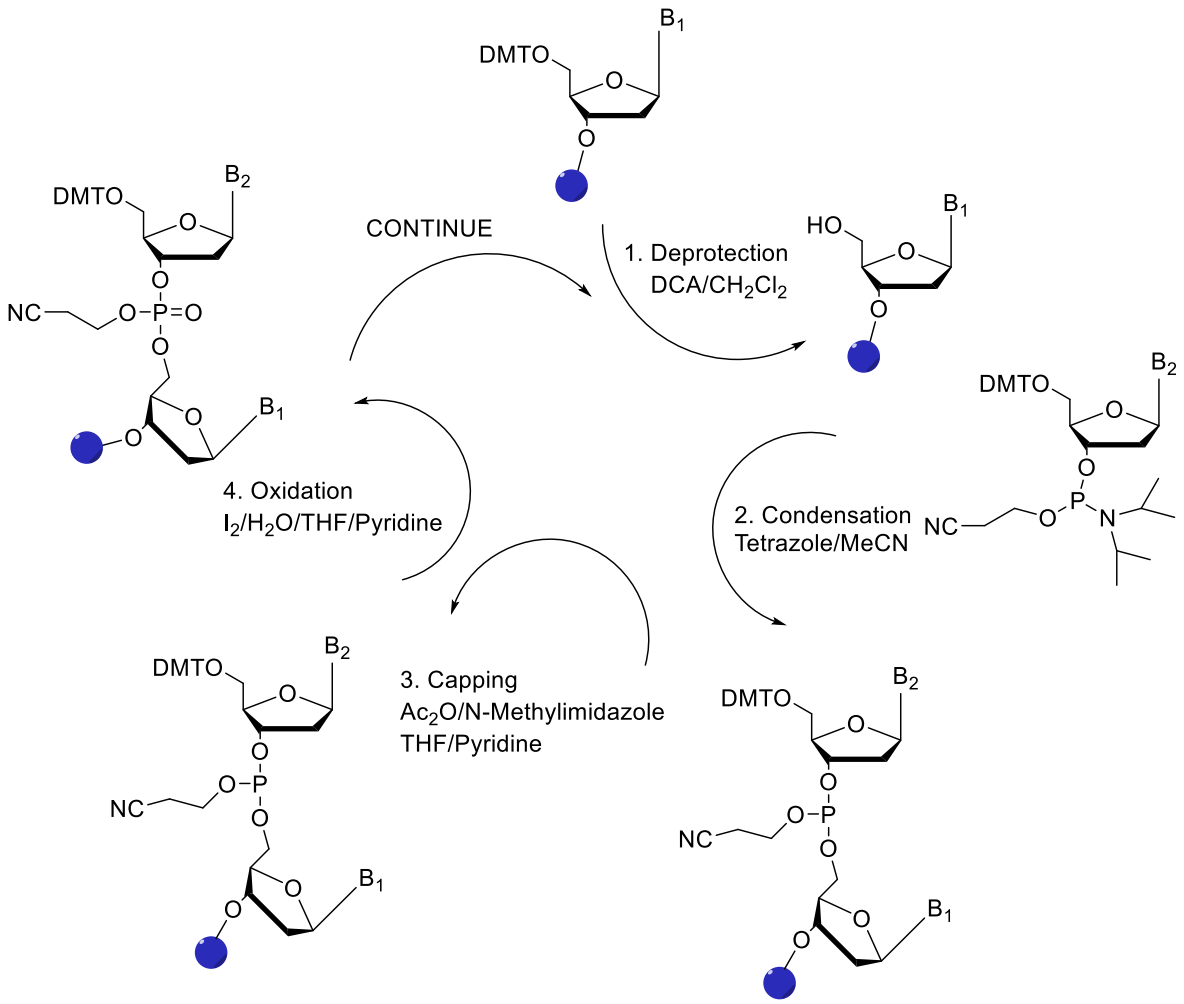

Figure 4. Oligonucleotide synthesis via the Phosphoramidite method.

In the last step of the synthesis, the last trityl group is deprotected (or kept for a first trityl-on purification). Thereafter, the oligonucleotide is cleaved from the solid support and at the same time the exocyclic amino groups are deprotected. The cyanoethyl group is also deprotected on the phosphate triester to form the final 3´-5´phosphate linkage. This is often done with ammonia. Optimization of ON protection methods is continuously under progress(36), especially when the goals are to be able to perform the syntheses on a large scale with few side reactions and with a product of higher purity and yield. Large scale synthesis of ONs has for a long time been a major challenge, where new synthesis methods are constantly being evaluated. New synthesis methods where solid phase synthesis has been departed from, to carry out the reactions in solution have also been developed (40-43).

Side reactions during oligonucleotide synthesis:

Several different parts of the synthesis steps can give side reactions such as depurination upon the acidic deprotection of the 5´-OH group. Depurination takes place most easily on deoxyadenosine and deoxyguanosine but can be prevented by choosing a protecting group on the exocyclic-NH_2 amino function. Alternative protecting groups that gives less depurination has been developed(36). Another side reaction during the ammonolysis step is the substitution of the exocyclic amido function of Cytidine that converts the nucleobase to Uridine. This can be avoided by choosing the right nucleobase protecting group (e.g., acetyl). In the coupling reaction, other side reactions can take place such as branching via the exocyclic Guanosine-O^6 function during phosphitylation and reactions such as acylation of exocyclic amino groups. The end result depends largely on the type of synthesis method, protecting group strategy and reagents that are being used. If side reaction occurs within each cycle, when a new building block is connected or deprotected, even small changes can cause a low overall yield of the final ON product. Even side reactions that occur at less than 1% can be devastating, as the sum of the results is multiplicative. The importance of minimizing side reactions is then of great importance.

Modified Oligonucleotides:

A large number of different modifications of oligonucleotides have been developed (5). The desire to be able to synthesize modified ONs grows with the applications. Depending on the modification, changes in known synthesis methods are often required, so that they work with the intended modification. A rough division into the type of modifications would be: modification of the sugar ring, nucleobases and the phosphate diester backbone. In ON synthesis, different types of modified building blocks can be combined in one and the same sequence, which can then carry a combination of properties that the different modifications exhibit. The modifications must exhibit good interactions with complementary strands so that

there is no steric hindrance or blockage in binding to the complementary nucleic acid strand. The modified ONs should also have good solubility and not create aggregation in blood plasma and in the cytoplasmic fluid. The ONs may interact with other types of biological molecules and cell membranes, other than what they are aimed to target, which can be either positive or negative for their action. The early properties that the ON therapy research focused on where, stability against nucleases and stronger binding to complementary RNA. Examples of some important ON modifications from the earliest to later developed are, 2´-O-alkyl(5), PNA(44), 2´-O-MOE(45), LNA(46), PMO-Morfolino(47), Amino-LNA(48) (Figuire 5), but there are many more.

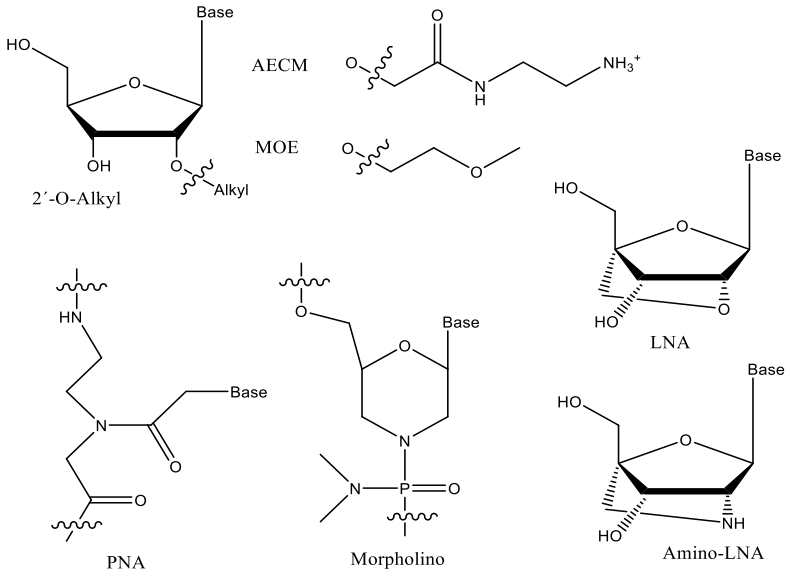

Figure 5. Examples of some important ON modifications.

Further development of ONs to enhance the cellular uptake or give them the ability to penetrate cells is most desirable. Several different modifications of ONs developed to, or known to, enhance the ability to enter cells have been found (Figure 6). Examples of these modifications are 2´-O-PivOM(18), phosphorothioates (PS) (23) and the cationic backbones, BCNSs(20), PNHBuGua(21) and tetramethyl phosphoryl guanidine (Tmg)(24).

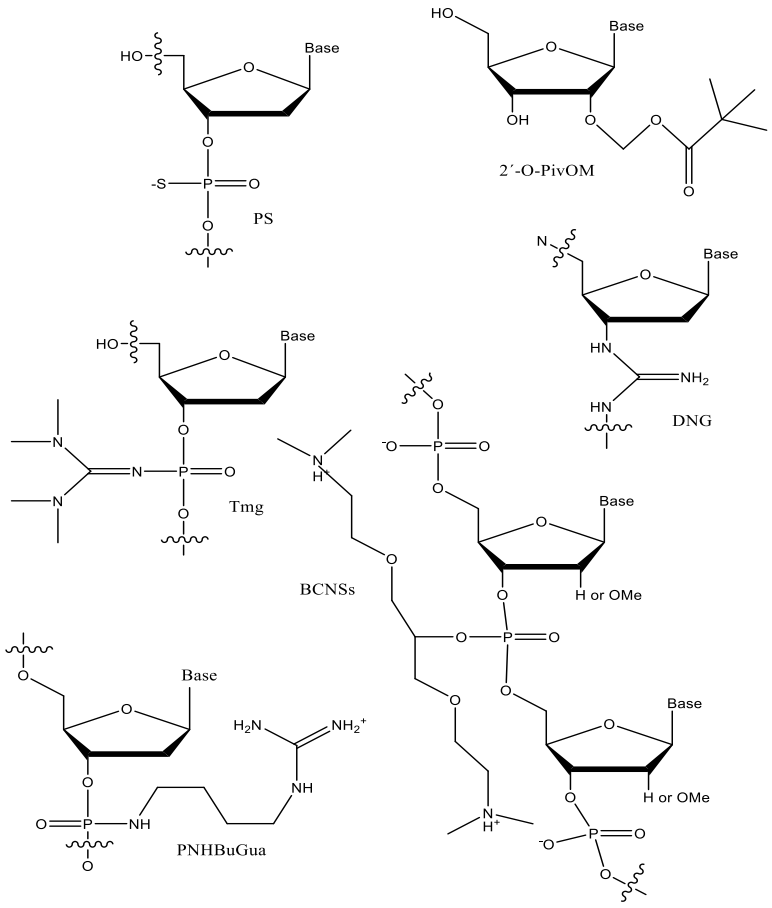

Figure 6. Examples of ON modifications with enhanced cell penetrating properties.

One of the more complex tasks in ON research, is to give oligonucleotides the ability to find the cells intended to be targeted and treated *in vivo*. These modifications are often a so-called oligonucleotide conjugate. The conjugate consists of the ON chemically linked to the target seeking molecule. The conjugated molecule can have the ability to bind to a specific receptor or cell surface that mainly the intended target cells carry. There are also types of ON conjugates that are intended to exert their effect inside the cells, such as a 5´-Cap which, for example, can induce translation of RNA (often used in, for example, RNA vaccines), or to be internalized in the cell nucleus(49) or the m3G-Cap that promote transport into the nucleus(50, 51).

