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„Synthesis of 2'-thioethyl modified adenosine and uridine and their use for generating peptide oligonucleotide conjugates“

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Abstract

Therapeutic oligonucleotides, such as ASONs or siRNA, are promising alternatives in treating illnesses and diseases. Most important in this context are genetic disorders, viral diseases, cancer and orphan diseases.

Although the concept of using oligonucleotides therapeutically roots back almost three decades, just very few compounds have won approval so far. To date, there are still some major hurdles concerning pharmacokinetic and pharmacodynamic parameters, which slow down the success of oligonucleotide therapy. Hence, several chemical modifications, conjugations and specialized transport systems were developed over the last decades.

During this diploma thesis, thiol-linker modified nucleotides were synthesized and incorporated into oligonucleotides for coupling with peptides in order to increase cellular uptake, which again would lead to improved efficacy.

At first, the nucleotides, uridine and adenosine building blocks with protected thiol linkers for automated oligonucleotide synthesis were prepared, and their structure was verified by NMR and HRMS spectrometry.

In line with this diploma thesis a synthesis route for the modified nucleotide was elaborated. Thereby, most effort was devoted to the selection of a suitable protection group for the thiol-linker, and the S-trityl function was identified to be most suitable out of several evaluated protection strategies. At first, uridine was transformed to anhydrouridine, followed by attachment of the alkyl chain to the 2'-hydroxy group. After protecting the 5'-hydroxyl group a phosphoramidite was attached at the 3'-OH, resulting in the final product.

The synthesis of adenosine, starting from 3',5'-O-(tetraisopropylidisiloxane-1,3-diyl)adenosine, involved the introduction of a methylthiomethyl chain at the 2'-hydroxyl group as well as an acetyl-protection group at the amino-group of adenine. Next, the silyl-bridge was removed using fluoride and the 5'-OH was protected using DMTrCl.

Next, phosphorothioates were synthesized and oligonucleotides containing these amended nucleotides, were prepared. Hereupon, a methodology for purifying the ONs, removing the trityl-protection group argentometrically and regenerating the sulphur of the uridine and thereby restoring an unprotected thiol

function, was established. Subsequently, developing a suitable method for coupling them with lysine-rich peptides was achieved, as proven by the results of gel electrophoresis.

Zusammenfassung

Therapeutisch genutzte Oligonukleotide, z.B. ASONs oder siRNA, stellen eine vielversprechende Möglichkeit für die Therapie von Krankheiten dar. Im Zentrum der Forschung stehen dabei virale oder genetische Krankheiten, sowie Krebs oder Erkrankungen mit geringer Prävalenz.

Obwohl das Konzept therapeutisch nutzbarer Oligonukleotide vor mittlerweile 30 Jahren vorgestellt wurde, sind bislang nur wenige Vertreter zugelassen worden. Bis heute gibt es einige Hürden, die es zu überwinden gilt, bevor therapeutisch genutzte Oligonukleotide breite Anwendung in der Medizin finden können. Dazu gehören vor allen pharmakokinetische und pharmakodynamische Hindernisse, wie beispielsweise kurze Halbwertszeiten, geringe Zellaufnahme und Zellspezifität sowie unerwünschte Nebenwirkungen.

Deshalb wurden in den vergangenen Jahrzehnten zahlreiche chemische Modifikationen, Konjugationen und Transportsysteme für Oligonukleotide entwickelt. Diese Diplomarbeit beschäftigt sich mit der Konjugation von Oligonukleotiden mit Peptiden durch Einführung eines geeigneten Thiol-Linkers in die Nukleotide. In weiterer Folge wird dadurch eine Verbesserung der Zellaufnahme in Verbindung mit einer Wirkungssteigerung erwartet.

Dabei wurden zuerst die Nukleotide, Uridin und Adenosin, chemisch so verändert, dass sie in der Lage sind, kovalent an Peptide zu binden. Im Anschluss daran wurden mittels RNA-Synthese Oligonukleotide, die diese chemisch veränderten Nukleotide enthalten, hergestellt.

Es gelang ein geeignetes Syntheschema für die Herstellung der modifizierten Nukleotide zu entwickeln. Dabei lag der Schwerpunkt auf der Verwendung geeigneter Schutzgruppen für den Thiol-Linker. Von mehreren getesteten Schutzgruppen erwies sich dabei die S-trityl Gruppe als am besten geeignet.

Zuerst wurde Anhydrouridin aus Uridin gewonnen, an das anschließend die Alkylkette in 2'-Position angehängt wurde. Nachdem die 5'-OH Gruppe mittels DMTrCl geschützt worden war, wurde in 3'-Position das Phosphoramidit angefügt, wodurch das angestrebte Endprodukt erhalten wurde.

Für die Synthese des Adenosin Bausteins wurde 3',5'-O-(tetraisopropyldisiloxane-1,3-diyl)adenosine an der Aminofunktion des Adenins acetyliert und in

2'-Position mit einer Methylthiomethyl Gruppe geschützt. Anschließend wurde die Silylspange mittels Fluorid entfernt und die 5'-OH Gruppe mit DMTrCl geschützt.

Im Anschluss daran wurden Phosphorothioate synthetisiert. Es konnte ein erfolgreiches Verfahren zur Kopplung dieser Oligonukleotide mit den Peptiden, sowie deren Aufreinigung, Entschützung und Regeneration entwickelt werden, was durch die Ergebnisse der Gel-Elektrophorese bestätigt wurde.

1. Introduction

1.1 Antisense oligonucleotides

Antisense oligonucleotides (ASOs) are a class of therapeutics made of short pieces of nucleic acids. They are synthetic DNA/RNA-like oligonucleotides, typically composed of 16-21 nucleotides, which display their effect by binding sequence-specifically to RNA by Watson-Crick base pairing⁴. Even before and ever after the first ASO, fomivirsen sodium, was introduced into the market in 1998, many others were designed following the same active principle. With high specificity they attenuate or correct the expression of discordantly regulated genes, thereby offering a new way of fighting previously incurable diseases, as well as rare diseases^{1,2}.

Despite the fact that the concept of ASOs has by now been introduced into science more than three decades ago and a lot of effort has been devoted to their development, only a few substances have made it onto the market yet. Due to their challenging development, poor nuclease stability, binding affinity and pharmacokinetics, including distribution and cellular uptake, systemic widespread application has been limited.

In 1978, Zamecnik and Stephens were first to demonstrate the potential of antisense therapy. These first oligonucleotides (ONs) showed good antiviral in vitro activity against the Rous sarcoma virus. Soon after, chemically modified ONs were designed aiming to increase nuclease resistance, binding affinity and bioavailability, upon which phosphorothioate modification was first used to maximise efficacy in vitro. Although several other chemical modifications were developed in the meantime only five different substance classes made it into clinical trials or were tested in vivo. Even though the concept of ASOs is promising, there are still significant hurdles to overcome before broad clinical use can be accomplished^{1,3}.

1.2 Hurdles and problems of ASON-therapy

Because of their unique structural properties and targets, ASONs show fairly different PK and PD parameters compared to small molecules, which represent most drugs.

The concept of ASONs involves new challenges to overcome. Ubiquitous nucleases rapidly degrade the phosphodiester backbone of wild-type oligonucleotides, which prevents systemic effects. Moreover, poor plasma protein binding and high renal elimination confine the potency of these drugs²⁷. Another limiting factor is cellular uptake. ASONs are large, polar molecules that cannot readily pass cellular membranes²⁸.

Furthermore, some ASONs show unspecific binding. Ideally, ASONs should modulate the expression of one single gene but unspecific binding to other mRNAs or proteins may cause adverse effects, which can be reduced at least partially by accurate sequence selection²⁹.

To overcome these odds chemical modifications as well as distinct transport systems and ligands have been developed. Although major improvements have been made over the last decades, further advancements are still required to achieve broad clinical use.

1.3 Biological effects of ASON-therapy

1.3.1 Treatment of DMD using ASON-based therapy

ASONS are being used in different medicinal areas to date. The various biological applications of ASONS can be explained by different mechanisms of action, responsible for their effects. A very popular field concerning ASON-research is Duchenne muscular dystrophy (DMD). Because of ASONS providing an alternative therapeutic approach, DMD has emerged as a genetic disorder for understanding and overcoming the challenges associated with developing more advanced ON therapies⁵.

DMD is an X-linked, incurable progressive muscular disorder which is caused by the absence of functional dystrophin, a protein vital for normal muscle function. Thus, mutations in the dystrophin gene lead to non-functional or truncated proteins, which results in premature death due to respiratory and/or cardiac failure typically by age of 30⁵.

DMD is most likely caused by duplication, non-sense- or frame shift mutations⁶, which lead to altered pre-mRNA. Pre-mRNA consists of introns and exons, with introns getting removed by splicing, thus producing mRNA. The process of splicing can be manipulated by ASONS, which is called exon skipping. Thereby, some of the exons get skipped selectively which results in altered proteins. These products are internally truncated but since the terminal parts of the dystrophin protein are instrumental for binding and function, this can thereby counter the symptoms of DMD⁹.

RNA- splicing:

The process of pre-mRNA splicing is pivotal in the body to produce all of its proteins. In fact, there are fewer genes in the human DNA than there are proteins in the body, which can be explained by alternative splicing. 95% of human pre-mRNA is alternatively spliced¹⁰. Thereby one gene can encode several proteins with very different, sometimes even antagonistic effects or it can lead to the introduction of a stop codon which results in the suppression of the gene and thereby the proteins it encodes. This flexibility is important in healthy individuals and contributes the clockwork function of human biology¹². In contrast, incorrect splicing can cause severe diseases, which makes it an interesting point of action for drug development. Thereby, ASONS bind specifically to the pre-mRNA

and block splicing sites, restoring canonical splicing¹². To accomplish sufficient efficacy, high target specificity and binding affinity is required as well as good PK/PD parameters.

Besides that mutations can cause sequences in the pre-mRNA that are normally not used as splicing sites, so called cryptic slicing sites, to be activated and either inactivate an existing splicing site or generate a new splicing site, where one has not existed before. As a result non-functional proteins are produced. These cryptic slicing sites display another target for ASONs. Blocking the access of the spliceosome to these sites can restore correct splicing^{11,12}.

Another way of using ASONs is to knock down the function of undesired genes, thereby inhibiting the expression of a certain gene. This can be achieved by inducing exon skipping and producing mRNA isoforms that encode non-functional proteins or trigger decay of mRNA¹³. However, RNase H activating oligonucleotides, RNA interference or translation inhibition are more commonly used if knockdown is desired.

If exons are deleted from the mRNA via splice switching, the open reading frame (ORF) has to be maintained, otherwise non-functional proteins would be the result¹².

Clinical use:

Research has shown that exons 45-55 cover mutations related to 40-45% of DMD patients⁸. Because of this, skipping this hotspot region is a promising approach towards a treatment for this disease.

The requirements for ASONs altering splicing vary from ASONs used to achieve gene downregulation⁷. They must not activate RNase H, which would lead to the destruction of the pre-mRNA and they should be able to access the pre-mRNA within the cell nuclei. Several chemical modifications fit these criteria. For example 2'-O-Me, PS, LNA and ASONs with PMO based backbones are RNase H inactive and display a high affinity for target sequence and are nuclease resistant as well. But still delivery issues and off-target-effects slow the progress of these drugs¹⁶. Yet data from a series of clinical trials, investigating ASON-based exon skipping therapy of DMD is highly promising⁵.

Several substances have so far made it into clinical trials and are currently under evaluation.

Eteplirsen, a PMO splice switching ON has recently completed a phase II trial. It is tolerated well, however poor bioavailability and cellular uptake in some muscle tissues, especially the heart, are major concerns.

Drisapersen, a 2'-O-Me ASON, is an investigational antisense oligonucleotide drug candidate for the treatment DMD. It induces the skipping of dystrophin exon 51. In applicable cases, skipping this exon allows for the production of a truncated but functional dystrophin protein. Thus, it potentially provides a therapeutic benefit to DMD patients for whom skipping of exon 51 restores the proper dystrophin reading frame. This affects approximately 13% of DMD patients. Although data from clinical trials suggests modest efficacy, it has been submitted to the FDA and EMEA for market approval for treatment of DMD amenable to exon 51 skipping recently³⁴.

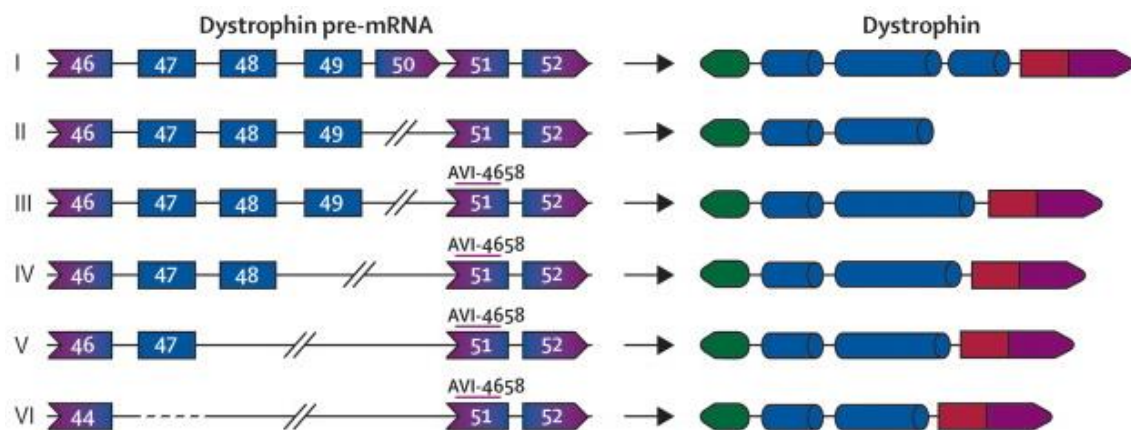


Figure 1.01: Exons of the dystrophin pre-mRNA. Deleting some of the exons leads to the production of differently truncated proteins, which, in some cases, are still partially functional.