2´-O-alkyl modifications:

One of the more explored areas in ON modifications is the 2´-O-alkyl modification. Relatively early around 1960, it was discovered that the naturally occurring 2´-O-Methyl modification existed. From a synthetic point of view, it is understandable to use ribonucleotides as starting material with relative ease, where the free 2´-OH hydroxyl can be used as reactive group. The 2´-OH position has been shown to be an excellent starting point for altering the properties of oligonucleotides and their interactions with complementary RNA and DNA sequences. Many 2´-O-alkyl oligonucleotides have a stronger binding to complementary RNA and to DNA as

well(5, 17). The duplex stability is usually performed by measuring the melting point of the modified ON with its complementary RNA or DNA in a UV-spectrophotometer. One of the explanations for the stronger binding is that the conformation of 2´-O-alkyl sugar pucker through the gauche effect favors the North (C3´-endo) conformation which in turn strengthens the possibility of being able to form a more stable A-form double helix with RNA. Mention should also be made here of LNA, which is a form of 2´-O-alkyl, where the 2´-oxygen is bound to the 4´-carbon via a methylene bridge. This constricted conformation, locks the sugar ring in a near North (C3´-endo) conformation and has shown to greatly increase the stability of complexes with RNA in a double helix(46). The 2´-O-alkyl group also generally provides protection against degradation by nucleases(52, 53). It has been found that with a 2´-O-alkyl-amino modification an effective inhibition of degradation by nucleases is obtained, which was explained by that the protonated 2´-alkylamino function binds to a Zink pocket of the enzyme and blocks its mechanism(54, 55). Another interesting observation is that some 2´-O-alkyls can reach the minor groove of an A-form double helix and there, depending on the functional groups, be able to stabilize this with hydrophilic groups and hydrogen bonds. In addition, with a sufficiently long functionalised 2´-O-alkyl group it is possibly to reach across the minor groove to the opposite phosphate group and thereby stabilize the entire double helix(56). Other uses for 2´-O-alkylamines are as a position to link conjugates to the ON, such as target recognizing molecules of extracellular target proteins and receptors, ex: Steroids, sugars, peptides and artificial nucleases.

Synthesis and protecting group strategy for alkylation of the 2´-OH:

Synthesis of 2´-O-alkyladenosines:

Adenosine can be alkylated on the 2'-hydroxyl directly in solution using a strong base and an alkyl halide in equivalent amounts(4, 57). This is most easily explained by the fact that the pKa for 2´-OH is the lowest of all free protons in adenosine(58). However, the selectivity between 2´-OH and 3´-OH is not large, at best 70/30 (59). The solubility of adenosine is also low in solvents such as DMF or Dioxane. However, with the introduction of a Trityl group in the 5´-O position, the solubility in aprotic solvents such as THF increases. THF can be easily obtained dry by distillation over Calcium Hydride or LAH. which is of great importance in alkylation, as otherwise water forms hydroxide ions which can consume alkylating reagents. The trityl group (monomethoxytrityl, MMT or dimethoxytrityl, DMT) also increases the selectivity between 2´-OH and 3´-OH to about 90/10% (55, 59). Other advantages of using the trityl group are that, column chromatography of the substance is facilitated as volatile solvents can be used. The most obvious advantage of using a trityl group is that, it will be introduced into the building block for the upcoming oligo synthesis. There are, of course, other alternatives if one strives

for even higher selectivity for 2´-OH. The most commonly used method is to protect 3´-OH and 5´-OH simultaneously, in order to then be able to selectively alkylate the free 2´-OH. However, this requires more specialized protecting group reagents such as TIPS-Cl_2, MDPS-Cl_2 or di-tert-butylsilylene-chloride (60, 61) and requires more synthesis steps (Figure 7).

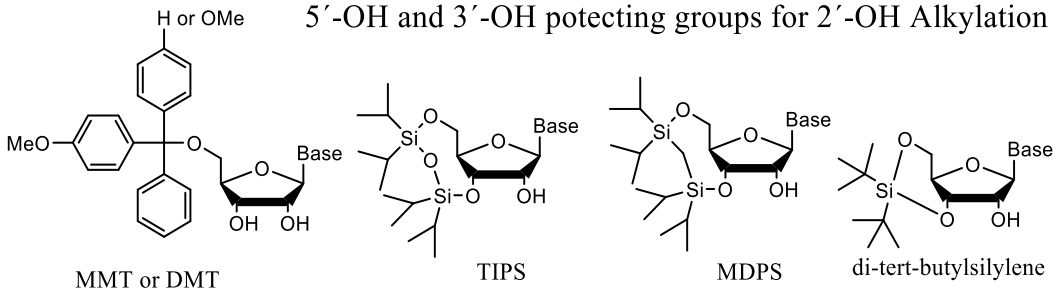

Figure 7. Protecting groups used in 2´-OH alkylation.

When using TIPS as a protecting group, one must also use dry conditions as hydroxyl ions tend to cleave the protecting group. In addition, it is common to use relatively expensive non-nucleophilic bases, such as phosphazene bases, which are costly and difficult to regenerate. However, the TIPS protecting group helps to increase the solubility and simplify the chromatography. Thereafter, the protecting group can be easily removed using triethylamine trihydroflouride, and from there one can continue further with the unprotected 2´-O-alkylatedadenosine. Anotheralternative for creating 2´-O-alkyl nucleosides (Not covered in detail here), is by coupling a heterocyclic base with an electrophilic sugar, transglycosylation or the Silyl-Hilbert-Johnson reaction, where a pre-2´-O-modified sugar is used as the reagent and together with the nucleobase reagent forms the desired product. Sometimes these methods require several reaction steps to synthesize the starting material (the modified sugar).

Synthesis of 2´-O-alkyl Uridines:

Direct alkylation of Uridine with a base and alkyl halide results in Uridine-3N-alkylation as the Lactam-3N-H proton has a lower pKa than the 2´-OH proton(58, 62, 63). Early work alkylating unprotected Uridine, uses a organo-tin reagent and a alkyl halide (64), or Diazomethane with stannous chloride as a catalyst(65). There are several other approaches to choose from, to synthetize a 2´-O-alkyl modified Uridine(66). One of the more commonly used syntheses method is based on 2,2´-anhydrouridine as starting material (Figure 8).

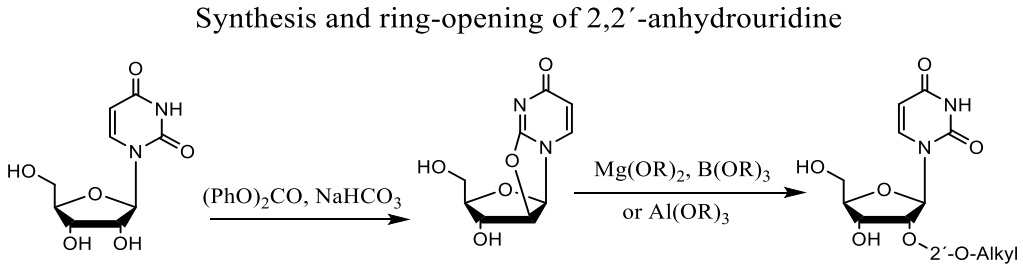

Figure 8. Synthesis of 2,2´-anhydrouridine and the ring-opening with magnesium, aluminum alcoholates and borate esters.

A reagent consisting of a magnesium, aluminum alcoholate or a borate ester is used, where the alcoholate/ester acts as a nucleophile at the 2´-position and results in a 2´-O-alkylated product(67). There are, of course, restrictions on the types of alcohols that can be prepared and used to form these alcoholates/esters. It depends on which functional groups these alcohols carry, such as amides, carboxylic acids, halogens, etc, which can be unsuitable for this type of reagent. This methodology is best suited for simple alkyl groups. Even with the use of 2,2´-anhydrouridine, it is advantageous to use the 5´-O-tritylated nucleoside which increases the solubility and yield. One similar reaction using 2,2´-anhydrouridine, is instead with BF_3 and a silyl-ether(68, 69). Another approach is to selectively protect the Lactam group with the more acidic pKa than the 2´-OH. What is required of such a protecting group is that it should be able to withstand the nucleophilic attack of hydroxide ions and strong base(70-72). In addition, the protecting group (Figure 9) must be able to be deprotected under conditions that retain the desired functionalities, such as the 5´-O-Trityl group.

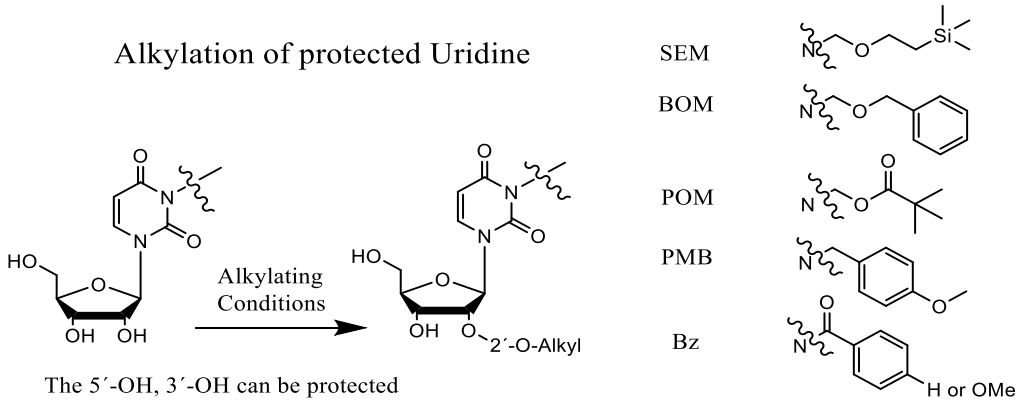

Figure 9. Lacatm-3N-protecting groups of Uridine, used in the 2´-OH alkylation.

One such alternative is to protect the Lactam group with a base stable acetal group or acyl group(37, 65, 73). An attractive candidate for this is the TOM group, which can be selectively

deprotected with fluoride(37), which allows one to use a pre-5´-OH tritylated Uridine which is then alkylated with strong base and alkylating reagent. An alternative to the trityl group is to protect the 3´-5´-OH with the TIPS and the lactam group with the TOM group. Both the TIPS and the TOM group could be deprotected simultaneously with Fluoride and result in a 2´-O-alkylated product. In addition, both protecting groups would provide an easily soluble product in many solvents suitable for alkylation.

Synthesis of 2´-O-alkyl Guanosines:

Guanosine is probably the most complicated nucleoside of the natural nucleosides in terms of 2'-O-alkylation. Guanosine is quite insoluble in most solvents but slightly soluble in solvents such as DMF and hot dioxane. The Lactim-O6-H/Lactam-N1-H functionality of Guanosine has a lower pKa (9.2-9.5) than the 2´-OH (pKa 12.5) hydroxyl group (similar to Uridine). Which makes it necessary to modify Guanosine in order to perform efficient 2´-O-alkylation. Many strategies have evolved that results in 2´-O-alkylated Guanosine. Alkylation from 2-amino-6-chloropurine riboside or starting from 2,6-Diamino-purine which can be alkylated directly (74), or with various protecting groups in the 5´-O and 3´-O positions. Then direct treatment or removal of the protecting groups, followed by treatment with Adenosine deaminase (ADA). The enzyme converts the 6N-amino function to the purin-6O functionality, which gives the Guanine base(75). The ADA enzyme appears to tolerate a large number of variations of 2´-O-alkyl modifications and different types of solvents in addition to H_2O. Another alternative during 2´-OH alkylation of Guanosine is to protect the Lactim-O6-H function in order to then be able to alkylate the 2´-OH hydroxyl(74, 76, 77). One such alternative is the use of 2-amino-6-chloropurine riboside(74) This requires modifications that increase the solubility in aprotic solvents. One used protecting group of the Lactam-N1-H function is N1-benzyl-Guanosine (Figure10).

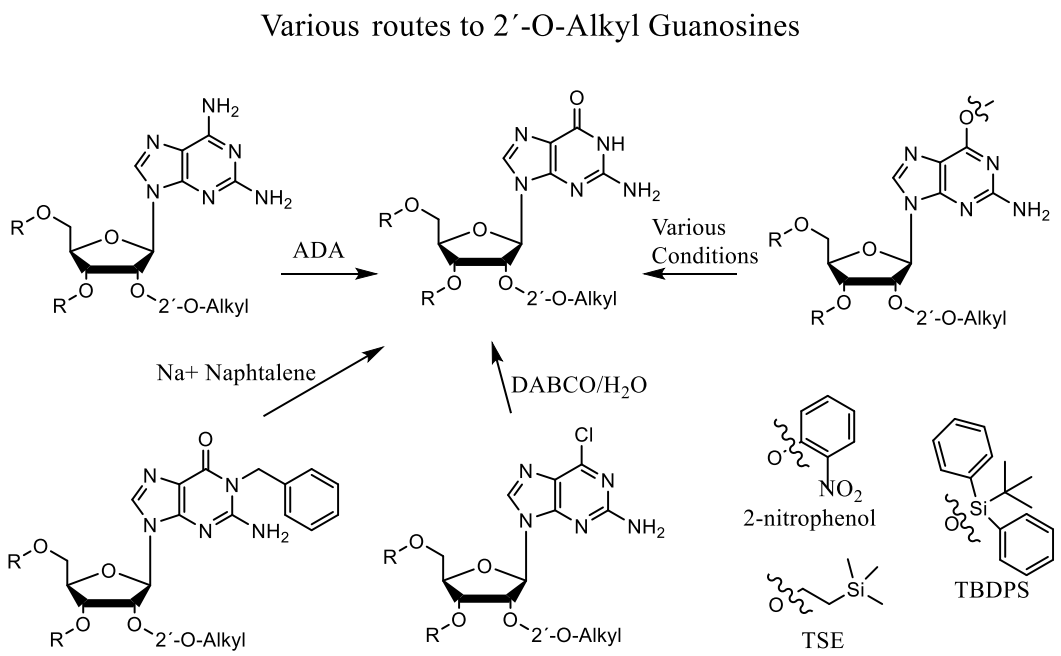

Figure 10. 2´-O-alkylated precursors that can be used in the synthesis strategy of 2´-O-alkyl-Guanosines.

The use of NaHMDS as base, can protect the 06-function in situ during an alkylation reaction of unprotected Guanosine together with an electrophilic alkylating reagent to give the 2´-O-alkylated Guanosine directly. This could be explained by considering that HMDS reacts initially at the 6O-position of the nucleobase to give the O6-TMS ether, that protects the lactam function from alkylation(78). A not fully explored strategy for this purpose is the TOM group, also for the Uridine protection (Lactam), that can be selectively deprotected with fluoride or maintained to the final step of deprotection of the oligonucleotide.

Synthesis of 2´-O-alkyl Cytidines:

It is possible to carry out a direct 2´-O-alkylation of Cytidine with a strong base such as NaH and an alkyl halide(79), but this usually gives low yields. The earliest synthesis methods for 2´-O-alkylation of cytidine used alkyl halides and AgO as reagents(80). A frequently used method is to use an already 2´-O-alkylated Uridine that can be chemically converted to Cytidine(67, 81). The synthesis begins with the creation of 4-(1H-1,2,4-triazol-1-yl)uridine with, for example, triethylamine, triazole and POCl3. The triazole derivative can then be substituted with NH_3 in dioxane to give the 2´-O-alkyl uridine derivative (Figure11).

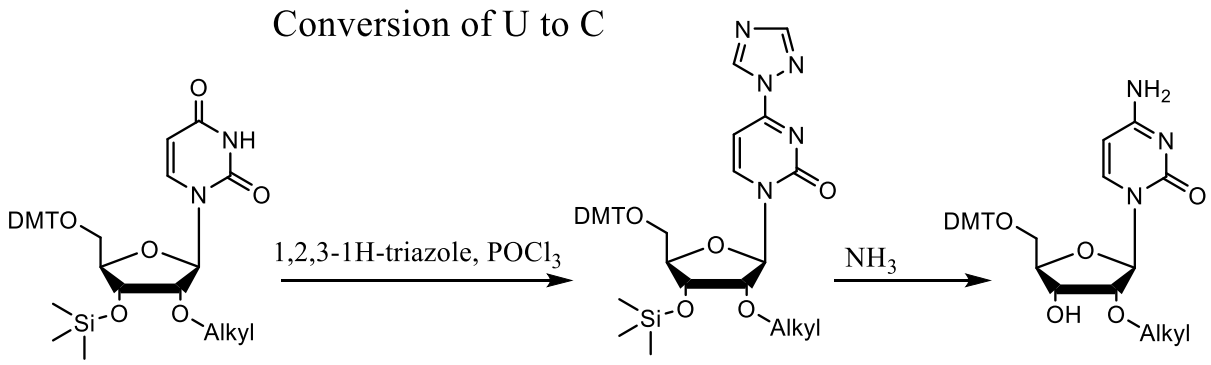

Figure 11. Synthesis of a 2´-O-alkylated-Uridine via a 4-(1H-1,2,4-triazol-1-yl)uridine derivative to a 2´-O-alkyl-Cytidine.

An alternative to the triazole is ammonolysis of a 4-nitrophenol substituted Uridine to get the 4-NH_2 function of Cytidine(67). Another method starts with, synthesis of anhydrocytidine (82). Cytidine and $POCl_3$ and then water gives anhydrocytidine. Anhydrocytidine can then be reacted with, for example, $Mg(O\text{-alkyl})_2$ reagents to generate 2´-O-alkyl-Cytidine derivatives (United States, US5739314 A 1998-04-14). As with anhydrouridine, this method is of course limited by the types of which $Mg(O\text{-alkyl})_2$ reagents are possible to start from.

2 RESULTS

Chapter 1:

Synthesis of a 2´-O-carbamoylmethyl (CM) modified Adenosine building block and synthesis of a CM containing dinucleotide, as well as a study of the dinucleotide with respect to chemical and enzymatic stability.

Many 2´-*O*-alkyl oligonucleotides have been shown to increase RNA affinity and thus increase duplex stability (83, 84). This gave us the interest to study a 2´-*O*-alkyl group with the properties of being a small electronegative hydrophilic group. The 2´-*O*-carbamoylmethyl group possesses these properties. The 2´-*O*-carbamoylmethyl group has previously been reported to give a higher melting point + 3 °C / modification (85). The aim was to find a method to be able to synthesize this modification in as simple and clean a way as possible. More importantly, we also wished to study the stability of the modification toward chemical and enzymatic degradation (69, 92c, 95) so that conditions can be found to obtain a relevant product while minimizing side reactions and get an idea about the potential use.

Paper I:

In the first study we explore the synthesis of a 2´-*O*-carbamoylmethyl modified Adenosine (85). The first attempts started by using unprotected Adenosine **1** with NaH and Iodoacetamide in DMF, to obtain the desired 2´-*O*-carbamoylmethylated adenosine **2**. The use of unprotected Adenosine in DMF resulted in a relatively low yield 20% of the desired 2'-*O*-alkylated product and an isomeric mixture corresponding to 2´-*O* and 3´-*O*-substituted products (in about 6/4 ratio). Adenosine has a relatively low solubility in most aprotic solvents and, e.g., THF is then not an option, but Adenosine is to some extent soluble in polar solvents such as DMF and DMSO, that are also suitable for strong bases that can be used for deprotonation of the 2´-OH. One of the most important things during alkylations is to work under dry conditions that otherwise highly affects the yield of the reaction. Alkylation of unprotected Adenosine with iodoacetamide gave a product that was even more polar than Adenosine itself, making it very difficult during chromatography to perform separation of the two isomers. The mixture of isomers was then used in the next step where the exocyclic 6-NH_2 group was protected **3** (Figure 12).

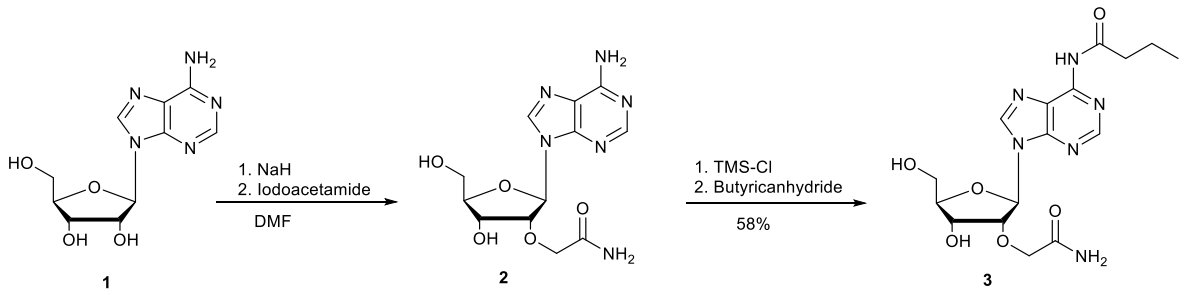

Figure 12. Alkylation of Adenosine and protection of the exocyclic 6-NH_2 function.

Although product could be obtained through alkylation of unprotected Adenosine, the yields were not impressive, so we decided to change the order of protection and optimize the 2´-OH alkylation experiments. The Alternative procedure was to first protect the 5´-OH group with 4-methoxytriphenylmethyl chloride (monomethoxytrityl-Cl, MMT-Cl), to increase the solubility and hopefully lead to some steric hindrance at the 3´-OH position. In the first attempts of alkylating 5´- MMT-O-Adenosine **4**, dimsyl sodium was used as base and iodoacetamide as alkylating agent in THF. This increased the isomeric ratio to 2´ vs 3´ to about 9/1 and the isomers where possible to separate at this stage by column chromatography. In an additional procedure we developed a one pot method where MMT-5´-O-A **4** was alkylated with methyl bromoacetate and potassium tert-butoxide **5** (both commercially available) followed by ammonolysis with NH_3/MeOH saturated at -20^{o}C which gave the 2´-O-carbamoylmethyl product **6** in high yield (Fig 13). THF gives an additional advantage in that it is easily prepared dry, by distillation over $LiAlH_4$ and it is a solvent where 5´- MMT-O-Adenosine **4** is readily soluble.

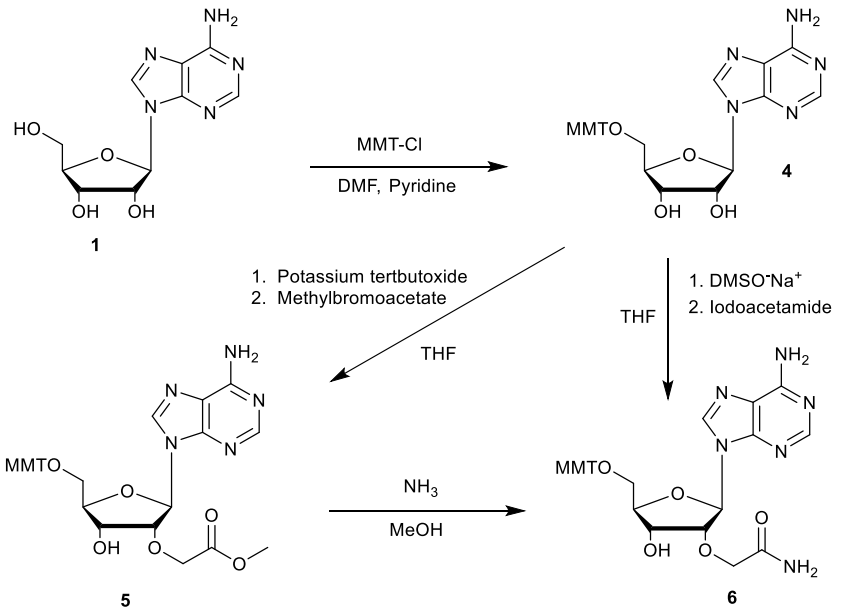

Figure 13. Improved alkylation steps of the MMT-5`-O-adenosine intermediate to the 2`-O-CM modification.