However, ASONs can lead to adverse and toxic effects, heavily related to the respective modification. For PS-ONs, prolongation of the activated partial thromboplastin time, reactions at the injection site, pro-inflammatory actions and activation of the alternative complement pathway have been reported as well as elevation of AST and ALT, which indicates liver injury^{21,22}. These effects occur as a result of sequence-unspecific actions due to high ON concentrations and can, therefore, be managed well by adjusting the dose scheme and application of slow i.v. infusions²³.

1.3.2 Treatment of other diseases using ASON-based therapy

Besides that, other potential medical fields where ASONs can provide potent therapies are neurodegenerative diseases, cancer, viral and microbial infections, metabolic diseases, inflammatory diseases and other rare diseases¹.

Since the introduction of ASONs three decades ago different mechanisms have been reported. Unlike most other drugs that target proteins in the human body the basic drug-receptor interaction for ASONs is the Watson-Crick hybridization of complementary DNA or RNA strands. This interaction can result in different mechanisms, proposed as explanations for the effects of ASONs.

mRNA degradation:

ASONs can induce strand scission by RNase H. This is a ubiquitously expressed ribonuclease that degrades preferentially DNA-RNA duplex, where it affects the RNA strand of the duplex. The ability of activating RNase H varies between different chemical classes of ONs and can be used selectively, depending on whether RNase H activation is desired or not¹⁴.

Steric block:

Poor activators of RNase H are believed to unfold their effects on protein biosynthesis via steric block. In this mechanism, physical drug occupation of the target RNA prevents the factors involved in splicing, translation and metabolism from accessing the RNA. The so called translational arrest is the most common way of steric block, at which ASONs prevent the recruitment of the translational machinery or the progress of translation by hybridizing with the complementary sequence¹.

Clinical use:

Mipomersen is a 20mer phosphorothioate-2'-methoxyethyl-gapmer targeting Apo B-100 and has successfully passed a phase III clinical trial and is currently used as a treatment for familial hypercholesterolemia, a disease caused by a mutation of the LDL receptor, which leads to increased plasma levels of LDL. It has been approved in the US in 2013, however approval in Europe has been denied due to safety issues.

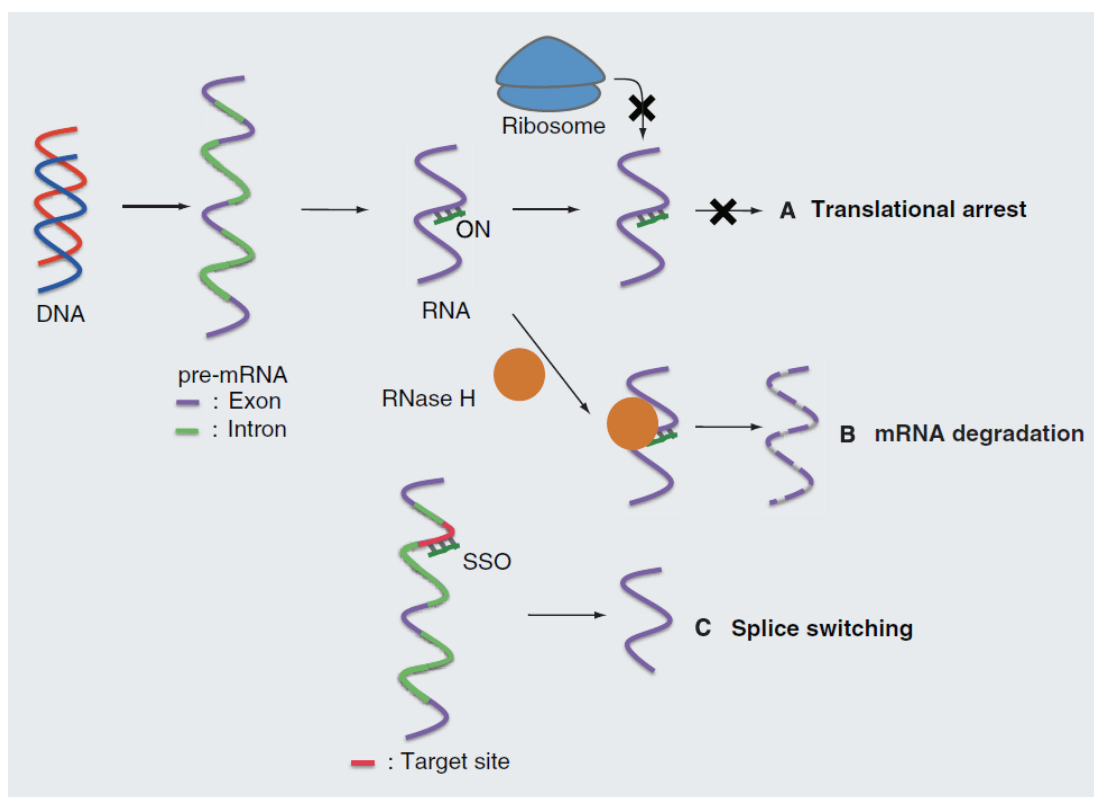


Figure 1.02: Three representative antisense mechanisms. A: The binding of ASONs prevent the ribosomes from interacting with mRNA, thereby inhibiting the expression of a protein. B: RNase H gets induced by the ASON bound to mRNA and degrades it. C: The complementary binding of the ASON results in altered splicing of the RNA and thereby in the expression of different proteins with altered 3D structures.

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1.4 Chemical modifications

Wild type ONs show several unfavourable properties, including poor plasma protein binding and organ distribution, rapid degradation by blood nucleases and quick renal elimination¹⁷. Thus, several chemical modifications have been developed, of which five have made it into clinical trials yet: phosphorothioates (PSs), 2'-O-methoxyethyl modified ONs (2'-MOEs), 2'-O-methyl modified ONs (2'-O-Me), locked nucleic acids (LNAs) and phosphorodiamidate morpholino oligomers (PMOs)¹.

ASONS average approximately 20 nucleotides and their molecular weight is approximately 6-7 kDa. Because of their inherent chemical properties they are predominantly applied parenterally or subcutaneously². They consist of a nucleobase, a negatively charged phosphodiester backbone and a ribofuranose or deoxyribose. Modifications can be applied to all three of these elements.

1.4.1 Backbone modified ONs

The most prominent example for backbone modified ONs are the often used phosphorothioates. They are referred to as first generation ASONS and were the first to be clinically tested^{1,2}. Thereby, a non-bridging oxygen atom of the phosphodiester linkage is replaced by a sulphur atom, thus making it somewhat more hydrophobic and acidic compared to wild type DNA¹⁵. Furthermore, the replacement creates chirality at the phosphorus centre. As a result, the synthesis of PS, which is almost always done in a non-stereoselective manner, produces 2^{n-1} stereoisomers¹.

PS show a slightly reduced affinity to RNA but dramatically increased nuclease resistance which has proven to be sufficient for parenteral administration. This is believed to be caused by small conformational changes due to the larger size of the sulphur atom, compared to oxygen^{1, 2}.

Furthermore, this modification reduces urinary excretion, which results in a 10-fold slower plasma clearance², improves tissue distribution and prolongs residence time in cells, mainly as a result of increased protein binding.

Following parenteral admission, PS-ONs are rapidly absorbed into the plasma and distributed widely among tissues, whereat, as seen at other modifications as well, kidney, liver, spleen, heart and skeletal muscle show highest concentrations. Hardly any ON can pass the blood-brain-barrier^{1,2}.

When ASONs get taken up into cells a productive pathway, which leads to functional ONs and a non-productive one, which fails to induce a pharmacological effect, can be distinguished. It is speculated that a satiable non-productive pathway has to be overcome to facilitate productive uptake, for example by bolus injection⁴.

Finally, ONs are mainly eliminated by the kidney after degradation by exonucleases². Because of this, serum and tissue half-lives, which can last up to several weeks, are dependent on nuclease stability of the nucleotides located at the edges of an ON. Thus, using PS-ONs or 2'O-modified ONs flanking an ASON is a legitimate way to extend half-lives and reducing adverse side effects at the same time⁴.

Hence, PS-ASONs have become very popular, even if their clinical use is still limited because of poor PD properties, safety issues and off-target-effects¹⁶.

Other examples of backbone modified ONs would be phosphorodithioates, at which both oxygen atoms of the phosphodiester linkage are replaced by sulphur atoms or boranophosphates. Here, an oxygen atom of the phosphodiester linkage is replaced by a borane¹. However, these modifications have not made it into clinical trials yet.

Like other modifications, PS-ONs can lead to adverse and toxic effects as well. These events can be managed relatively well by adjusting the dose schema and proper sequence selection.

Besides that, other modifications have been developed concerning not just the backbone, but also the ribose.

Backbone modified ONs were first generation drugs, which showed important improvements compared to wild-type ONs. The plasma protein binding is essential for achieving meaningful effects and since this is not induced by other modifications, the PS backbone is used in conjunction with newer modifications, even if their clinical use is still limited because of poor PD properties, safety issues and off-target-effects^{1,2}.

1.4.2 Sugar modified analogues

Thus, further modifications were developed, representing second generation ONs with modifications not only limited to the backbone but also additionally including structural changes of the ribose. The goal of these substances was, once again, modulating protein binding and increasing binding affinity as well as nuclease resistance¹⁸.

2'-O-methoxyethyl modified ONs and 2'-O-methyl modified ONs are clinically used examples for these drugs. They show increased nuclease stability and increased binding affinity. They are often combined with PS-modification, which is necessary for plasma protein binding and thus prevents renal elimination and induces a certain degree of cellular uptake. It has been shown in rats that intestinal permeability can be increased by 2'-MOE-PS gapmers by two- to fourfold which is most likely due to their increased stability and steric factors¹⁹.

These gapmers show similar distribution profiles compared to PS-ONs, plasma protein binding of about 90% and significantly increased tissue residence time, compared to first generation ONs²⁰. Another example of an ON with a modified sugar are locked nucleic acids (LNA) which feature a methylene bridge between 2'-O and 4'-C of the ribose. Due to steric factors, they exhibit high nuclease stability and binding affinity, which increases their melting temperature compared to wild-type DNA. They are typically used in gapmers in conjugation with a PS backbone¹.

1.4.3 Other modifications

PMOs in which riboses are substituted by morpholino rings can be used as splice-switching ONs. Although trials performed in mice showed higher potency of PMOs compared to 2'-OMe-PS-ONs, their success is restricted due to their poor cellular uptake. Like other ONs they are primarily distributed to the liver and the kidneys followed by spleen and muscles. PMOs are stable against enzymatic degradation. They are less bound to plasma proteins than PSs and are, therefore, eliminated by the kidneys to a higher extent¹.

Due to their non-ionic character, which prevents drug interactions with cellular components, PMOs show little adverse effects and are tolerated well^{24,25}.

Besides that, there are some modifications trying to improve cellular uptake. One approach is to incorporate ONs into polymeric carriers, nanoparticles or

liposomes². Another method is to create molecular scale conjugations. Thereby, ONs are attached covalently to ligands that are enabled to bind highly specifically to cell surface receptors. Thus, entry via the endocytic pathway is enabled³⁰. Therefore ONs can be covalently attached to cationic peptides²⁶. Most cationic peptides bind to cell-associated glycosaminoglycans and are taken up into the cell by endocytosis. The detailed mechanisms involved, however, remain controversial. Furthermore, it has been shown that sequestration and degradation in endocytic vesicles severely limits the efficiency of cationic-peptide mediated delivery.