Further protection of the exocyclic 6-NH_2 group was chosen from one of the protecting group strategy used in H-Phosphonate oligonucleotide synthesis (26). The reaction was performed with butyric anhydride in pyridine and after presilylation with trimethylsilylchloride to afford the resulting compound **7**. The 3´-OH group was subsequenctly phosphonylated with

PCl_3/Imidazole to give the H-phosphonate building block 2´-O-carbamoylmethyl modified-5'-O-(4-monomethoxytrityl) Adenosine 3´- H-Phosphonate triethylammonium salt **8** (86) (Fig 14).

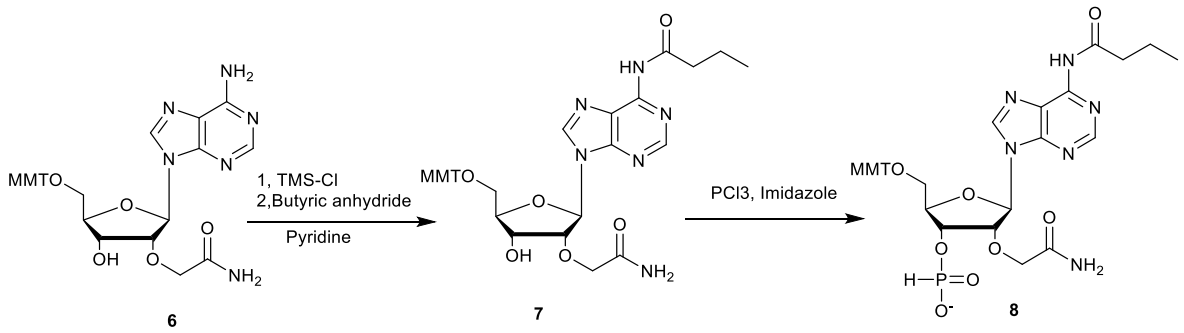

*Figure 14. Further protection of **6** and phosphonylation to give the H-phosphonate **8**.*

The H-Phosphonate building block **8** was then coupled to pre-prepared 3´-O-MMT-thymidine **9** and further oxidized to give the protected dinucleoside phosphate **10** (87). The dinucleotide was detritylated with DCA and then subjected to ammonolysis in saturated NH_3/MeOH to give the final product **11** (Fig 15).

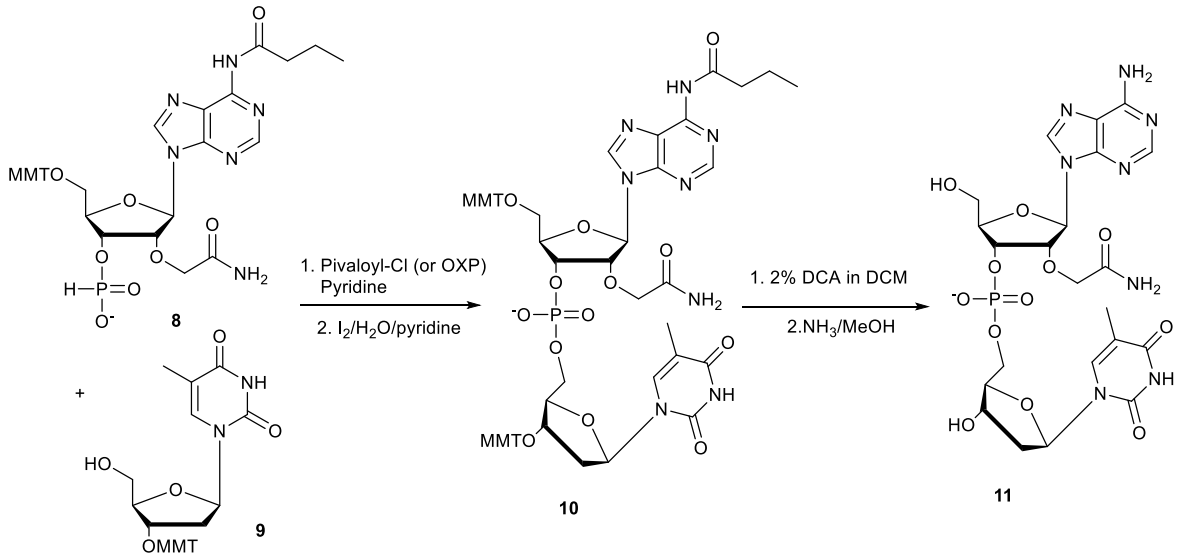

*Figure 15. Synthesis of the 2`-O-CM modified di-nucleotide **11**.*

Paper II:

This work aimed to explore the stability of the 2´-*O*-carbamoylmethyl (CM) group under different condition that an oligonucleotide containing the 2´-*O*-CM group may meet during deprotection (85). In addition, we wished to investigate the stability of phosphodiesters vicinal to the 2´-*O*-CM group towards enzymatic degradation.

To be sure that the 2´-*O*-carbamoylmethyl groups would keep their amide functionality intact, it is crucial that the modification survives the chemical conditions of the oligonucleotide synthesis. The most critical step is the stability of the 2´-*O*-carbamoylmethyl group during ammonolysis that is used in the last stage of the oligo synthesis when the exocyclic nucleobase -NH_2 protecting groups are being cleaved of, as well as the solid support. The most common conditions for this include concentrated ammonium hydroxide at elevated temperature.

It is also important to remember that in the 3´-end of a fully modified oligonucleotide, the 2´-*O*-carbamoylmethyl can possibly be affected via a intramolecular hydrolysis assistance via the free 3´-OH. The first indication of the stability of the 2´-*O*-carbamoylmethyl amide moiety against hydrolysis during ammonolysis was from a nucleoside monomer **3** with a free 3´-OH. In this compound we obtained the indication that that the amide was hydrolyzed to a large extent in concentrated NH_4OH but not in saturated NH_3 in MeOH, compound **2** and **3** (Fig 16).

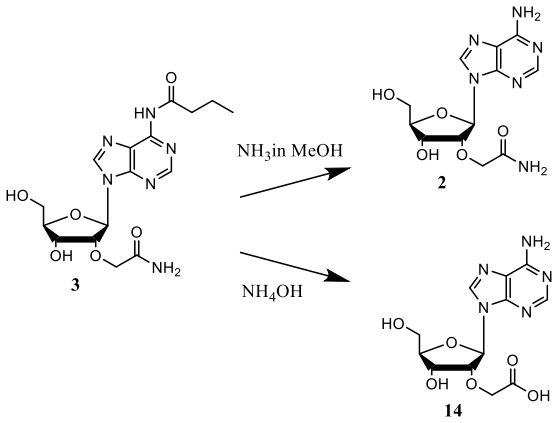

Figure 16. Hydrolysis of the CM-amide function during ammonolysis with a vicinal 3´-OH.

After these results we followed up with a study of the carbamoylmethyl moiety vicinal to a phosphodiester. The previously synthesized 2´-*O*-carbamoylmethyl dinucleotide (Paper I), was then used in this work as a model compound **11** (88). A reference for the hydrolyzed 2´-*O*-carbamoylmethyl dinucleotide was prepared (2`-*O*-Carboxymethyl-ApT, **15** COMAT) and used in the following HPLC analysis.

The dinucleotide 2`-*O*-Carbamoylmethyl-ApT **11** (CMAT) was subjected to various ammonolysis conditions, that are used in the final deprotection step in oligonucleotide synthesis, *i.e.*, Conc. Ammonia 25% or 32% / 24-48h, NH_3 (sat) in MeOH / 48h, ethylenediamine / EtOH, and 32% aq. ammonia / EtOH. It turned out that the conc. ammonia solutions gave a large proportion of hydrolyzed 2´-*O*-carboxymethyl product while other conditions did not (Figure 17). In the condition of NH_4OH 25% at 55ºC gave 19% hydrolysis after 24h and these conditions are commonly used in standard oligonucleotide synthesis (for at least 16h). Saturated NH_3 in MeOH at 20ºC for 48h did not show any hydrolysis at all. These results are promising since it should be possibly to make oligonucleotides containing fully or partially modified with carbamoylmethyl functionalities without compromising purity of the modified oligonucleotide.

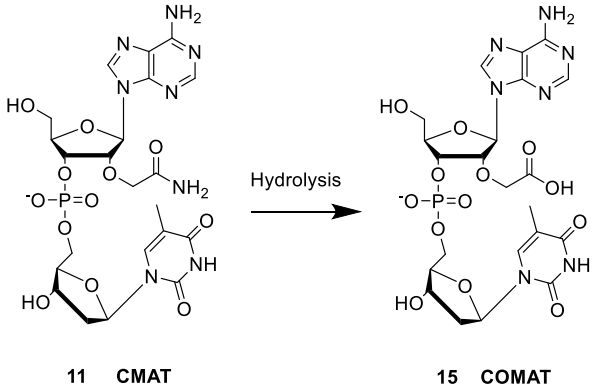

Figure 17. Hydrolysis of the CM-amide function during ammonolysis of 11 (CMAT) with a vicinal 3´-O-phosphate diester.

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One other important factor for an oligonucleotide is the enzymatic stability in biological fluids (*in vitro* for cell experiments and *in vivo*). Although phosphorothioate (PS) linkages are quite stable towards enzymatic cleavage and commonly used is oligonucleotides, they are associated with some negative issues such as toxicity (107) and PS are also degraded even if this is at a reduced rate compared to phophodiesters. If highly stable oligonucleotides with PS linkages are required a combination with additional modifications are typically desired, and there is also a development to try to reduce the number of PS linkages in oligonucleotides to reduce toxicity and to balance protein binding. Modifications that stabilize the phosphodiester (and also the corresponding PS linkage) are there most interesting for future development of oligonucloetide therapeutics. Thus, we decided to investigate how the CM modification influences the ability to survive enzymatic hydrolysis of the phosphate diester. The **11** CMAT dinucleotide was exposed to two different nucleases, Phosphodiesterase I from Crotalus adamanteus venom and Phosphodiesterase II from Bovine spleen. As a reference compound a dApT dinucleotide was used. HPLC analysis after incubation with the respective nucleases revealed that the 2´-*O*-carbamoylmethyl modification has a strong protective effect on the stability against these

nucleases (Figure). The stability of the modified dinucleotide showed great stability even after 6 days against PDA I where 30% **11** CMAT was still remaining and with PDA II there was not really any significant cleavage (~99% remaining), while the reference dApT was cleaved within 2 and 4h respectively (Figure 18).