After a conjugated ON is taken up by a cell via endocytosis, releasing it into the cytosol is a critical steps which currently attracts research attention²

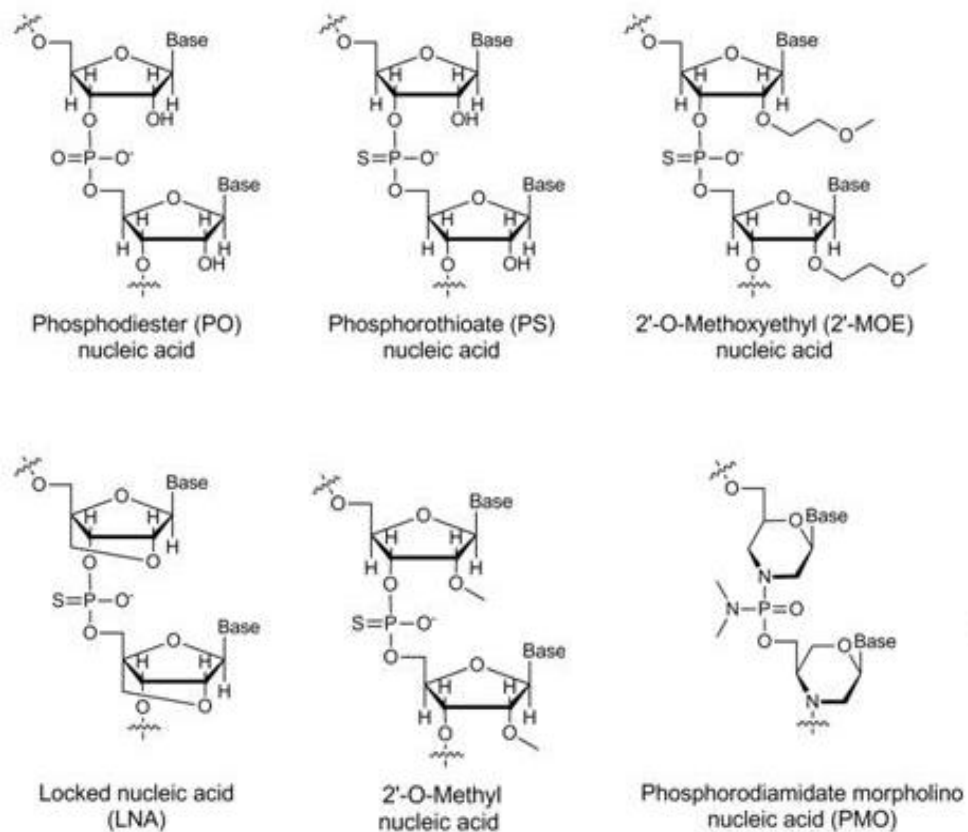


Figure 1.03: POs represent the backbone of wild-type RNA. PSs, 2'-MOEs, 2'-O-Mes, LNAs and PMOs represent clinically used chemical modifications.

Modified from: Expert opinion on biological therapy 2013 Jun; 13(6): 875-888

2. Aim

ASOs are a highly promising approach towards treating diseases. Poor cellular uptake, however, is one of the big hurdles, which still slows down the progress of clinical use. Hence, new ways of improving cellular uptake are invented, such as coupling oligonucleotides with cationic peptides.

The purpose of this diploma thesis was the synthesis of chemically modified nucleotides and their incorporation into oligonucleotides. Therefore, we introduced an ethylthiol chain at the 2'-OH of a uridine, with the sulphur masked with a suitable protection group. Furthermore, for oligonucleotide synthesis, the 5'-hydroxyl group should be protected with a DMTr-group, and a phosphoramidite was to be introduced at the 3'-OH.

It was planned to use this nucleotide for the synthesis of oligonucleotides via the standard methodology of solid phase synthesis. Adequate methods compatible with the thiol protection strategy for purifying, deprotecting, regenerating and coupling the 18mer oligonucleotides with peptides were aimed to be developed. Successful deprotection, coupling and purity of the product were to be examined by MS, HPLC and gel electrophoresis.

3. Materials and methods

^1H - and ^{13}C -NMR spectroscopy were performed on a Varian 500 MHz or Bruker 200 MHz machine.

Silica gel pore size 60 Å 35-70 µm (Acros) was used as a stationary phase for column chromatography. Silica gel on TLC aluminium plates was obtained from Merck (silica gel 60 F 254 nm, Al foil 20x20 cm). The development of the TLCs was performed in conventional, saturated developing chambers with solvents DCM/MeOH, PE/MTBE, DCM/n-hexane/TEA. For detection UV shadowing under a 254 nm light source was used. Some compounds were also stained by spraying them with Seebach Reagent (2.5% phosphomolybdic acid, 1% $\text{Ce}(\text{SO}_4)_2$, 6% sulphuric acid, 90.5% water).

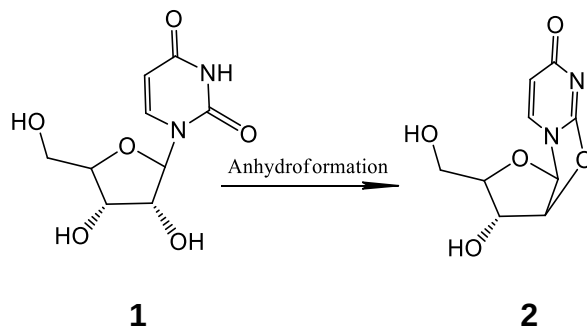
The reagents and solvents for the syntheses of the nucleotides were obtained from Sigma-Aldrich and Serva. The reagents and solvents for the synthesis, purification and modification of the oligonucleotides were obtained mostly from Sigma-Aldrich, Serva, Acros, Gibco, Fluka, PAA and Thermo Fisher Scientific.

The quantification of the oligonucleotides was performed on a NanoDrop spectrophotometer ND-1000 (NanoDrop, Thermo Fisher) based on the Beer-Lambert-Law using the extinction coefficient calculated from the sequence of the oligonucleotides (molar extinction coefficient of: adenine= 15400, cytosine= 7300, guanine= 11700, uracil= 8200)

$$E_{\lambda} = -\ln\left(\frac{I}{I_0}\right) = c \cdot \epsilon_{\lambda} \cdot l$$

3.1 Syntheses of the uridine

3.1.1 2,2'-anhydro-1-(β -D-arabinofuranosyl)uracil



Uridine (**1**, 10 g, 40.95 mmol) and diphenyl carbonate (9.56 g, 44.63 mmol) were dispersed in anhydrous DMF (16.7 ml) and stirred at 80°C until the materials were completely dissolved (ca. 2 hrs). Sodium hydrogen carbonate (0.26 g, 3.07 mmol) was added and the mixture was stirred at 110°C. The reaction was monitored by TLC. After 3 hrs, the reaction mixture was cooled to room temperature and the precipitate was filtered off. The white solid (**2**) was recrystallized and dried under vacuum to gain the title compound as white crystals in 87% yield (8.06 g).

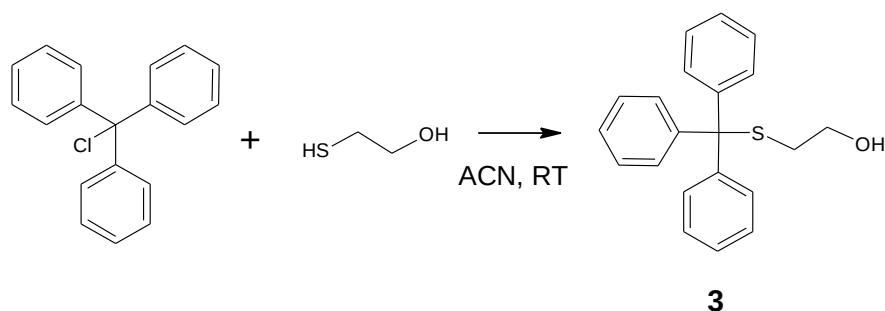
^1H NMR (500 MHz, DMSO- d_6) : δ = 7.84 (d, J = 10.0 Hz, 1H, H-6), 6.31 (d, J = 5.0 Hz, 1H, H-5), 5.89 (s, 1H, OH-3'), 5.84 (d, J = 10.0 Hz, 1H, H-1), 5.20 (d, J = 5.0 Hz, 1H, H-3'), 4.99 (s, 1H, OH-5'), 4.38 (s, 1H, H-4'), 4.07 (m, 1H, H-2'), 3.28 (m, 1H, H-5'/1), 3.19 (m, 1H, H-5'/2).

^{13}C NMR (125 MHz, DMSO- d_6) : δ = 171.20 (C-4), 159.82 (C-2), 136.87 (C-6), 108.61 (C-5), 90.03 (C-1'), 89.22 (C-4'), 88.74 (C-2'), 74.72 (C-3'), 60.83 (C-5').

ESI-HRMS: mass calculated for $\text{C}_9\text{H}_{12}\text{N}_2\text{O}_6\text{Na}$ $[\text{M}+\text{Na}]^+ = 249.0482$, found 249.0487.

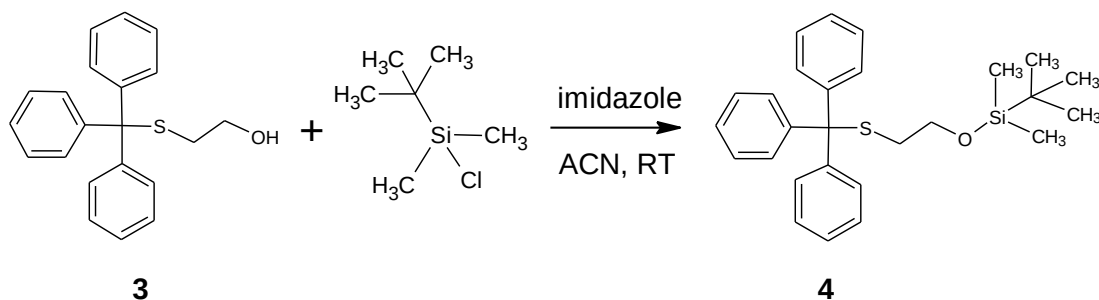
R_f: 0.36 (DCM/MeOH 9/1)

3.1.2 O-dimethyltert.-butylsilyl-S-trityl-2-mercaptoethanol



Triphenylmethylchloride (10.26 g; 36.8 mmol) was dissolved in 120 ml acetonitrile under argon. Then, mercaptoethanol (2.1 ml; 20 mmol) was added. After 18 hrs of stirring at RT the reaction, which was monitored using TLC, was completed. The red-brown, cloudy solution, which contained (**3**) (S-trityl-2-mercaptoethanol), was used for the next step without further purification.

R_f: 0.58 (DCM/MeOH 40/1 + Seebach reagent).



Therefore, imidazole (6.12 g; 89.9 mmol) and TBDMSCl (9 g; 59.71 mmol) were added. The reaction was again monitored by TLC. After 20 hrs the solvent was removed under vacuum at 40°C, the residue was dissolved in EtOAc (300 ml), transferred into a separation funnel and washed with water (3 x 300 ml), saturated aqueous NaHCO₃ (1 x 300 ml) and brine (1 x 300 ml). Then, the organic layer was concentrated to a small volume under vacuum and purified via

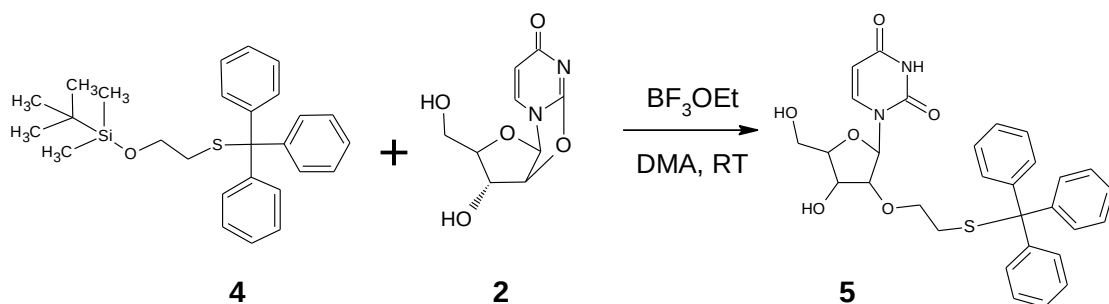
column chromatography at which PE/MTBE 40/1 was used as the eluent to produce 10,7 g (82,3 %) of **(4)** as a viscous oil.

^1H NMR (500 MHz, CDCl_3) : δ = 0.10 (s, 6H, Me-Si), 0.98 (s, 9H, tert.-But-Si), 2.48-2.55 (t, J = 0.035 Hz, 2H, $\text{CH}_2\text{-S}$), 3.52-3.59 (t, J = 0,035 Hz, 2H, $\text{CH}_2\text{-O}$), 7.24 (m, 15H, Tr-S).

^{13}C NMR (500 MHz, CDCl_3) : δ = 5.16 ($\text{CH}_3\text{-Si}$), 18.45 (tert.-But), 26.04 ($\text{CH}_3\text{-C}$), 34.87 ($\text{CH}_2\text{-S}$), 62.03 ($\text{CH}_2\text{-O}$), 126.73 (Ph-C-4), 127.98 (Ph-C-2,6), 129.81 (Ph-C-2,4), 145.13 (Ph-C-1).

R_f: 0.51 (PE/MTBE 20/1).

3.1.3 2'-O-(2-(triphenylmethylthio)ethyl)uridine



The whole reaction was performed under argon and exclusion of water.

Anhydrouridine (**2**, 2.624 g; 10.75 mmol) was added to 22 ml DMA and dissolved using a supersonic bath. Next, BF_3OEt_2 (boron trifluoride diethyl etherate, 2.139 ml; 17.33 mmol) was added dropwise and under vigorous stirring to the cloudy mixture. After five minutes of stirring at RT a portion of (**4**) (S-trityl-O-tert-butyltrimethylsilylmercaptoethanol, 7 g; 16.3 mmol) was added to the solution. Stirring was continued for 16 hrs at 120°C using a reflux condenser.

After the reaction mixture had been allowed to cool to RT, it was transferred dropwise into a separation funnel filled with 100 ml saturated aqueous NaHCO_3 and was diluted with 100 ml EtOAc. The organic solution was washed with saturated aqueous NaHCO_3 (1 x 100 ml) and brine (1 x 100 ml). The layers were separated, dried over Na_2SO_4 , filtered and then the solvent was removed under vacuum. For the chromatography the residue was redissolved in a small volume of DCM and eluted with a gradient of DCM/MeOH from 30/1 to 20/1. The fractions were collected and monitored by TLC. Finally, the combined fractions that contained product were dried under vacuum to produce 1.14 g (19.4 %) of (**5**) as a white foam.