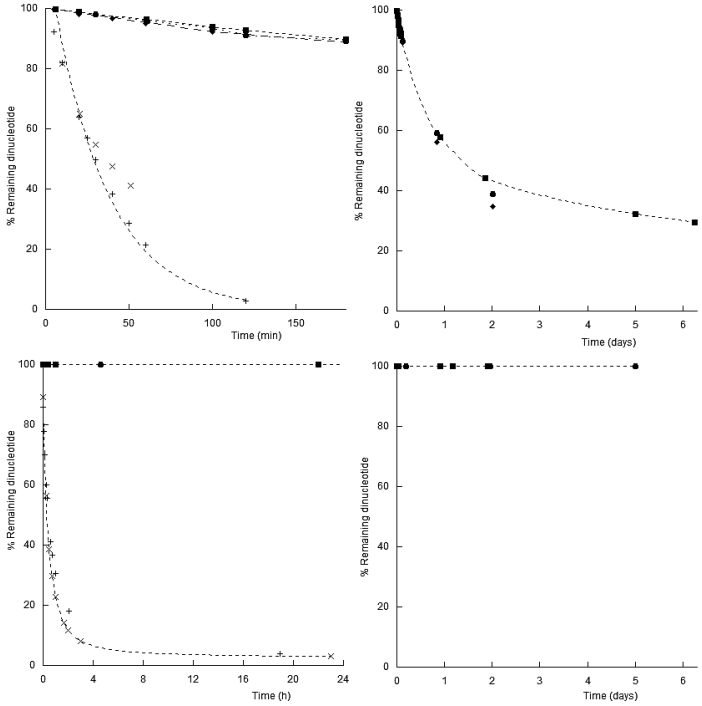

Figure 18. RP-HPLC analysis of enzymatic stability of CMAT ■ *and the reference dApT+ . Upper: with (PDE I). Lower: with (PDE II).*

These results show that it is possible to deprotect compounds with a phosphodiester vicinal to the 2`-O-carbamoylmethyl functionality, but that conditions should be chosen to avoid hydrolysis (e.g., not conc ammonia) to keep the modification intact. In addition the CM modification imposes an increased stability towards nucleases which can be particularly useful in therapeutic settings (69, 92c, 95). The modification is also very promising with the respect of previously reported results of the 2`-O-carbamoylmethyl modification giving a substantial increase of the melting temperature (tm) in duplexes with RNA (77, 85).

Chapter 2:

Evaluation of lactam protection for synthesis of 2´-O-alkylated Uridines.

Many different types of strategies have been used in the synthesis of 2´-*O*-alkyluridine (64-69). Direct alkylation of uridine using strong bases and alkyl-halides, in most cases results in the alkylation of the lactam function on the nucleobase. In this work, the intention is to evaluate two different types of protection group families for the lactam group of the Uracil nucleobase.

where the goal is for them to be stable against strong bases such as potassium tert-butoxide. It should also be possible to remove the protecting group after synthesis of the 2´-*O*-alkyluridine has been performed (70). An alternative is also that the protecting group is kept during oligonucleotide synthesis and removed in the final deprotection.

Paper III:

Two different classes of protection for the uridine lactam function were evaluated. These are aroyl (methoxy substituted benzoyl) protections and different acetals (hemiaminal ethers). As starting materials both Uridine and 5´-*O*-MMT-Uridine were used. The 5´-O-MMT has the advantage of greater solubility in dry organic solvents. Several different substituted benzoyl and acetal modified nucleosides **16 a-c** where synthesized according to known procedures (65) (Figure 19). The benzoyl-lactam protection would have the advantage that it could be kept until the last step of a oligonucleotide synthesis and be removed under the same conditions as the rest of the other nucleoside exocyclic amine protecting groups. In the study of the aroylated compounds it was clear that the strong basic conditions used during the alkylation step to a great extent removed the substituted benzoyl even with two methoxy groups in the orthospositions (73). The benzoyl protection was abandoned at that moment.

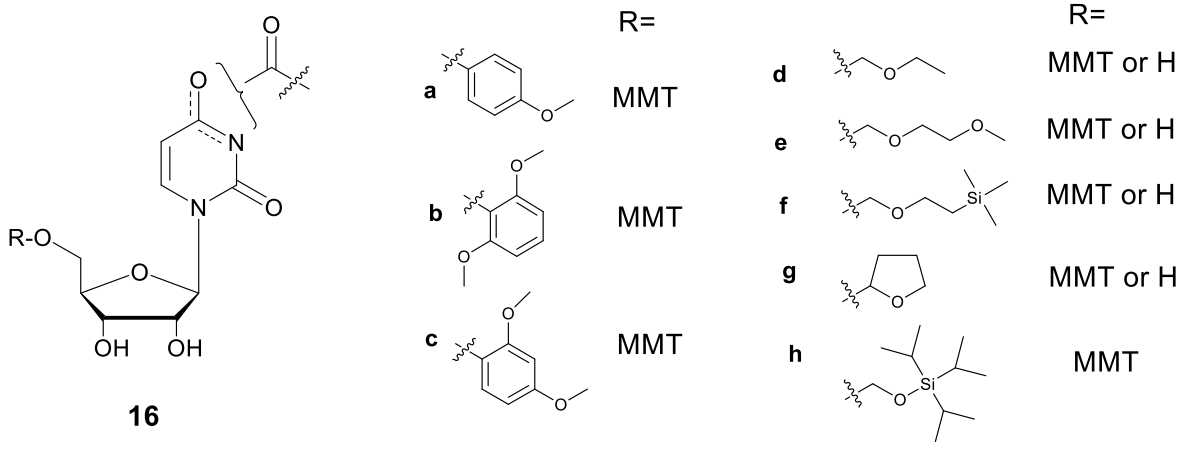

*Figure 19. Two different classes of protecting groups, aroyls **a-c** and hemiaminal ether derivatives **d-h** where synthesized and evaluated for 2´-O-alkylation.*

The lactam acetal protection was introduced using DBU or diisoropylethylamine as base and the corresponding alkoxymetyl chloride or tetrahydrofuranyl chloride in THF, DMF or dichloromethane with good yields (compounds **16 d-h)**. The tetrahydrofuranyl protecting group when introduced to Uridine in DMF with DiPEA as base gave about 50% yield as a racemic mixture, compound **16 g**. It was possible to remove in 80% acetic acid/H_2O. In

particular, the triisopropylsiloxymethyl (TOM) protection appeared to be a most promising lactam protecting group for use in synthesis of 2´-*O*-alkyl-uridines (compound **16 h**). TLC experiments showed that it can withstand the basic alkylating conditions with potassium tertbutoxide and it can be removed in less than 2h with a few drops of triethylamine trihydrofluoride, 1 ml CH_2Cl_2. Interestingly from 15N-1H and 13C-1H NMR correlation spectroscopy (HMBC) experiments it was clear that the TOM group is positioned at the oxygen of the lactam function. All the other lactam-acetal protecting groups where not possible to remove under mild enough conditions, despite many trials especially with the SEM group **16f** (71) (Figure 20).

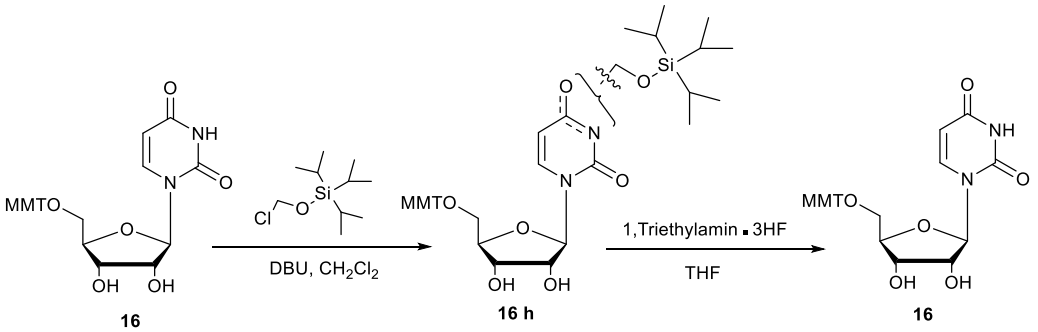

*Figure 20. Protection with the TOM-group of the lactam function of MMT-5`-O-uridine **16** and deprotection.*

One interesting discovery not mentioned in the publication was in the procedure for the preparation of the SEM protection using (2-trimethylsilylethoxy)methyl chloride. Depending on what base was used, different products were obtained. DBU in CH_2Cl_2 gave the best selectivity for the lactam function, DiPEA almost the same result, while potassium tertbutoxide in THF gave predominantly 2´-O-SEM product. One could speculate that the harder counter ion to a greater extent coordinate to the 2´-OH than to the lactam function, although the lactam function has a lower pKa than the 2´-OH. This study has so far not led to the use of the TOM group for further work on 2'-*O*-alkyluridines in our research group but laid the foundation for use of other acetal protection (pivaloyloxymethyl and benzyloxymethyl) for this purpose (70).

Chapter 3:

Synthesis of nucleotide building blocks and studies of Di- and Oligo- nucleotides bearing the 2-O-[N-(Aminoethyl)carbamoyl]methyladenosine modification.

In the previous work presented in this thesis, the carbamoylmethyl (CM) modification synthesis started with alkylation to give the carboxymethyl ester. The ester functionality opens up for the possibility to use a large number of amines to create new interesting modifications(53, 59, 70,

92). We chose ethylenediamine to obtain a modification that would still keep it relatively small and give it a new feature of having a terminal amino functionality. The pKa of the terminal amino function would give an additional positive charge at neutral pH but be close enough to be partially deprotonated in some environments. The resulting modification incorporated in an oligonucleotide would together with the negative charge of the phosphate diester backbone give the interesting feature of a charge neutral oligonucleotide if fully modified.

Paper IV:

This work started with the introduction of the 2`-*O*-[*N*-(2-aminoethyl)carbamoyl]methyl (AECM) modification in Adenosine and further transformation to the corresponding H-phosphonate building block. The protecting groups should survive the conditions used in oligonucleotide synthesis and be removable at the end of an oligonucleotide synthesis, preferably in the same step used to cleave the oligonucleotide from the solid support. 5'-*O*-MMT-Adenosine was chosen as the starting nucleoside since it can be straightforwardly alkylated. In the first approach dimsyl sodium was used as base and allyl bromoacetate as the alkylating reagent (108). During the chromatography step of the compound using DCM/MeOH and a few drops of triethylamine a transesterification to the methyl ester happened. The ester **5** was subjected to ethylenediamine and formed the corresponding amide **17** via aminolysis (Figure 21).

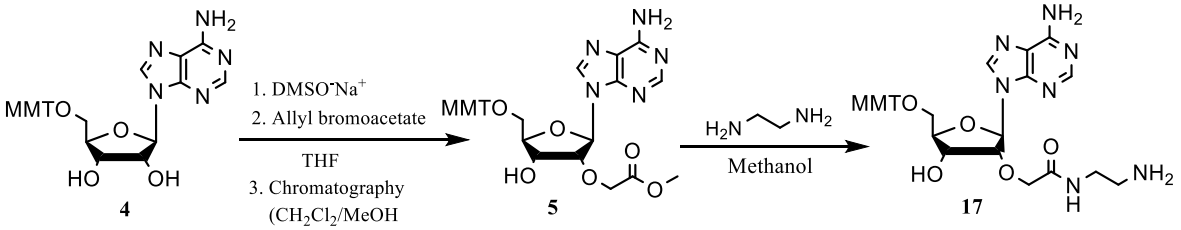

*Figure 21. Alkylation of MMT-5`-O-Adenosine **4** to the 2´-O-carboxymethyl ester 5 and aminolysis to the 2´-O-AECM modified **17**.*

For the terminal -NH_2 of the AECM modification trifluoroacetyl was chosen as protecting group, since it can be removed during the last stage ammonolysis step and it protects against any side reactions during the oligonucleotide synthesis. The trifluoroacetyl protection was introduced by using trifluoroacetic anhydride and triethylamine in CH_2Cl_2 and gave compound **18**. To obtain a more efficient route an alternative method to **18** was developed and performed in a three step one pot procedure. For the exocyclic -NH_2 butyryl was chosen as protection since it is cleaved off fast enough during ammonolysis and survives sufficiently during the steps of oligonucleotide synthesis. There are also many other protecting groups that could be used depending on the sensitivity of the oligonucleotide to different treatments (65, 91). The

protection of the exocyclic -NH_2 was done with butyric anhydride after presilylation with trimethylsilyl chloride in pyridine to give **19** followed by deprotection of the silyl ether with triethylamine trihydroflouride that gave **20**. In the last step of making the 3`-H-phosphonate, phosphonylation using PCl_3 and imidazole was used to get the building block **21** (26, 86) (Figure 22).