^1H (500 MHz, CDCl_3) : δ = 9.23 (s, 1H, NH), 7.65 (d, J = 9.8 Hz, 1H, H-6), 7.39 (d, J = 5.0 Hz, 6H, Ph-2,6), 7.28-7.24 (m, 6H, Ph-3,5), 7.22-7.14 (m, 3H, Ph-4), 5.68 (d, J = 5.0 Hz, 1H, H-5), 5.59 (d, J = 4.2 Hz, 1H, H-1'), 4.19 (t, J = 5.0 Hz, 1H, H-3'), 4.05-4.01 (m, 1H, H-4'), 3.99-3.96 (t, J = 5.0 Hz, 1H, H-2'), 3.96-3.91

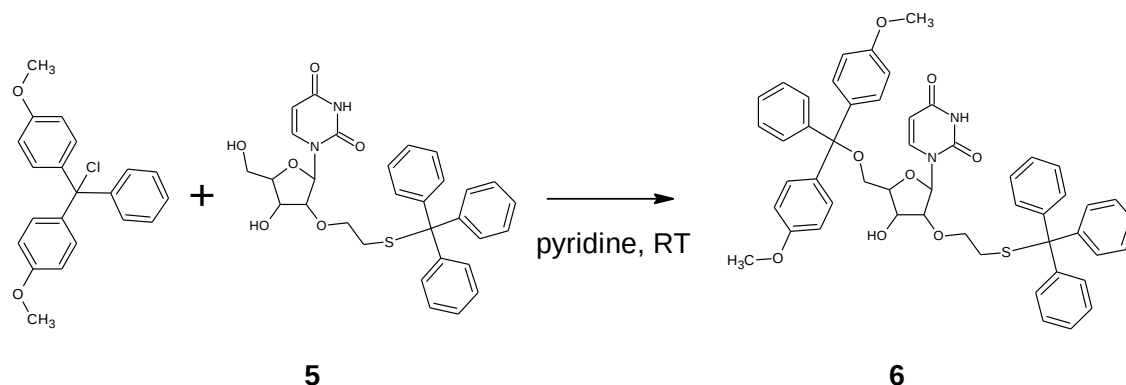
(m, 1H, H-5'/1), 3.81-3.75 (m, 1H, H-5'/2), 3.23-3.15 (m, 1H, H-1''/1), 2.90-3.03 (m, 1H, H-1''/2), 2.53 (ddd, $J = 10.3, 5.6, 4.7$ Hz, 1H, H-1''/1), 2.43-2.35 (m, 1H, H-1''/2).

^{13}C NMR (125 MHz, CDCl_3) : $\delta = 163.49$ (C-4), 150.30 (C-2), 144.56 (Ph-1), 142.02 (C-6), 129.64 (Ph-3,5), 126.95 (Ph-4), 102.45 (C-5), 90.51 (C-1'), 85.23 (C-2'), 81.13 (C-4'), 69.46 (C-3'), 69.34 (C-1''), 68.79 (C-2''), 67.10 (Tr-C), 61.46 (C-5'), 32.18 (C-2'').

ESI-HRMS m/z $[\text{M}+\text{Na}]^+$: calculated for $\text{C}_{30}\text{H}_{30}\text{N}_2\text{NaO}_6\text{S}$ 569.1717, found 569.1720.

R_f: 0.22 (DCM/MeOH 20/1).

3.1.4 5'-O-(4,4'-dimethoxytrityl)-2'-O(2-(thiophenylmethylthio)ethyl)uridine



After **(5)** (1.14 g; 2.09 mmol) had been co-evaporated three times with pyridine, it was dissolved in 10 ml anhydrous pyridine and DMTrCl (1.265 g; 3.73 mmol) was added. The mixture was allowed to react for 48 hrs at RT under argon.

The reaction was quenched by adding 2 ml MeOH. After 20 minutes the solvents were removed under vacuum. The residue was taken up in MTBE, washed with water (3 x 50 ml), saturated aqueous NaHCO₃ (1 x 50 ml) and brine (1 x 50 ml). The organic layer was separated, dried over Na₂SO₄, filtered and concentrated to a small volume. Then, it was purified via column chromatography.

For this, silica gel was pretreated by shaking gently with 10% TEA in n-hexane for 10 minutes, filtered and dried under vacuum. Then, it was poured into the column and packed with DCM/MeOH 50/1. The sample was loaded in DCM and eluted with a gradient of DCM/MeOH from 50/1 to 30/1. The fractions were collected and analysed by TLC. Those that contained product were combined and dried under vacuum to produce 1.383 g (79.4%) of **(6)** as a pale yellow foam.

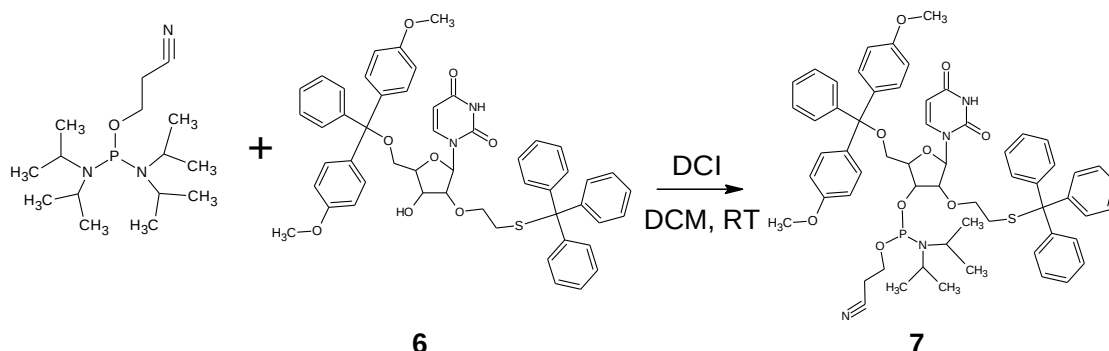
¹H (200 MHz, CDCl₃) : δ = 8.57 (s, 1H, NH), 7.63 (d, J = 9.6 Hz, 1H, H-6), 7.67-7.41 (m, 8H, Tr-2,6 and Ph-2,6), 7.42-7.18 (m, 12H, Tr-4 and Ph-4 and Tr-3,5 and Ph-3,5), 6.91 (d, J = 9.8 Hz, 4H, Ar-3,5), 5.89 (d, J = 5.0 Hz, 1H, H-5), 5.31 (d, J = 12.3 Hz, 1H, H-1'), 4.44 (t, J = 5.0 Hz, 1H, H-3'), 4.09-4.01 (m, 1H, H-4'), 3.94-3.89 (m, 1H, H-2'), 3.86 (s, 6H, OCH₃), 3.82-3.76 (m, 1H, H-1''/1), 3.64-3.56 (m, 2H, H-1''/2 and H-5'/1), 3.44-3.30 (m, 1H, H-5'/2), 2.78-2.59 (m, 2H, H-2''/1,2).

^{13}C NMR (50 MHz, CDCl_3) : δ = 163.53 (C-4), 160.12 (Ar-4), 150.61 (C-2), 143.91 (Tr-Ph-1), 141.30 (C-6), 136.10 (Ph-1), 133.32 (Ar-1), 129.12 (Tr-Ph-3,5), 128.00 (Ph-4), 127.93 (Tr-Ph-2,6), 127.53 (Ar-2,6), 127.50 (Ph-2,3,5,6), 126.93 (Tr-Ph-4), 113.75 (Ar-3,5), 102.34 (C-5), 94.84 (Tr-C), 88.12 (C-1'), 86.09 (C-2'), 85.78 (C-4'), 75.14 (C-3'), 71.40 (C-1''), 68.90 (C-1''), 63.85 (C-5'), 56.33 (DMT-OMe), 28.90 (SCH₂).

ESI-HRMS m/z $[\text{M}+\text{Na}]^+$: calculated for $\text{C}_{51}\text{H}_{48}\text{N}_2\text{NaO}_8\text{S}$ 871.3024, found 871.3011.

R_f: 0.43 (DCM/MeOH 30/1).

3.1.5 5'-O-(4,4'-dimethoxytrityl)-2'-O-(2-(thiophenylmethylthio)ethyl)uridine-3'-O-(2-cyanoethyl-N,N'-diisopropyl)-phosphoramidite



A portion of (**6**) (550 mg; 0.648 mmol) was dissolved in 5 ml DCM and cooled to 0°C. Then, 2-Cyanoethyl-N,N,N',N'-tetraisopropylphosphordiamidite (302.5 μ l; 0.952 mmol) was added slowly and under vigorous stirring. Next, DCI (2.43 ml 3% (G/V) in ACN; 0.617 mmol) was added. The reaction was allowed to warm to RT and was stirred for 20 hrs under argon. The whole reaction was performed under argon and monitored by TLC.

After the reaction had been completed, the mixture was diluted with 5% TEA in EtOAc (46 ml) and transferred into a separation funnel. The mixture was washed with saturated aqueous NaHCO₃ (3 x 46 ml) and dried over Na₂SO₄. The organic layer was separated and the solvents were removed under vacuum.

The residue was redissolved and loaded in DCM after the column had been packed. For this, silica gel (40 g) was swirled in 5% TEA in n-hexane and neutralised for 30 minutes. Then, the column was packed and loaded. The sample was eluted with DCM/n-hexane/TEA 5/4.5/0.5. The fractions were collected and concentrated to give 495.1 mg (72.8 %) of (**7**) as a white foam.

The product was subsequently used for the oligonucleotide synthesis.

Diastereomers: ¹H (500 MHz, CDCl₃) : δ = 8.01/7.92 (d, J = 9.8 Hz, total 1H, H-6), 7.43-7.32 (m, total 8H, Tr-2,6 and Ph-2,6), 7.32-7.15 (m, total 12H, Tr-4 and Ph-4 and Tr-3,5 and Ph-3,5), 6.86-6.74 (m, total 4H, Ar-3,5), 5.91/5.85 (d, J = 5.0 Hz, total 1H, H-5), 5.20/5.18 (d, J = 5.8 Hz, total 1H, H-1'), 4.51-4.47 and 4.38-4.33 (each m, total 1H, H-4'), 4.22-4.12 (m, total 1H, H-3'), 3.91 (dd, J = 10.0 and 5.0 Hz, total 1H, amidit-OCH₂), 3.84-3.80 (m, total 1H, H-2'), 3.78 and

3.77 (s, total 6H, OCH₃), 3.72-3.71 (m, total 1H, H-1''/1), 3.68-3.63 (m, total 1H, H-1''/2), 3.62-3.59 (m, total 1H, H-5'/1), 3.58-3.55 (m, total 1H, H-5'/2), 2.82-2.67 (m, total 2H, amidit-*ipr*-CH), 2.57-2.45 (m, 2H, H-2''/1,2), 1.28-0.98 (m, total 12 H, amidit-*ipr*-CH₃).

¹³C NMR (125 MHz, CDCl₃) : δ = 163.03/162.94 (C-4), 158.85/158.84 (Ar-4), 149.98/149.91 (C-2), 144.91/144.84 (Tr-Ph-1), 144.42/144.29 (Ph-1), 140.29-140.22 (C-6), 135.34/135.25 (Ar-1), 130.37/129.73 (Tr-Ph-3,5), 128.37/128.13 (Ph-4), 127.35/126.84 (Tr-Ph-2,6), 127.53 (Ar 2,6), 127.50 (Ph-3,5), 126.93 (Tr-Ph-4), 117.88/117.60 (amidite-CN), 113.35/113.24 (Ar-3,5), 102.14/101.94 (C-5), 88.07/87.87 (C-1'), 87.24/87.05 (Tr-C), 82.47/82.43 (C-2'), 81.61 (C-4'), 69.76/69.72 (C-3'), 69.55/69.15 (C-1''), 61.48 (C-5'), 58.04 (amidite-OCH₂), 55.39 (DMT-OMe), 43.44/43.34 (amidite-*ipr*-CH), 32.19/32.07 (amidite-α-CH₂), 24.80/24.63 (SCH₂), 23.12/23.02 (amidite-*ipr*-CH₃).

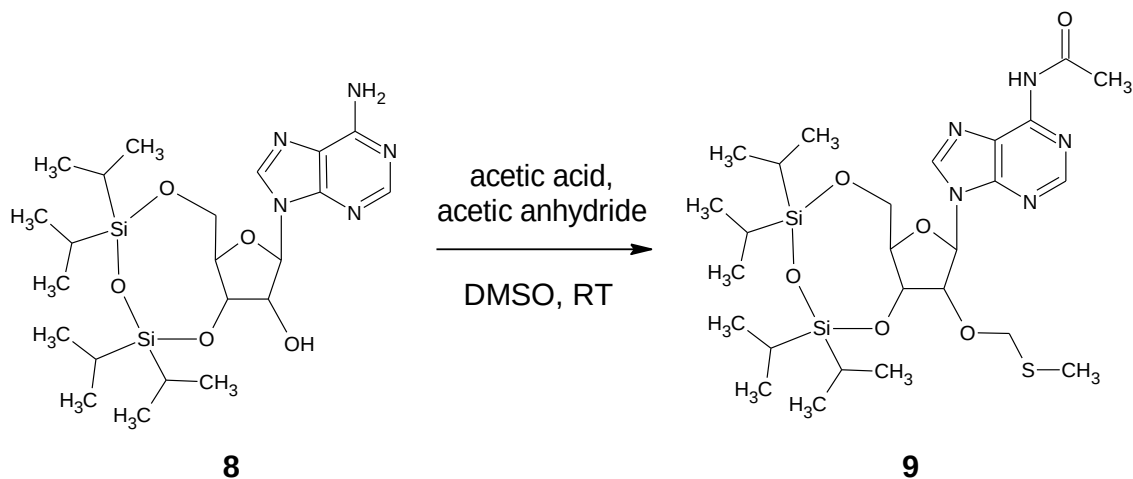
³¹P NMR (202 MHz, CDCl₃): δ= 150.52, 150.43.