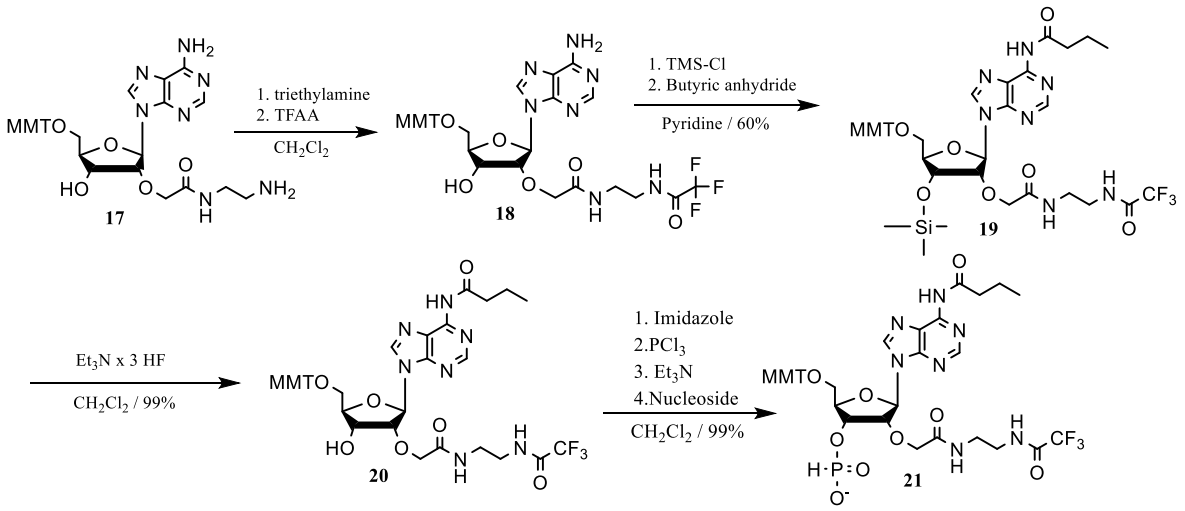

*Figure 22. Synthesis of the 2´-O-AECM modified H-phosphonate building block **21**.*

As in the study of the carbamoylmethyl (CM) modification an AECM modified dimer was synthesized in order to study the chemical and enzymatic stability of the modification and the vicinal phosphodiester. The H-Phosphonate building block **21** and 3´-*O*-MMT-thymidine **9** (87) was then coupled with Bis(2-oxo-3-oxazolidinyl) phosphinic chloride (OXP) in pyridine giving **22** according to TLC. The H-phosphonate diester **22** was further oxidized *in situ* by adding Iodine and H_2O to give the protected dinucleoside phosphate. The dimer was detritylated with 80% acetic acid and then subjected to aminolysis with 20% ethylenediamine in MeOH to give the crude final product dinucleotide **23**. The deprotected product was then purified by reversed-phase high performance liquid chromatography (RP-HPLC) to give the pure AECM-AT dimer **23** (Figure 23).

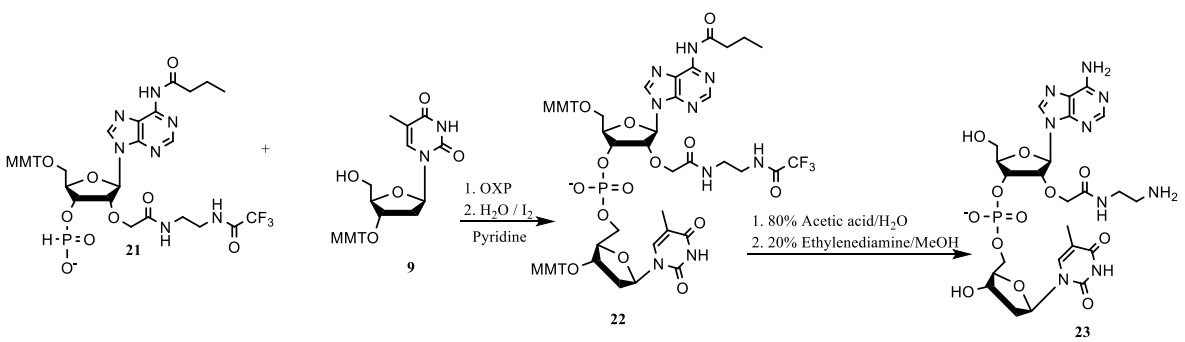

*Figure 23. Synthesis of the 2`-O-AECM modified di-nucleotide **23**.*

To be certain of the chemical stability during conditions possible for the final deprotecting step used in oligonucleotide synthesis we subjected the new dinucleotide to different conditions. If necessary, we could also modify the deprotecting step so that it is directly suited for the AECM modification and keeps the modification intact in a final modified oligonucleotide. The AECM-AT dimer **23** was subjected towards different chemical condition that can be used to remove the TFA protection as well as different acyl protecting groups from the heterocyclic bases. After neutralization the reactions were analyzed by RP-HPLC (Scheme 3). With concentrated ammonium hydroxide 55ºC 24-48h a few % of the modification was hydrolyzed to the carboxylate. Although only a few percent cleavage was observed this would mount up if the modification would occur throughout an oligonucleotide. The results point to that the safest approach would be 20% ethylenediamine in methanol, but also non-aqueous ammonia solutions are possible.

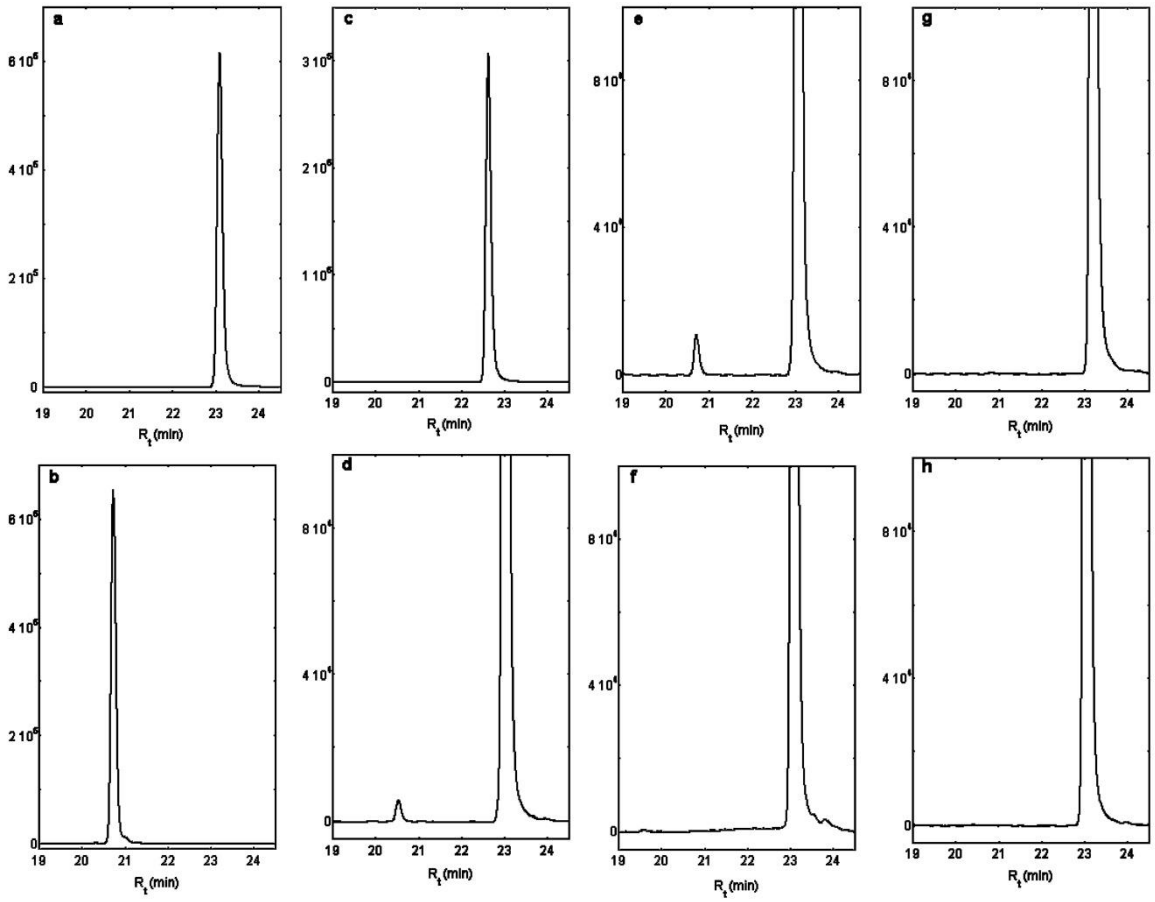

*Figure 24. RP-HPLC analysis of (a) 2'-O-AECM-AT **23**, (b) 2'-O-COM-AT ref **15**, (c) 2'-O-CM-AT ref **11**;(chromatograms) **23** with different aqueous or alcoholic ammonia or ethylenediamine solutions: d) conc. NH3 (aq) at 55°C, 24 h, e) conc. NH3 (aq) at 55°C, 48 h, f) 20% ethylenediamine in methanol , RT, 48 h, g) Methanol saturated with NH3, RT, 48 h, h) NH3 (aq) – Ethanol (3:1), RT, 24h.*

A second important aspect of the AECM modification is to what extent it infers stability against phosphodiester hydrolysis by nuclease enzymes. Certain 2´-*O*-modifications which carries a terminal -NH2 amino function has previously shown that they are resistant to exonucleases (54,

55). The AECM modified dinucleotide **23** was treated with two different nucleases, Phosphodiesterase I (PDE I) a 3`-exonuclease and Phosphodiesterase II (PDE II) a 5´-exonuclease. As a reference compound a dApT was used. It was found that 2´-O-AECM has a very strong protective effect towards degradation by these nucleases. The modified dinucleotide showed great stability and even after 7 days treatment with PDE I ~70% of the dinucleotide was intact and with PDE II cleavage was barely noticeable, while the reference dApT was cleaved within 2 and 4h respectively (Figure 24).

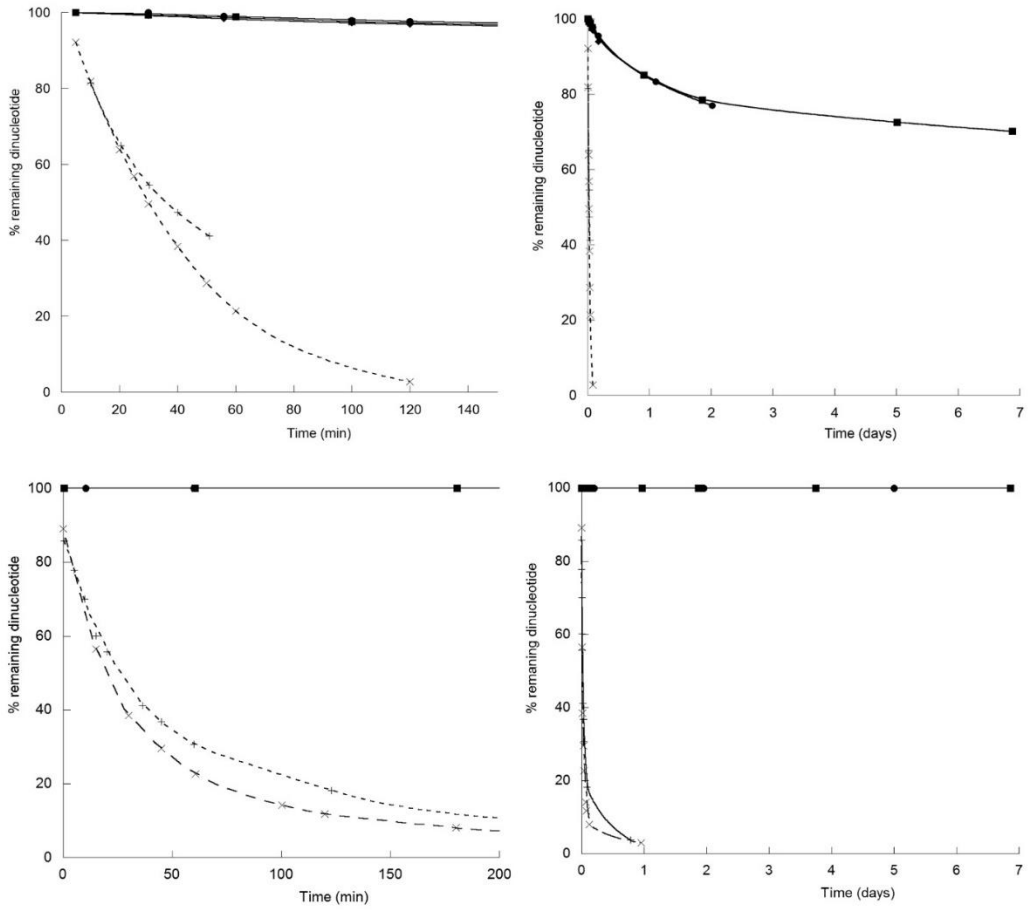

*Figure 24. Enzymatic stability of the 2´-O-AECM-AdT Compound **23** + the reference DNA dAdT. Upper:Phosphodiesterase I (PDE I). Lower: (PDE II).*

These results show that it is possible to synthesize a fully modified dinucleotide carrying the AECM modification and that different conditions are usable for deprotection of bases and the trifluoroacetyl used to protect the AECM modification, but conc aqueous ammonia should be avoided when incorporating several AECM modifications. The AECM modification was shown to have an exceptional stability against degradation by nucleases (69, 92c, 95). This points in the direction for a good candidate to continue studying and to synthesize the modification on all nucleobases and to explore the possible use in oligonucleotide therapeutics.

Paper V:

Having found suitable conditions for deprotection and that the AECM infers high resistance to nucleases we wished to continue to explore and investigate the properties of the 2´-*O*-AECM modification when incorporated in oligonucleotides. Above, we synthesized an AECM modified H-phosphonate building block. Since there is a larger variety of commercially available nucleoside phosphoramidites we decided to synthesize the AECM modified building block for oligonucleotide synthesis as a phosphoramidite that then can be used in common synthesizers (29-32). This was then used to synthesize several sequences where the AECM modification is mixed in with unmodified DNA, as well as fully modified charge neutral oligonucleotides.

From the previously reported synthesis (55) of the AECM modified starting material compound **18** benzoyl protection of the -N^6 amino function was performed. The synthesis was carried out in CH_2Cl_2 by adding triethylamine and first presilylate with TMS-Cl, followed by addition of a catalytic amount of DMAP and Benzoyl chloride. After extraction and evaporation, the crude compound was dissolved in ethanol and ammonium hydroxide was added to remove the second benzoyl from material that was N^6 bis-benzoylated to receive the mono benzoylated compound **24** which was purified by chromatography. **24** was then dissolved in dry THF and triethylamine was added before the reagent 2-Cyanoethyl N,N-diisopropylchlorophosphoramidite chloride. The reaction mixture was concentrated, extracted and purified to give the final 2´-O-AECM modified phosphoramidite building block **25** (Figure 25).

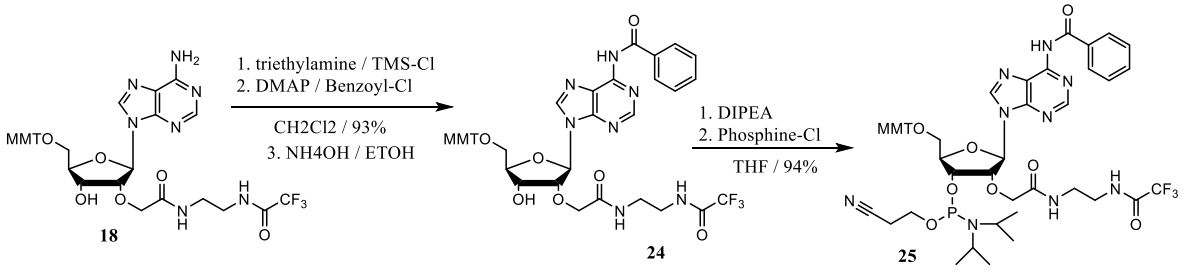

*Figure 25. Synthesis of the 2´-O-AECM modified phosphoramidite building block **25**.*

The 2´-*O*-AECM modified phosphoramidite **25** together with other commercial nucleoside phosphoramidites where then used to synthesize a number of oligonucleotides using a Applied

Biosystems 392A DNA/RNA synthesizer. ON:s containing AECM modifications, sequences O1, O2, O3 and O4) including the corresponding oligodeoxynucleotides , sequences dO1–dO4) (Table 1). The oligonucleotides where deprotected using 20% ethylenediamine in methanol for 24 h at room temperature and purified with reverse phase HPLC.

Sequnce no.	Sequence[a]
O1 and dO1	GGaCCGGaaGGTaCGaG
O2 and dO2	GaaGaaaGaGaGGaGG
O3 and dO3	CaaaGaaCaCCaG
O4 and dO4	aaaaaaaaaaaA

Table 1. Sequences for evaluation of hybridization of AECM containing oligonucleotides. [a] In all sequences A, C, G , T= 2'-deoxyribonucleotides, In sequences O1-O4 a = 2'-O-AECM-adenosine and in dO1-dO4 a = 2'-deoxyadenosine.

Sequnce no.	Tm (°C) with Complementary RNA	ΔTm (°C) for Complexes with RNA	Tm with compl. DNA (°C)	ΔTm (°C) for Complexes with DNA
dO1	58.4		61.8	
dO2	40.2		51.0	
dO3	38.6		47.0	
dO4	5.6		30.0	
O1 (5 mod)	61.1	+0.5	60.2	-0.3
O2 (7 mod)	56.6	+2.3	60.0	+1.0
O3 (7 mod)	44.6	+0.9	46.0	-0.1
O4 (12 mod)	25.3	+1.6	32.7	+0.2

Table 2. Thermal melting (Tm) of oligonucleotides in 100mM Na+, 10 mM phosphate, 0.1 mM EDTA, pH 7 at 4 microM strand concentration.

Thermal melting of duplexes of oligonucleotides **O1–O4** and **dO1–dO4** with complementary RNA as well as with complementary DNA (complementary RNA and DNA was of commercial origin) was then determined by use of UV spectroscopy (9). The resulting melting points (Tm) values in (Table 2). The AECM modification sequences **O1–O4** compared with the DNA sequences **dO1–dO4** towards complementary RNA showed a general increase in melting points T_m of +0,5 to +2,3^{o}C per modification. And The AECM modification sequences **O1–O4** compared with the DNA sequences **dO1–dO4** towards complementary DNA gave only small differences -0.3 to +1.0 ^{o}C per modification. It is interesting to see that there apparently is some sequence dependence on the effect of the AECM modification on melting temperature and that RNA is a preferred target rather than DNA.

To investigate the origin of the increased thermal melting point with O2 we ran thermal melting at different salt concentrations (Figure 26). The 2´-O-AECM modified oligomer **O2** and the DNA **dO2** were studied as heteroduplexes with complementary DNA and RNA. The range of the salt concentration was in the range of 0.05–0.50 M. The **O2**-DNA duplex shows an increase in T_m at low salt concentrations that is absent at higher salt concentration compared to the **dO2**-DNA duplex. This suggests that the low stabilization (+1 °C) at low salt is due to an electrostatic effect by the positively charged AECM group. On the other hand, the **O2**-RNA duplex shows an increase of the T_m in all salt concentrations compared to the **dO2**-RNA duplex. This suggests that stabilization of the duplex with RNA is mainly through non-electrostatic effects (98, 99).

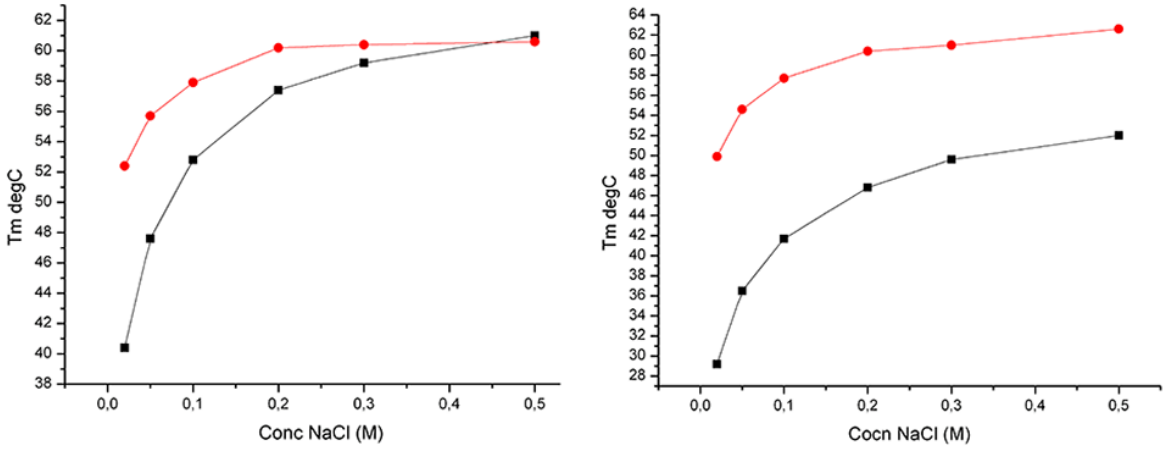

Figure 26. Salt dependence of the thermal melting points (Tm) for the duplexes: ***O2*** *(red lines AECM) and* ***dO2*** *(blue lines DNA) with complementary DNA (left panel) or RNA (right panel).*

To understand the influence of the 2´-*O*-AECM modification has on the conformation of a heteroduplex, the duplexes were investigated by CD-spectroscopy (4-6) (Figure 27). The 2´-*O*-AECM modified sequence **O2** and the DNA sequence **dO2** were studied in combination with the complementary DNA and RNA sequences (Fig. 3). At 270 nm the AECM sequence **O2** in combination with RNA shows the highest absorbance and strong negative bands at 210 nm indicating that the heteroduplex has adopted the structure of an A-form double helix, while the **dO2** DNA-DNA double helix suggests a B-form double helix as expected. These results suggest that the AECM modification drives the heteroduplex towards a more A-like structure (9).