ESI-HRMS *m/z* [M+H]⁺: calculated for C₆₀H₆₆N₄O₉PS 1049.4283, found 1049.4280.

R_f: 0.38 (DCM/*n*-Hexane/TEA 6/3.3/0.5).

3.2 Synthesis of the adenosine

3.2.1 3',5'-O-(tetraisopropylidisiloxane-1,3-diyl)-2'-O-(methylthiomethyl)-N⁶-acetyladenosine



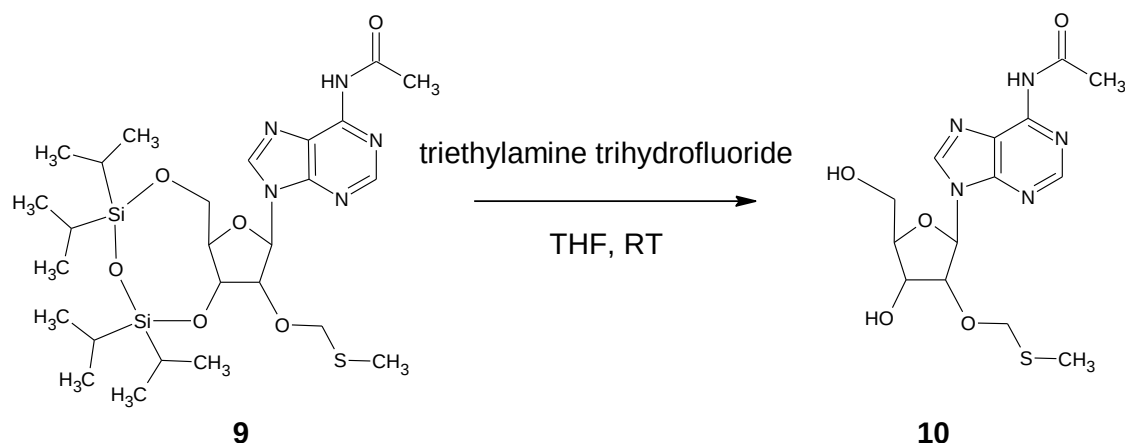
A portion of (**8**) (1 g; 1.63 mmol) was dissolved in DMSO (2.29 ml; 32.24 mmol). Then, acetic acid (2.29 ml; 32 mmol) and acetic anhydride (1.515 ml; 16.1 mmol) were added. The reaction was heated to 40° C and stirred for 48 hrs. After that, the reaction was cooled down to RT and a solution of K₂CO₃ (4.71 g; 34.08 mmol) in 20.85 ml demineralized water was added dropwise under vigorous stirring, during which the product precipitated. Next, the product was filtered and dissolved once again in a small volume of THF, reprecipitated with demineralized water and filtered again. Finally, the product was dried under vacuum and purified via column chromatography using DCM/MeOH as an eluent to produce 0.414 g (34.38 %) of (**9**).

¹H (500 MHz, CDCl₃) : δ = 8.65 (s, 1H, H-2), 8.33 (s, 1H, H-8), 6.09 (s, 1H, H-1'), 4.64 (d, J = 4.7 Hz, 1H, H-2'), 4.68 (dd, J₁ = 4.7 Hz, J₂ = 9.45 Hz, 1H, H-3'), 4.18 (d, J = 8.85 Hz, 1H, H-4'), 4.27 (d, J = 13.55 Hz, 1H, H-5'1), 4.03 (d, J = 15.75 Hz, 1H, H-5'2), 2.61 (s, 3H, Ac-CH₃), 5.06 (d, J = 11.35 Hz, 1H, SCH₂O), 5.01 (d, J = 11.35 Hz, 1H, SCH₂O), 2.20 (s, 3H, SCH₃), 1.16-0.9 (m, 24H, iPr-H), 1.16-0.9 (m, 4H, iPr-CH₃).

^{13}C NMR (125 MHz, CDCl_3) : δ = 152.45 (C-2), 150.12 (C-4), 122.04 (C-5), 148.98 (C-6), 140.82 (C-8), 88.53 (C-1'), 77.58 (C-2'), 68.68 (C-3'), 81.83 (C-4'), 59.56 (C-5'), 170.23 (Ac-COO), 25.67 (Ac-CH₃), 74.70 (SCH₂O), 13.40 (SCH₃), 13.40, 12.90, 12.80 and 12.56 (iPr-H), 17.44, 17.33, 17.28, 17.04, 17.02, 16.98 and 16.87 (iPr-CH₃).

R_f: 0.4 (DCM/MeOH 30/1).

3.2.2 2'-O-(methylthiomethyl)-N⁶-acetyladenosine

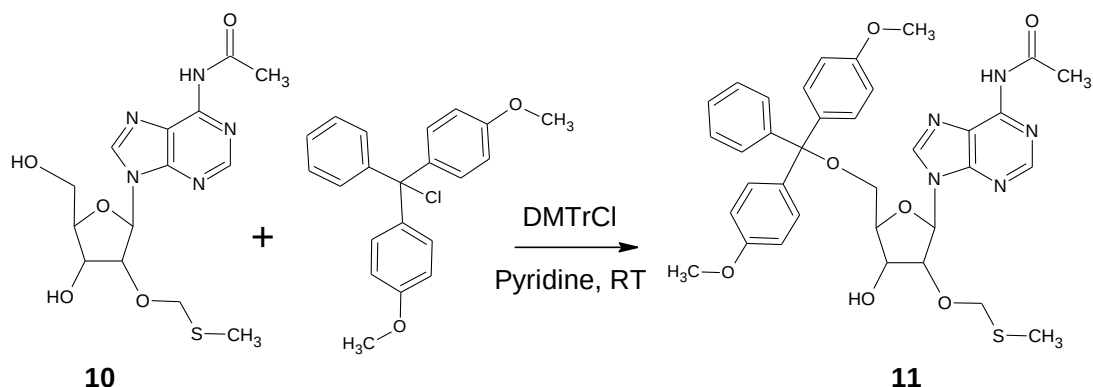


A portion of (**9**) (374 mg; 0.56 mmol) was dissolved in THF (2 ml). Triethylamine trihydrofluoride (546 μ l; 3.39 mmol) was added and the mixture was allowed to react for 3 hrs at RT and under argon.

After 3 hrs the solvent was removed under vacuum. The residue was taken up in 2 ml EtOAc, transferred into a separation funnel, diluted and washed with water (2 x 25 ml), saturated aqueous NaCl (1 x 25 ml) and saturated aqueous NaHCO₃ (1 x 25 ml). Then, the organic layer was separated, dried over Na₂SO₄ and was finally dried under vacuum. The result was (**10**, 106.03 mg; 44.46 %) which was used without further purification for the next step.

R_f: 0.15 (DCM/MeOH 30/1).

3.2.3 5'-O-(4,4'-dimethoxytrityl)-2'-O-(methylthiomethyl)-N⁶-acetyladenosine



A portion of (**10**, 92.9 mg; 0.218 mmol) was co-evaporated 3 times with pyridine before it was dissolved in 2 ml pyridine. DMTrCl (115 mg; 0.3052 mmol) was added and the reaction was stirred for 20 hrs at RT under argon.

Then, 1 ml EtOH was added to the reaction. After 20 minutes the solvents were removed under vacuum and the residue was taken up in 20 ml MTBE/water 1/1. It was washed with water (4 x 25 ml), saturated aqueous NaCl (1 x 25 ml) and saturated aqueous NaHCO₃ (1 x 25 ml). The organic layer was dried over Na₂SO₄ and the solvent was removed under vacuum. No further purification steps were performed.

¹H (200 MHz, CDCl₃) : δ = 8.88 (s, 1H, H-2), 8.10 (s, 1H, H-8), 7.45-7.31 (m, 5H, Ph-2,3,4,5,6), 7.30-7.20 (m, 4H, Ar-2,6), 6.90 (d, J = 9.9 Hz, 4H, Ar-3,5), 6.04 (d, J = 7.3 Hz, 1H, H-1'), 5.15-5.03 (m, 1H, H-3'), 4.91-4.86 (m, 1H, H-4'), 4.70 (s, 2H, S-CH₂), 3.86 (s, 6H, OCH₃), 3.20-3.13 (m, 1H, H-5'/1), 3.04-2.76 (m, 2H, H-2' and H-5'/1), 2.34 (s, 3H, S-CH₃), 2.07 (s, 3H, Ac-CH₃).

¹³C NMR (50 MHz, CDCl₃) : δ = 166.50 (Ac-C), 158.86 (Ar-C-4), 147.44 (C-6), 139.64 (C-4), 134.83 (C-6), 133.11 (C-2), 130.18 (C-8), 129.22 (Ph-3,5), 127.92 (Ph-2,6), 127.19 (Ph-4), 122.43 (C-5), 113.25 (Ar-3,5), 98.91 (C-1'), 91.62 (Tr-C), 88.11 (C-2'), 84.10 (C-4'), 73.22 (S-CH₂), 71.11 (C-3'), 62.41 (C-5'), 55.27 (O-CH₃).

R_f: 0.24 (DCM/MeOH 30/1).

3.3 RNA-synthesis

The synthesis of the oligonucleotides was performed on a PolyGen 10 column DNA/RNA synthesiser, which enables full automation, using the phosphoramidite method. Phosphoramidite oligonucleotide synthesis proceeds in 3'- to 5'- direction (in contrast to the 5'- to 3'-direction of DNA biosynthesis in DNA replication). One nucleotide is added per synthesis cycle. As a solid phase, controlled pore glass (CPG) was used, to which the first nucleotide, an adenosine, had already been pre-attached. The nucleotides used for the synthesis of the oligo-nucleotides had a silylether group in 2'-position, a DMTr- group at the 3'-position and a cyanoethyl function at the sulphur of the. Additionally, the uridine synthesized as part of this diploma thesis was used to replace some of the uridines.

For the coupling of the nucleotides the stronger catalyst dicyanoimidazole was used instead of the standard reagent tetrazole.

3.4 RNA cleavage and purification

After the last cycle of the oligonucleotide synthesis had been completed, the solid phase was transferred from the PolyGen Synthesizer slider into a vial.

Three mg CPG were transferred into a vial with 400 μ l ammonium hydroxide solution (28-30% NH_3) and heated for 16 hrs at 55°C. Next, the vial was allowed to cool to room temperature. Excessive NH_3 was removed by blowing air at the surface of the liquid. Then, the supernatant was transferred into a sterile tube with a pipette using a glass frit as a filter to prevent carryover of the resin. The residual solid phase was rinsed twice with 0.1 M AA in RNase-free water, which was added to the supernatant. The solvent was removed under vacuum using a SpeedVac (Christ). The residue was taken up in 50 μ l 0.1 M AA in RNase-free water and the concentration was determined by recording the absorption at 260 nm using a NanoDrop spectrophotometer.

Purification of the oligonucleotides was performed by precipitating them from iPr. Therefore, 1 μ l RNase-free water was added to the dried product. Then it was suspended in 100 μ l iPr. After sonicating the mixture for 15 minutes it was stored at -20°C for 20 minutes to complete precipitation. Then, it was centrifuged at 5°C and 14.000 rpm for 15 minutes. The iPr supernatant was decanted and the pellet was dried under vacuum using a SpeedVac.

The whole process was performed three times to obtain pure product. After the last purification step the pellet was dissolved in 50 μ l 0.1 M AA in RNase-free water and the concentration was determined using a NanoDrop spectrophotometer at 260 nm.

The purified oligonucleotides were stored at -20°C.

3.5 Deprotection and regeneration of the thiol linker

Before coupling the oligonucleotides with peptides the trityl-protection group of the sulphur had to be removed.

Therefore, AgNO₃ (100 µl of a 102 mM aqueous solution) was added to a solution of oligonucleotide (20 nm) in 1500 µl dd water. After shaking the solution gently for 90 minutes at RT, DTT (30 µl of a 0.5 M aqueous solution) was added. The mixture was again incubated for 90 minutes at RT while shaking it gently before centrifuging it for 15 minutes at 14 000 rpm. The supernatant was decanted and dried under vacuum. To increase the yield of the process, the pellet was then again suspended in 200 µl 0.1 mM AA in RNase-free water, sonicated for 20 minutes and centrifuged at 14 000 rpm for 15 minutes. The supernatant was once again decanted and dried under vacuum. Thereafter, the pellets obtained by drying the supernatants, were combined. Then it was redissolved in 50 µl dd water and the concentration was determined using a NanoDrop. Subsequently, the oligonucleotides were purified as described in RNA cleavage and purification.