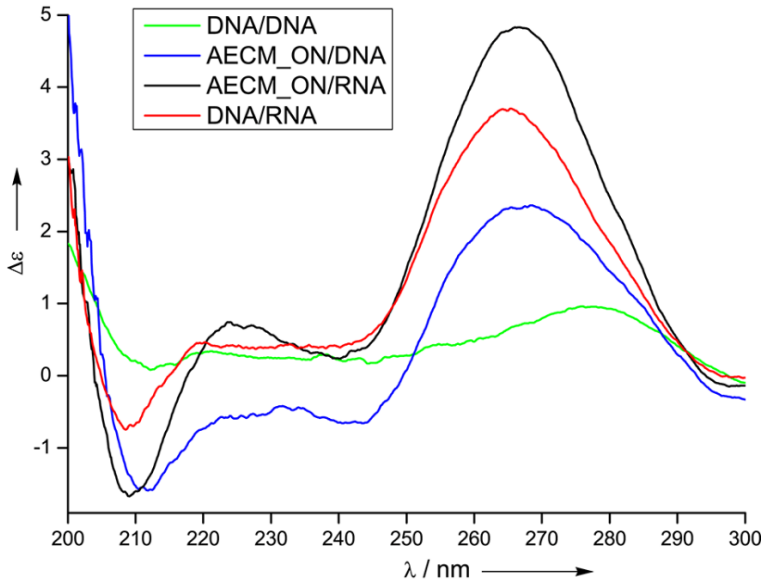

Figure 27. Circular dichroism spectroscopy: O2 (blue and black lines) AECM dO2 (green and red lines) DNAwith complementary DNA or RNA.

Our previous results showed that the 2´-*O*-AECM modification has outstanding stability toward the nucleases PDE I and PDE II (56, 69, 94-97). We decided to test the charge neutral 2´-*O*-AECM modified sequence **O4** together with the DNA sequence **dO4** in human serum. The DNA sequence is as expected degraded fast while the 2´-O-AECM modified sequence **O4** is still intact even after 48h incubation (Figure 28).

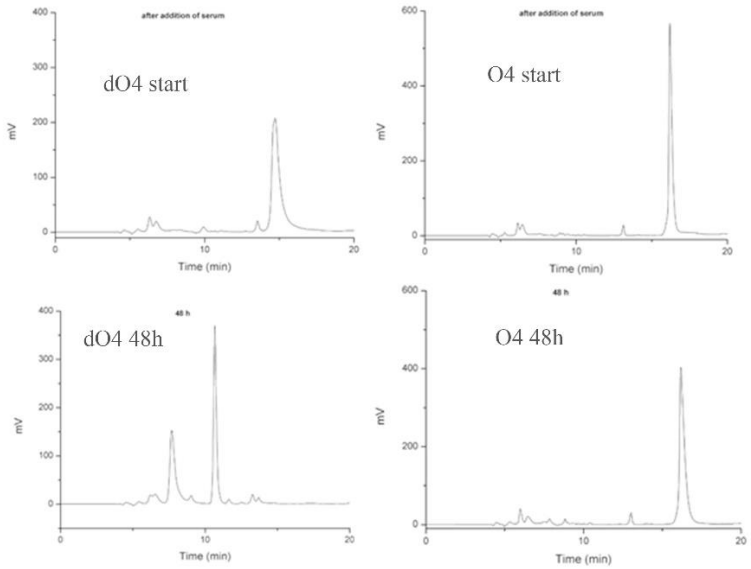

Figure 28. Serum stability of a the fully AECM modified oligonucleotide O4 (AECM-A12-dA) and dO4 (dA13) DNA.

Oligonucleotides are often transfected into cells with various transfecting agents that facilitate the cellular uptake (93). During a HPLC purification of the charge neutral 2´-O-AECM modified sequence **O4 a** small drop of the compound prior to injection was accidently spilled on the hand that resulted in a stinging feeling deep under the skin. The idea came into mind that it might be so, that the compound itself penetrated the skin. The next step was to investigate if the modification can promote cellular uptake. A solid support with a fluorescein linker was used for the synthesis of a 2´-*O*-AECM modified 10-mer **O5** oligonucleotide and a reference DNA 10-mer **dO5**. The two fluorescein labeled oligomers where incubated with U2OS cells and studied using confocal microscopy (Figure 29). The 2´-*O*-AECM modified **O5** displayed significant cellular uptake while no uptake was seen at all with the DNA **dO5**. The spotted pattern may suggest that the AECM oligonucleotide mainly resides in endosomes, but it is quite remarkable how much oligonucleotide that is taken up (possibly triggering endosomal engulfment) (44-48).

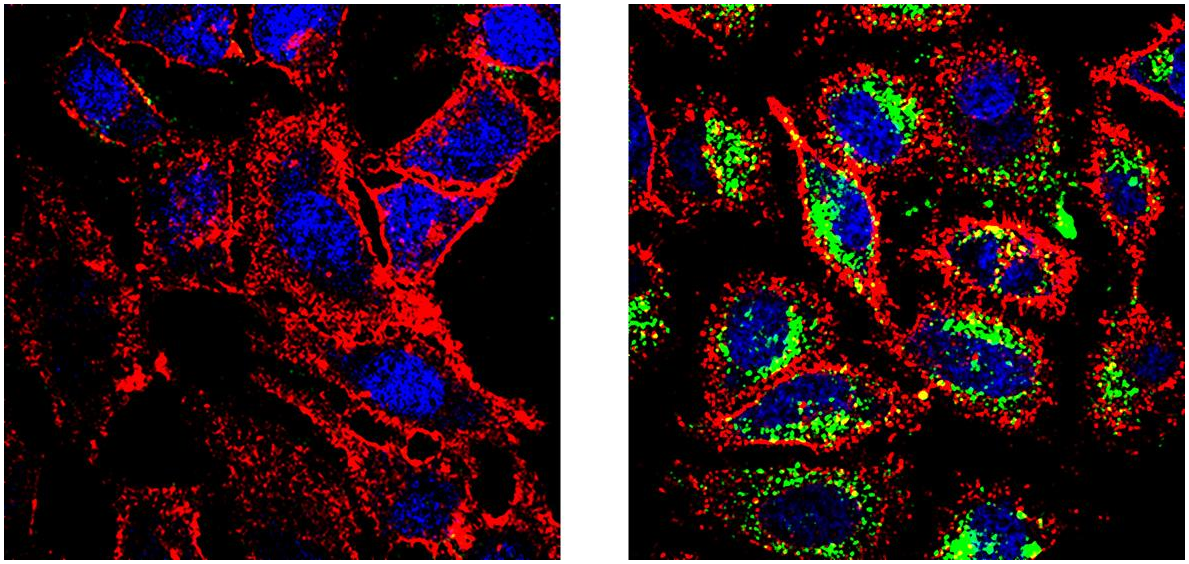

Figure 29. Confocal microscopy of U2OS cells with Left: Fluorescein labelled 10-mer adenosine DNA and Right: Fluorescein labelled 10-mer 2´-O-AECM oligonucleotides.

These results may be of high significance since very few modifications has shown this ability. Much more studies need to be done to understand how the mechanism for the uptake functions and if it will lead to functional properties of the oligonucleotide. Together with stability against nucleases and the slight increase of the T_m the AECM modification may have quite some potential for use in oligonucleotide therapy.

2`-O-AECM modified phosphoamidite building block was synthesized and successfully used in the synthesis of AECM-modified oligonucleotides. The oligonucleotides provide an unusual combination of remarkable properties. This includes the combination of high resistance towards enzymatic degradation and the spontaneous cellular uptake of AECM oligonucleotides. Thermal melting of duplexes of oligonucleotides showed a general increase in melting of complexes with RNA (+0.5 to +2.3 degrees per modification), while in complexes

with DNA there was little difference compared to a DNA–DNA duplex (0.3 to +1.0 degrees per modification). CD spectroscopy curves for AECM containing oligonucleotide duplexes with both DNA and RNA suggests that the incorporation of AECM modifications pushes the duplex to adopt an A-conformation which is also consistent with the higher melting points (4-6). A fully modified oligonucleotide was incubated in human serum and appears to be completely stable after 24h. Fully AECM-modified oligonucleotides and the reference DNA sequence were synthesized with a fluorescein label. U2OS cells were then treated with the respective oligonucleotide and subjected to analysis by confocal microscopy. It appears as if there is massive uptake of the AECM containing oligonucleotide after the relatively short incubation time of 8 h whereas the corresponding native DNA oligonucleotide is not visibly taken up.

3 CONCLUSIONS AND PERSPECTIVES

Both the CM and AECM modifications have been shown to have many coveted properties such as nuclease stability and good binding ability to complementary DNA and RNA as well as the apparent cellular uptake of the AECM modification.

Of course, these modifications need to be studied further to explore their potential. New optimized and scaled-up syntheses of building blocks would be beneficial. The carbamoyl group opens up many new possibilities for changing the basic structure (92, 100). That can be achieved from the carboxylic acid ester, for example, and could lead to molecules with varying abilities/properties.

It would also be interesting to study where these molecules travel in animal and human studies. Using radiolabeling with sufficiently long-lived radionuclides such as 18-Fluorine, 45-Titanium 68-Gallium (or other radionuclides) could function in PET studies (101). This may give a good answer as to where these molecules travel inside the body and where they accumulate as well as how they leave the body, at least within the duration of the isotope detection time. This is an area that has been little explored in terms of oligonucleotides so far, but it would be valuable to answer this question. Another question that would be interesting to answer is how the mechanism for the cellular uptake of the AECM modification works. This is something that needs an answer.

A third interesting idea is whether it would be possible to alkylate uridine and guanosine, both of which carry the lactam function, selectively in the 2`-OH position without having to protect the lactam function before alkylation. If this could be done by using such conditions using different metal ions which can selectively coordinate the alkylation so that it takes place in the 2´-OH position and not on the lactam function. This would save many reaction steps. Also the leaving group of the alkylating agent can have an effect of where the alkylation will take place. Most alkylating reagents that are being used in the alkylation of the 2´-OH are alkyl halides but there are examples of sulfonic esters being used for this type of reactions (95). There are also examples of metals such as stannous reagents to coordinate the reaction towards the 2´-OH (64,102).

As a final speculative idea in the pursuit of generation of 2´-O- alkyl modified nucleotide building blocks that can be used in oligonucleotide synthesis. The idea would avoid many reaction steps where nucleosides must be protected and deprotected before they can have the final protecting groups intended for oligonucleotide synthesis. The question is if 2´-O -alkyl nucleotides could be prepared from 2',3'-O-alkyllidene compounds, that can be prepared in one step from all ribonucleosides (Figure 30). If the formed 2',3'-O-alkyllidene is reduced under a mild and simple procedure, employing sodium cyanoborohydride in the presence of boron trifluoride etherate in dry THF, for the reductive cleavage of the alkylidene to form the 2´-O-

alkylated product (if the nucleobase and ribose sugar moiety can stand the conditions). It would be interesting to see if it is possible and what the selectivity outcome would be with respect to the 2´-O and 3´-O alkylated products after reduction (103-106).

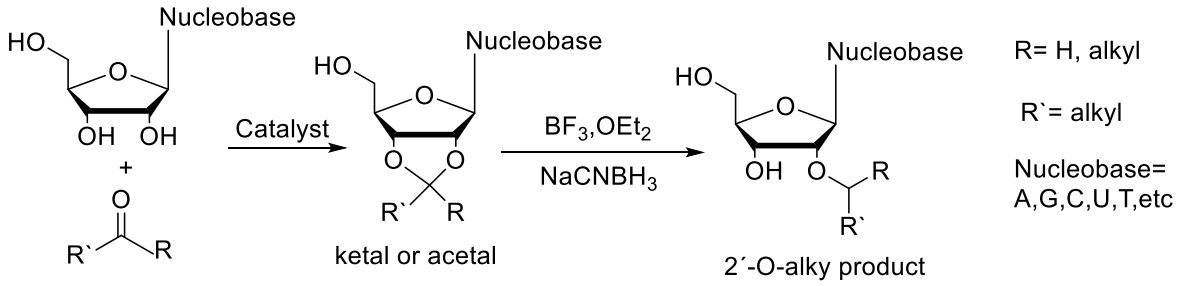

Figure 30. Possible synthesis pathway for 2´-O-alkyl nucleosides from 2',3'-O-alkyllidene compounds.

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