The purified oligonucleotides were stored at -20°C.

3.6 Coupling of the oligonucleotides with peptides

The deprotected and purified oligonucleotides were used for coupling them with peptides.

The deprotected oligonucleotide was taken up in tris-HCl/EDTA/NaCl (50 mM/1 mM/100 mM) buffer. The peptide, which was used as a 2 mM aqueous solution, was added (4 equiv.) and the reaction was shaken gently at 25°C for 16 hrs.

The coupled oligonucleotides were dried on the SpeedVac and stored at -20°C.

3.7 Polyacrylamide gel electrophoresis

Preparation of a 20% acrylamide gel

7.2 g urea were dissolved in 1.5 ml 10xTBE (tris-borate-EDTA-buffer) by sonicating it for several minutes. Hereafter, 7.5 ml acrylamide gel stock solution (40%, acrylamide/bis-acrylamide, 29:1) were added.

Then the mixture was heated in the microwave for several seconds until the viscous liquid became clear. After the clear solution had been cooled down, 75 µl APS and 7.5 µl TEMED were added to induce polymerisation and the mixture was immediately poured into a gel-forming sandwich (Bio-Rad). Ten slots were formed with a comb. Then the gel was allowed to polymerize for 2 hours at RT. The gel was pre-run in a Mini-Protean II chamber for 45 minutes at 150 V in 1 x TBE. The slots were washed using a Hamilton syringe before filling them with the samples.

Preparation of the samples

The oligonucleotides were precipitated from iPr. The concentration as well as the level of purification was measured via NanoDrop.

For the analysis of the samples one nanomol of each oligonucleotide was diluted with 10 µl formamide loading buffer. Then, the samples were heated for 3 minutes at 95 °C before they were immediately put on ice to cool down. Then, the samples were loaded onto the gel. The gel was run at 150 V until the tracking dyes (bromophenol blue and xylene cyanol) had reached about one third and two thirds of the gel distance, respectively, which took around 90 min.

Methylene blue staining and detection

After the run the gel was shaken in a 2% solution of methylene blue for 15 minutes. Afterwards, the blue oligonucleotide bands were visible on the gel. Hence, the gel background was destained with water (3 x 5 min) leaving the oligonucleotide bands coloured.

The bands were digitalized on a Calibrated Imaging Densitometer GS-710 (Bio-Rad). The pictures were analysed with Quantity One 4.6.3, 1-D Analysis Software (Bio-Rad).

4. Results and Discussion

4.1 Synthesis of the uridine

In the diploma thesis at hand, the nucleoside chosen to be chemically modified was primarily uridine.

The aim of the modification was the introduction of a thiol-linker at the 2'-position. The linker should be introduced into the molecule by attaching it covalently to the 2'-OH of the uridine's ribose, which is not employed in forming the backbone of the ON and attractive tethering position, as it can be introduced at any site within the oligonucleotide and allows attachment of ligands in the minor groove of the nucleic acid helix. Uridine offers the shortest synthetic route of all coding nucleotides, because of the lack of the need of protecting an exocyclic amine function of the nucleobase. Therefore, the modified nucleotides, containing the linker, first had to be synthesized and should then be integrated into oligonucleotides.

The synthesis route started with the activation of the 2'-OH moiety of uridine, resulting in anhydrouridine. 2,2'-anhydro-1-(β -D-arabinofuranosyl)uracil was formed starting from uridine in a published procedure using sodium bicarbonate and diphenyl carbonate as a basic catalyst for the transesterification between diphenyl carbonate and carbohydrates in N,N-dimethyl formamide solution.²³ It has been published that using other ribonucleosides instead of uridine in this reaction yielded 2',3'-cyclic carbonates. It may be suggested that either uridine 2',3'-carbonate or 2'-phenoxycarbonyluridine is a likely intermediate in the reaction.

The formation of anhydrouridine leads to an improved region-selectivity. When adding the alkyl chain to uridine, it is attached to the 2'-OH as well as to the 3'-OH. When using anhydrouridine instead of uridine the 2'-position is the preferred alkylation site to the 3'-position, which leads to an increased yield of the desired product.

4.1.1 Synthesis of the alkyl chain

For introduction of the thiol tether, a suitable side chain which contained the thiol-linker was designed, whereby the thiol function had to be protected. The selection of the –SH protection group proved to be the critical step of the whole synthesis. On the one hand it has to be stable during the following reactions but on the other hand it should be readily removable at a later point. Otherwise it would not be possible to couple the ON with peptides.

Thus, pyridine was used as a protection group first. The preliminary synthesis of the nucleoside was carried out starting with the alkylation of the 2'-hydroxyl function. The synthesis was continued by protecting the 5'-OH using DMTrCl. However, when introducing the phosphoramidite the pyridine protection group turned out to be inappropriate since the reaction did not result in the desired product, but instead gave rise to a mixture of several compounds. Presumably, the sulphur was deprotected during the reaction. This results in the formation of undesired side products. TLC analysis supports this idea, since a DMTr cleavage product was identified in the solution, which indicates that the protection group has been removed during the reaction, leaving the sulphur deprotected and able to partake in side reactions.

Next, tert.-butyl mercaptane was chosen to replace the pyridine by a tert.-butyl moiety. Therefore, tert.-butyl mercaptane was added to the solution of the nucleotide synthesized as described in 4.1.1.1. This strategy was abandoned, due to the challenging handling of tert.-butyl mercaptane.

The third protection group used was a trityl-group, which has already been used by Jin, Miduturo and Silverman³³. The reaction path started once again from the synthesis of the side chain, which then was attached to the 2'-OH of anhydro-uridine.

However, in this diploma thesis a novel approach towards the final product was elaborated. Thereby, the synthesis was achieved in fewer steps than it had been described previously. Jin et al. used a different 2'-moiety and decided to protect the 3'- and 5'-hydroxyl group of uridine simultaneously using a tetrai-

sopropylidisiloxane-1,3-diyl protection. Later on, the silyl-bridge was removed. Then the 3'-hydroxyl group was protected using DMTrCl and the phosphoramidite was attached to the 3'-hydroxyl group³³.

In this diploma thesis the side chain was activated and protected using TBDMS-Cl. Then the trityl reagent was added in large excess and used as a nucleophile for the ring opening reaction of 2,2'-cyclouridine. The reaction was catalysed using $\text{BF}_3 \cdot \text{Et}_2\text{O}$ as a Lewis acid.

This step produced the lowest yield of all reactions in the synthetic pathway (19%). Even though TLC analysis indicated an adequate level of purity, it was not possible to induce reaction of the entire amount of the educt. For this step, the 1.5-fold amount of alkyl chain was used in relation to the nucleotide. Adding the side chain in larger excess may increase the yield of the reaction. Moreover, refining the purification step via column chromatography, especially in regard to the composition of the eluent, may result in a higher yield as well through the reduction of mixed fractions.

4.1.2 Synthesis of the final nucleotide

After the 2'-OH side chain had been introduced into the molecule, the 5'-OH was protected. Therefore, DMTr- was used, which represents a well-established protection group due to its favourable chemical properties such as its removability in less than one minute, using 3% trichloroacetic acid in DCM during solid phase ON-synthesis.

The reaction was performed under argon. Nevertheless, it was not possible to induce reaction of the entire amount of the educt.

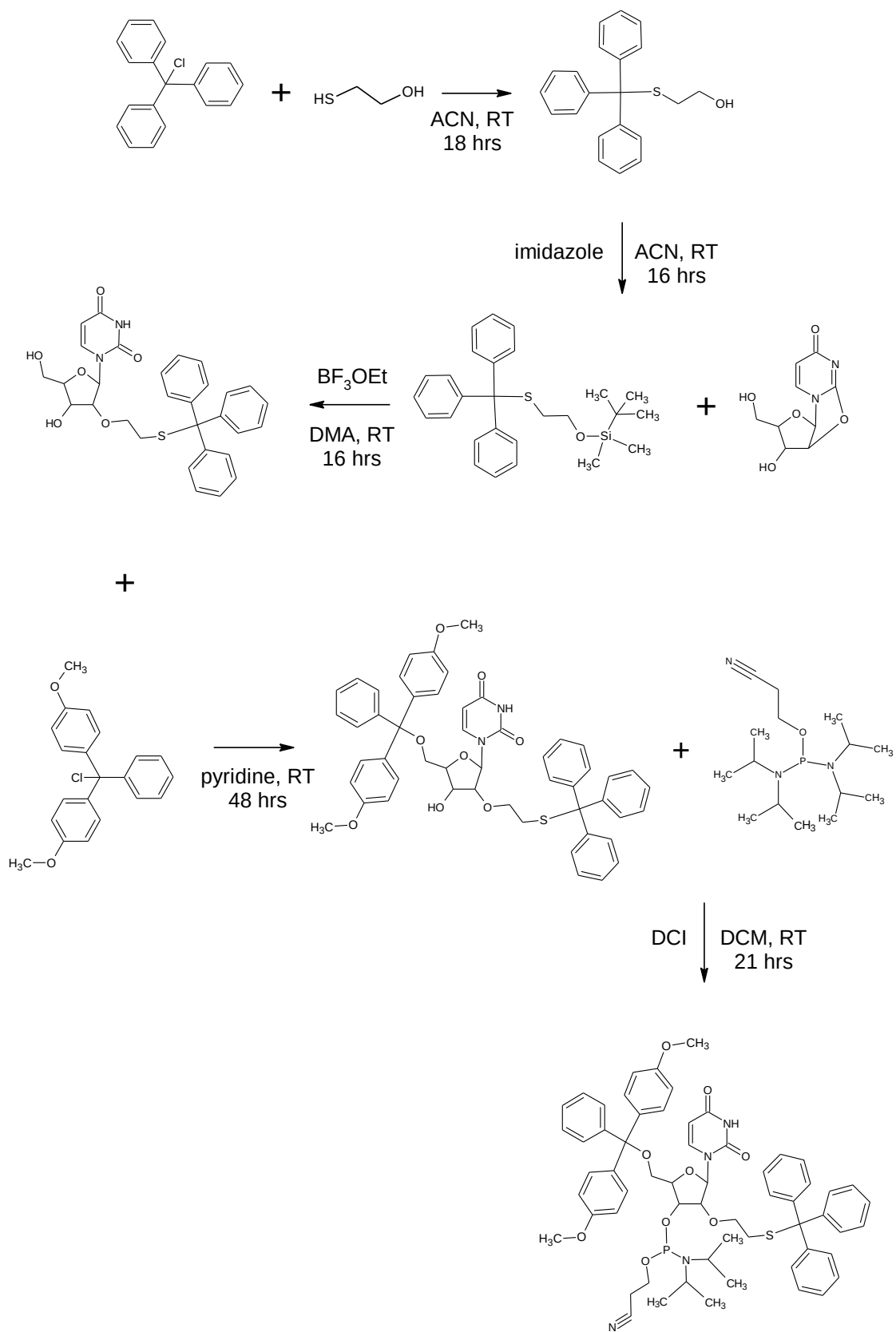
As the next step, the phosphoramidite was covalently attached to the 3'-OH. To this end, 2-cyanoethyl-N,N,N',N'-tetraisopropylphosphordiamidite was used and DCI served as an activator. Besides DCI, tetrazole was used as an activator as well. Although both activators produced comparable results concerning yield and purity DCI was first choice during the further procedure because of it being more nucleophilic and less acidic than tetrazole. Thereby, adding a large excess of phosphoramidite proved to result in higher yields without significant side product formation.

After each synthesis step the product had to be purified by column chromatography.

The synthesis of the final nucleoside provided an overall yield of 8%, at which the alkylation of the 2'-OH displayed the step with the lowest yield (19%), probably caused by a rather high lability of the silyl activated reagent due to its strict necessity of being performed free of water.

Except for the alkylation reaction, all steps provided a satisfactory yield.

Synthesis route:



4.2 Synthesis of the adenosine

In order to increase the available building blocks for thiol linker incorporation, the synthesis of a respective adenosine building block was designed. Thereby, a thiol-linker should be introduced into the molecule, as described for uridine previously.

The synthesis of this nucleotide started from 3',5'-O-(tetraisopropylidisiloxane-1,3-diyl)-adenosine³⁵. First, the amino-group of the adenine had to be protected. For this, an acetyl group was chosen. Further-more, a methylthiomethyl chain was attached to the 2'-OH. Both modifications were performed in one reaction using acetic acid, acetic anhydride and DMSO. To achieve sufficient yield, the reaction was heated to 40°C.

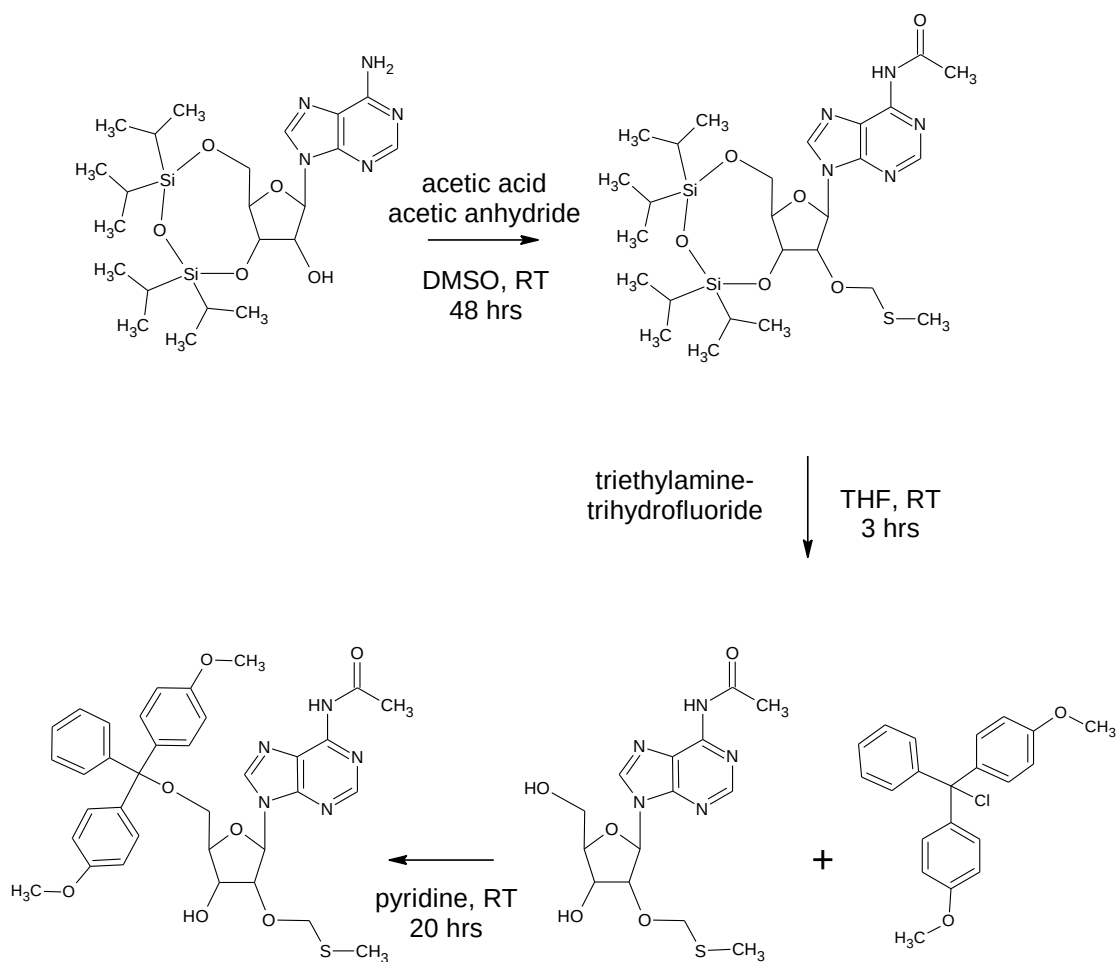
The reaction was performed at different temperatures and the reaction time was varied as well. Thereby, heating the reaction up to 40°C and allowing it to react for 48 hrs turned out to result in the highest yield.

During the whole synthesis process and after the reaction had been completed it was monitored using TLC. The resulting liquid turned out to be composed of a mixture of several compounds. Because of this, purification via column chromatography was essential before continuing with the next step of the synthesis. As a result of this, the yield of the product was diminished (34%) due to mixed fractions after the column chromatography. However, this alternative route offers the possibility of achieving two steps in one reaction. The yield of the process may be enhanced by optimizing the composition of the eluent used at the column chromatography. This way, separating single compounds could be accomplished more effectively.

After that, the silyl-bridge was cleaved with fluoride and the 5'-OH was protected using DMTrCl using the same reaction conditions as described for the uridine compound. The amount of DMTrCl added was optimized once again, starting with an educt/DMTrCl-ratio of 1/1 which was gradually increased during the reaction to 1/1.4. On the one hand, adding DMTrCl in excess proved to result in higher yield. On the other hand, adding larger amounts of DMTrCl increases the risk of reaction with secondary hydroxyl functions. The 3'-hydroxyl group was suspected to partake in the reaction as well, if DMTrCl was added extensively. Therefore, using 1.4 equivalents of DMTrCl was chosen to offer best results.

The final nucleotide, which contains a phosphoramidite at the 3'-OH, has not been synthesized in the course of the diploma thesis due to time restrictions.

Synthesis route:



4.3 RNA synthesis

The synthesis of the oligonucleotides was performed on a PolyGen DNA Synthesizer. The yield of the coupling was controlled after each consecutive step via trityl-analysis. Only 2'-O-methyl nucleotides were attached, and thus for all couplings, increased reaction times of 15 min were used. Thereby, sufficient yields were measured for all four synthesized sequences. Even the introduction of the modified nucleotide did not result in lower yields compared to unmodified nucleotides.

In this case, phosphorothioates were synthesized, at which, in difference to wild-type RNA, one oxygen of the phosphodiester linkage is replaced by a sulphur atom. Thus, they show an extensive increase in nuclease resistance and plasma protein binding as well as reduced urinary excretion. These effects are most likely to be caused by the conformational changes due to the larger size of the sulphur compared to the oxygen. Additionally, the replacement of the oxygen creates chirality at the phosphorus centre. Hence, the resulting product is a mixture of 2^{n-1} stereoisomers.

The cyclic prolongation of the oligonucleotides consists of four recurring steps:

1. Detritylation

At the beginning of each cycle the 5' hydroxyl group gets activated. Therefore, the DMTr protection group has to be removed, which is achieved in less than one minute using 3% trichloroacetic acid in DCM. For analysing the efficacy of this step the removed DMTr- cation can be quantified thanks to its orange colour. This allows predicting whether the prior cycle was achieving a sufficient yield or not.

2. Coupling

Following detritylation, the next nucleoside is added in the form of a phosphoramidite monomer. An excess of the appropriate nucleoside is mixed in situ with an activator (DCI), both of which are dissolved in acetonitrile. The diisopropylamino group of the nucleoside is rapidly displaced by nucleophilic attack creating

a phosphite triester. When 2'-OH modified nucleotides are used the coupling time needs to be prolonged because of the steric hindrance.

3. Sulfurization

TETD was used as a sulfurization reagent. Up to 5% of the phosphoramidite bond can be oxidised by the capping reagent. Therefore, this step has to be performed prior to the capping.

4. Capping

Expecting a yield of up to 99% still means that there will be a few unreacted 5'-hydroxyl groups on the resin-bound nucleotide. If left unchecked, these 5'-hydroxyl groups would be available to partake in the next coupling step, reacting with the incoming phosphoramidite. The resulting oligonucleotide would lack one base, accumulating with each successive cycle, and finally produce a complex mixture of oligonucleotides slightly shorter than the intended one which would be difficult to purify.

Thus, a capping step after the coupling reaction is introduced to block the unreacted 5'-hydroxyl groups. Therefore, two capping solutions are used: acetic anhydride and 2,6-lutidine in THF and N-methylimidazole in THF. The mixture of these reagents quickly acetylates the 5'-OH groups and renders them inert to subsequent reactions. The 2,6-lutidine ensures that the pH remains basic to prevent detritylation of the nucleoside phosphoramidite.

The synthesis lead to the desired ONs.

oligonucleotide	sequence
1	CCU-CUU-ACC-UCA-GUU*-AC(A)
2	CCU*-CUU-ACC-UCA-GUU*-AC(A)
3	CCU-CUU-ACC-U*CA-GUU*-AC(A)
4	CCU*-CU-ACC-U*CA-GUU*-AC(A)

Table 4.01: Synthesized ONs: A= adenosine, C= cytidine, G= guanosine, U= uridine, U*= modified uridine containing the thiol linker. ONs contain one to three modified uridines in different positions. ON 1 is modified once, ON 2 and ON 3 are modified twice with variation of the location. ON 4 is modified three times.

After the oligonucleotides had been synthesized successfully, they were used for further purification and analysis.

Purification via Sephadex and precipitation from iPr as well as from EtOH 96% were performed. However, precipitation from iPr showed best results concerning yield and level of purity. Hence, this method was chosen for most of the purification steps.

A disadvantage of iPr is the possibility of it being peroxidised during storage. That is why using it includes the risk of oxidation of the ON during purification steps. To lower this risk, these steps were performed at low temperature. iPr was cooled to -20°C and centrifugation was performed at low temperature as well.

Although phosphorothioates show beneficial PK/PD parameters compared to wild-type RNA, purification and analysis turned out to be more challenging. HRMS was performed using these compounds, however due to their unfavourable properties, including a high propensity for salt binding no data is available. The standard methods for desalting, such as repeated precipitation from ammonium acetate buffers proved to be insufficient, and improved methodology needs to be developed.

4.4 RNA cleavage and purification

The RNA sequences synthesized on the CPG had to be removed from the solid phase to allow consecutive work steps, such as deprotecting the thiol-group and coupling it with peptides.

To this end, NH_4OH was added to the CPG, which was then incubated at 55°C for 16 hrs. Although the cleavage of the RNA from the solid phase can be achieved by incubating the RNA for a much shorter period of time, the long incubation time is required to remove the protection groups of the nucleotides used for the ON-synthesis. If this process were not performed entirely the resulting ONs would still contain these protection groups, which may affect further analyses, such as HRMS, HPLC-analysis or biological testing.

NH_4OH was added in large excess. The yield of the process was measured using a NanoDrop. Thereby, the absorption of a solution is measured. Since ONs absorb light at a specific maximum of 260 nm this method can be used to determine the concentration of ONs in a solution.

The cleavage of the ON and its protection groups resulted in a sufficient amount of ONs for the following steps. Purification by precipitating the ONs from iPr was performed before continuing with the following steps.

Purification via Sephadex size exclusion chromatography and precipitation from iPr as well as from EtOH 96% were performed. However, precipitation from iPr showed best results, concerning yield and level of purity. Therefore, this method was chosen for most of the purification steps.

4.5 Deprotection and regeneration of the thiol linker

Next, the trityl-protection group of the uridine was removed argentometrically. Assuming that Ag^+ would bind to the thiol function as well as to the backbone of the ONs, it was added in a large excess (30-fold amount of nucleotides plus -SH groups). Thereby, the trityl-group was replaced by Ag^+ ions. To obtain a thiol group the sulphur had to be regenerated, which was achieved by using DTT. Thus, Ag^+ was replaced by hydrogen, resulting in the desired thiol- group. After that the solution had been processed, as described in 3.5, the concentration of the solution was determined using the NanoDrop. In addition to that, analysis via NanoDrop allows estimating the level of purification, achieved during the antecedent purification steps. Normally, the absorption maximum of a solution containing ONs is 260nm. Therefore, the highest peak of the absorption curve should be located at this position of the scale. Peaks at other parts of the spectrum indicate the presence of undesired components, in this case often residues of DTT used previously. If a curve showed impurities, further purification steps were performed.

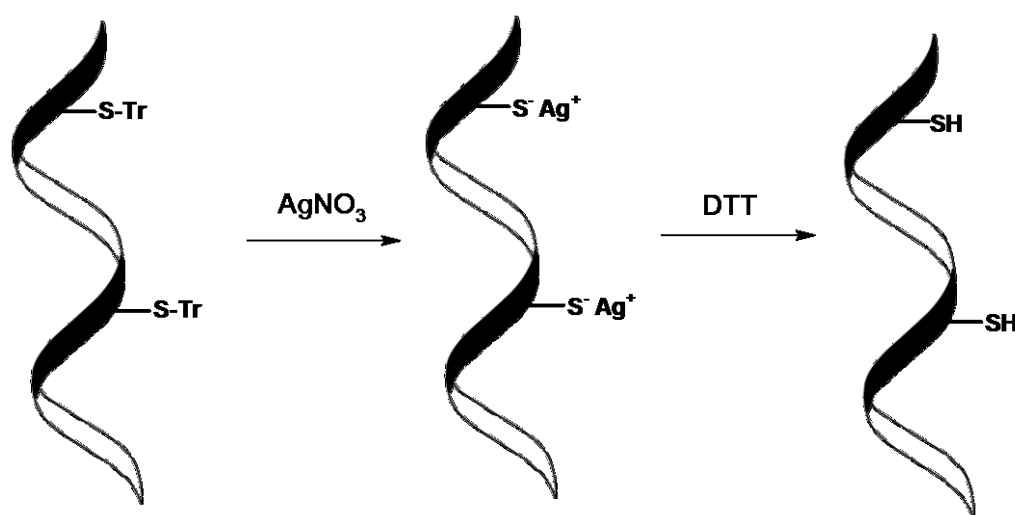


Figure 4.01: Schematic illustration of the argentometric deprotection of the thiol linker and its consecutive regeneration via DTT.

4.6 Coupling of the oligonucleotides with peptides

The deprotected ONs were coupled with peptides. Using peptides to enhance cellular uptake by basic, so-called cell penetrating peptides (CPPs), has been well established for various substances. When coupled with ONs, proteins rich in positively charged amino acids like arginine or lysine are used. Thus, the ON-peptide-conjugate's ability of passing the membrane of the target cell is increased. Besides entering the cell via endocytosis, releasing it in the cytoplasm from the endosomes without being degraded in lysosomes is pivotal for high concentrations inside the cell, needed to achieve the desired effects^{31,32}.

In this diploma thesis, lysine-rich peptides were used. They were formed out of 5-6 lysine molecules and one cysteine³⁶, which carried a thiosulfonate rest at the sulphur. Thereby, lysine contributed the positive charges, while the sulphur of cysteine was responsible for the coupling via disulphide exchange.

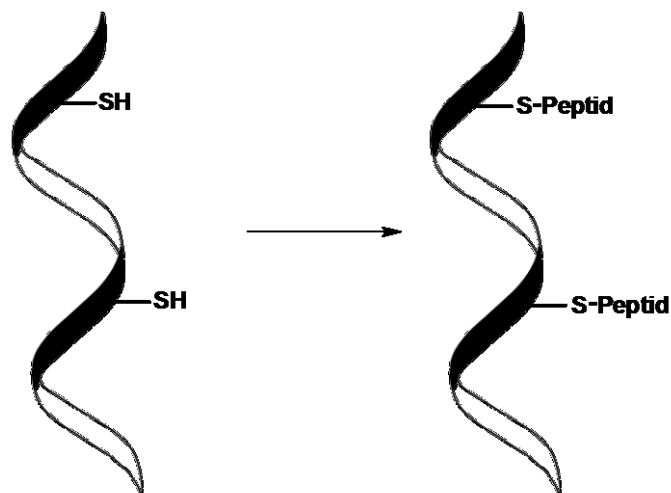


Figure 4.02: Schematic illustration of the coupling of the ON with a peptide. The thiol function of the cysteine is used to form a disulphide bond with the thiol linker of the modified ON.

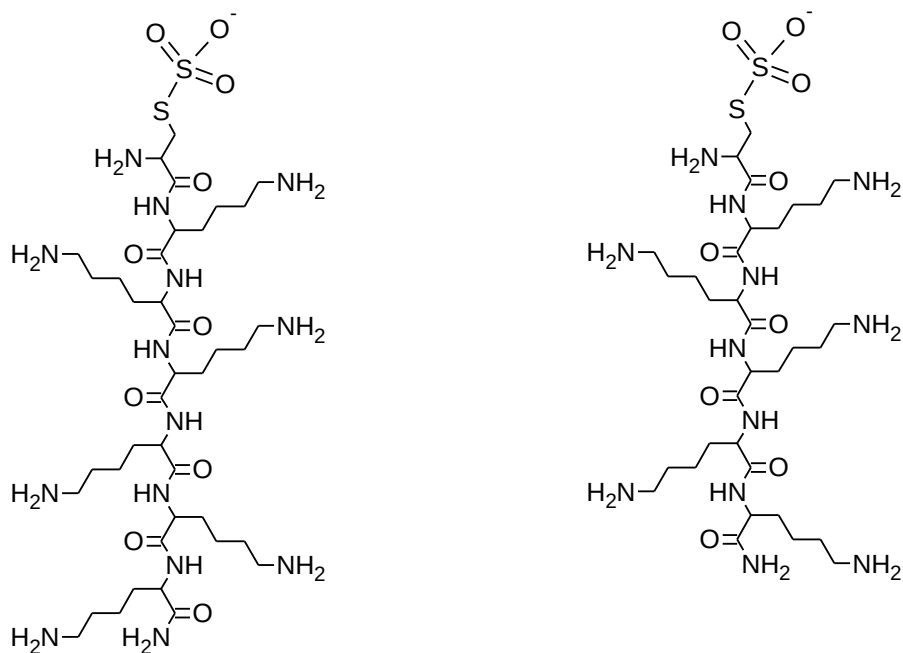


Figure 4.03: Structure of the peptides coupled with the synthesized ONs.

On the left: C6K: it consists of 6 lysines and one cysteine. At the sulphur of the cysteine a thio-sulfonate group had been attached. This allowed coupling the peptide with the thiol linker of the ONs via disulphide exchange.

On the right: C5K, made of 5 lysines and one cysteine carrying a thiosulfonate group.

4.7 Polyacrylamide gel electrophoresis

After the coupling process had been completed, the product was again purified by precipitation from iPr before analyses were performed.

For the characterisation of the oligonucleotides polyacrylamide gel electrophoresis was used. Therefore, the gel was loaded with 1 nmol of the ON. After the developing the gel and staining with methylene blue as described in 3.7, the migration of the samples was examined.

Gel electrophoresis separates substances according to their mass/charge-ratio. For unmodified oligonucleotides, the migration is dependent on the intrinsic negative charge of nucleic acids and thus the sequence length, and longer sequences migrate slower than shorter ones. In the case of the oligonucleotide conjugates with basic peptides, migration speed is influenced by the reduction of the net negative charge. As expected, the successful attachment is reflected by a marked reduction of migration and localization of the respective bands close to the top of the gel. The intensity of the bands allows estimating the amount of substance the respective band consists of.

A slight distortion of the gel was observed, that is possibly caused by varying salt content of the samples or by a certain degree of aggregation of the conjugates with basic peptides. Compared to lane 1 and 2, the bands of lane 3, 4 and 5 are significantly closer to the start, which implies that these molecules show a larger mass/charge-ratio compared to the uncoupled oligonucleotides of slot 1 and 2. The molecular weight of the uncoupled oligonucleotides averages around 6.5 kDa, whereas the formula weight of the oligonucleotides coupled with peptides averages around 7-8 kDa. Therefore, the spots of the oligonucleotides coupled with peptides can be expected closer to the start than those of the uncoupled oligonucleotides. However, this effect would only result in a slight reduction of the migration distance. Additionally, the net charge of the negatively charged molecules gets reduced by the binding of positively charged peptides. Therefore, the mass/charge ratio is getting diminished, which shortens the migration distance to a greater extent.

It is not possible to unequivocally deduce the exact ratio of peptide/oligonucleotide from the present analytical data. When examined closely, the band of lane 4 can be identified to be closer to the start than lanes 3 and 5.

This band represents the oligonucleotide **2**. Hence it can be expected to be successfully coupled with two peptides, while the others have only one binding site. This increases the molecular weight and reduces the net charge of the molecule and, therefore, decelerates its movement through the gel. The phosphorothioate backbone of the oligonucleotides may represent possible additional binding sites for peptides. This effect would result in oligonucleotides with larger molecular weights than expected. A higher number of peptides are potentially favourable concerning the biological efficacy.

However, the gel strongly indicates the successful coupling of the synthesized ONs with peptides.

In addition, further analyses of the coupled ONs including HPLC analysis and high resolution mass spectroscopy were performed. Due to the challenging performance of these methods the investigations were not completed during the course of this diploma thesis. Further investigations will be needed to elucidate the exact structure of the synthesized ONs and subsequently examine the pharmacological properties.

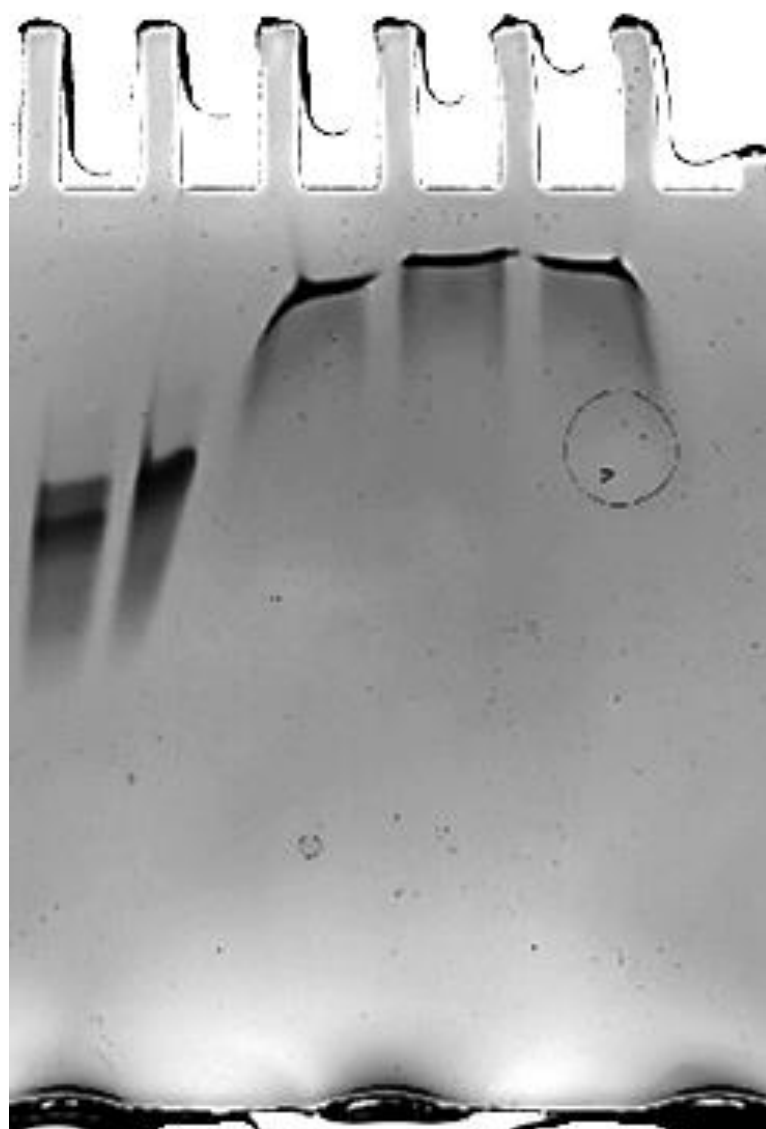


Figure 4.04: Urea-polyacrylamide-gel: slot 1: oligonucleotide **2**, slot 2: oligonucleotide **1**, slot 3: oligonucleotide **1** coupled with C6K (1x), slot 4: oligonucleotide **2** coupled with C5K (2x), slot 5: oligonucleotide **1** coupled with C5K (1x).

5. Conclusion

Coupling ONs with peptides is believed to improve cellular uptake, which still represents one of the major hurdles for ASONs. Labile thiol tethers are particularly attractive because of their ability to being cleaved in the endosomal environment. Before being able to do so, modified nucleotides, containing a thiol-linker with the capability of forming a disulphide bond with the –SH of cysteine, had to be developed. Successful synthetic schemes for 2'-thioethyl modified adenosine and uridine nucleotides were established, and in the case of uridine, a respective building block for standard automated oligonucleotide synthesis was prepared.

Employing this modified nucleotide into oligonucleotides via solid phase synthesis on a DNA-Synthesizer was successful and gave high yields.

To enable further work steps, such as coupling the thiol-linker with a peptide, protecting the –SH with a protection group was indispensable. Thereby, the selection of a suitable protection group turned out to be challenging. First, the protection group has to be stable while synthesizing the nucleotide and, later, during the synthesis of the oligonucleotide. Furthermore, it has to be efficiently removable before coupling the oligonucleotide with a peptide.

Different protection groups were evaluated, including pyridine and tert.-butyl. Finally, the synthesis was performed successfully using a trityl protection group. Due to their favourable biological effects, phosphorothioates were synthesized for generating potentially active oligonucleotide conjugates. Although synthesizing the compounds was unproblematic, purification and analysis, especially HRMS and HPLC analysis turned out to be more challenging than initially expected. After all, optimization of these steps is still required.

Coupling the oligonucleotide with peptides was performed efficiently. Analysis of the product via gel electrophoresis indicated a significant reduction in migration, caused by increase of the molecular mass and decrease of the net charge.

The biological effectiveness of the synthesized compounds remains to be explored. However, coupling ONs with peptides displays an attractive method of improving cellular uptake of these substances.

The developed modified nucleotides and the corresponding methodology for the preparation of oligonucleotide peptide conjugates is fully compatible with a large

number of chemical modifications of both oligonucleotide and peptides and of course available for any sequences of the two components. Thus, the building blocks and methods are attractive for further development toward optimized therapeutic oligonucleotides.

6. List of abbreviations

2'-MOE	2'-O-methoxyethyl modified oligonucleotides
AA	ammonium acetate
ACN	acetonitrile
ALT	alanine transaminase
APS	ammoniumpersulfate
ASON	antisense oligonucleotide
AST	aspartate transaminase
CPG	controlled pore glass
DCI	dicyanoimidazole
DCM	dichloromethane
dd	double distilled
DMD	Duchenne muscular dystrophy
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DMTrCl	dimethoxytritylchloride
DNA	deoxyribonucleic acid
DTT	dithiotreitol
EtOH	ethanol
FDA	food and drug administration
hrs	hours
iPr	iso-propanol
LNA	locked nucleic acid
MeOH	methanol
mRNA	messenger-RNA
MTBE	methyl tert.-butyl ether
ON	oligonucleotide
ORF	open reading frame
PD	pharmacodynamic
PE	petroleum ether
PK	pharmacokinetic
PMO	phosphorodiamidate morpholino oligonucleotide

PPB	plasma protein binding
PS	phosphorothioate
RNA	ribonucleic acid
RT	room temperature
TBDMSCl	tert.-butyldimethylsilyl chloride
TEA	triethylamine
TEMED	N,N,N',N'-tetramethylethylenediamine
TETD	tetraethylthiuram disulfide
THF	tetrahydrofuran
TLC	thin-layer chromatography

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Ich habe mich bemüht, sämtliche Inhaber der Bildrechte ausfindig zu machen und ihre Zustimmung zur Verwendung der Bilder in dieser Arbeit eingeholt. Sollte dennoch eine Urheberrechtsverletzung bekannt werden, ersuche ich um Meldung bei mir.

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