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DEVELOPMENT AND SYNTHESIS OF OLIGONUCLEOTIDE BUILDING BLOCKS AND CONJUGATES FOR THERAPEUTIC APPLICATIONS

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Development and Synthesis of Oligonucleotide Building Blocks and Conjugates for Therapeutic Applications

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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To my Family

ABSTRACT

The research and development of oligonucleotide therapeutics has been a topic of great importance in life sciences. The potential to treat rare genetic diseases can be demonstrated simply by highlighting the increasing amount of approved oligonucleotide-based drugs. However, the delivery and stability issues of oligonucleotides complicate the transition to clinic and hampers the development in the field. Numerous oligonucleotide modifications have been developed to improve their properties and to overcome these limitations. This thesis focuses on the development and preparation of selected compounds, which are designed to improve the oligonucleotide properties related to stability and delivery.

The first chapter (papers I and II) presents the development of a versatile synthetic platform for oligonucleotide-conjugate synthesis. The linkers, specifically developed for oligonucleotide conjugation, are compatible with automated oligonucleotide synthesis conditions and enable the incorporation of e.g., biologically active moieties at any position of the oligonucleotide sequence. Therefore, this approach enables the preparation of constructs with tailored properties. The development and synthesis of orthogonal linkers is described in this chapter together with the preparation of several different oligonucleotide conjugates, bearing different biologically active moieties/modalities. Furthermore, stability studies are performed on one of the linkers to evaluate its potential to be used during automated oligonucleotide synthesis in the future.

The second chapter (paper III) describes the upscaling and optimization for the synthesis of methyl-uridine and methyl-cytidine nucleosides bearing 2′-*O*-(N-(aminoethyl)carbamoyl)-methyl modification. Since this modification showed promising results in nuclease stability and cellular uptake, larger amounts of modified nucleosides are necessary for future evaluations. Therefore, the syntheses of 2′-modified MeC and MeU building blocks are developed and demonstrated at larger (up to 100 g) scales. The final nucleosides are prepared as phosphoramidites to allow for the direct incorporation into an oligonucleotide sequence during the automated oligonucleotide synthesis. Suitable work-up and alternative purification strategies to reduce the number of chromatographic steps are also explored in this chapter.

LIST OF SCIENTIFIC PAPERS

I. Dmytro Honcharenko[§], **Kristina Druceikaitė[§],** Malgorzata Honcharenko, Martin Bollmark, Ulf Tedebark and Roger Strömberg;
New Alkyne and Amine Linkers for Versatile Multiple Conjugation of Oligonucleotides.
ACS Omega 2021, 6, 579-593

II. **Kristina Karalė**, Martin Bollmark, Oswaldo Pérez, Ulf Tedebark and Roger Strömberg;
Synthesis and Stability of Bicyclo[6.1.0]nonyne Scaffold for Solid-Phase Oligonucleotide Synthesis.
Manuscript

III. **Kristina Karalė**, Martin Bollmark, Rouven Stulz, Dmytro Honcharenko, Ulf Tedebark and Roger Strömberg;
A Study on Synthesis and Upscaling of 2′-*O*-AECM-5-methyl Pyrimidine Phosphoramidites for Oligonucleotide Synthesis
Molecules 2021, 26, 6927

Papers not included in this thesis:
Olivia Luige, **Kristina Karalė**, Partha Bose, Martin Bollmark, Ulf Tedebark, Merita Murtola and Roger Strömberg;
Influence of sequence variation on the RNA cleavage activity of Zn^{2+}-dimethyl-dppz-PNA-based artificial enzymes.
Submitted manuscript

[§] Equal contribution.

CONTENTS

LIST OF ABBREVIATIONS

A	Adenine
ADIBO	Azadibenzocyclooctyne
AHP	Acute Hepatic Porphyria
ASCVD	Atherosclerotic Cardiovascular Disease
ATTR	Hereditary Transthyretin Amyloidosis
C	Cytidine
CMV	Cytomegalovirus
CpG	5′-C-phosphate-G-3′
DBCO	Azadibenzocyclooctyne
DCM	Dichloromethane
DIBAC	Azadibenzocyclooctyne
DIPEA	*N*,*N*-diisopropylethylamine
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSC	*N*,*N*′-disuccinimidyl carbonate
EDA	Ethylenediamine
EMA	The European Medicines Agency
equiv.	equivalents
EtOH	Ethanol
FCS	Familial Chylomicronemia Syndrome
FDA	Food and Drug Administration
G	Guanine
GC	Gas chromatography
HBTU	Hexafluorophosphate benzotriazole tetramethyl uronium
HeFH	Heterozygous Familial Hypercholesterolemia
HoFH	Homozygous Familial Hypercholesterolemia
iPrAc	Isopropyl acetate
LNP	Lipid nanoparticle
MeCN	Acetonitrile
MEK	Methyl ethyl ketone
MeOH	Methanol
MIF-1	Melanocyte-inhibiting factor

NMR	Nuclear magnetic resonance
PH1	Primary Hyperoxaluria
RNA	Ribonucleic acid
RNase H	Ribonuclease H
RP-HPLC	Reversed-phase high-performance liquid chromatography
SMA	Spinal muscular atrophy
ssDNA	Single-stranded DNA
T	Thymidine
TBABr	Tetrabutylammonium bromide
TEA	Triethylamine
THF	Tetrahydrofuran
TLC	Thin-layer chromatography
U	Uridine
VOD	Hepatic Veno-occlusive Disease

1 INTRODUCTION

1.1 OLIGONUCLEOTIDE THERAPEUTICS

Oligonucleotide (ON) therapeutics have been in active development for several decades and the growth rate of the research field is rapidly increasing to this day. Synthetic therapeutic ONs are comparatively short, single or double-strand sequences of modified, functional nucleic acids. This delineation covers antisense oligonucleotides (ASOs), microRNAs (miRNAs), small interfering RNAs (siRNAs), aptamers and unmethylated CpG-containing sequences.[1-4] ON-based treatments, depending on the mode of action, may result in decreased, increased, or restored protein expressions.[1-4] These effects come from one of the largest native advantages of ONs - the fact that they can be designed to hybridize to complementary targets via Watson-Crick base pairing. The hybridization can cause either steric blocking, altered splicing patterns or gene silencing. On the other hand, aptamers are somewhat of an exception, since they do not act via Watson-Crick base pairing,[1] but are recognized by target proteins by their three-dimensional structure.[3, 4] Despite the fact that there are currently 13 approved oligonucleotide-based treatments in the market (Table 1), as well as many others in the pharmaceutical development pipeline, the inherently unfavorable absorption, distribution, metabolism, excretion and toxicity properties are continuously challenging the development of nucleic acid based therapeutics.[1, 3] Also, nine out of the thirteen ON therapeutics have been approved later than 2018 showing great recent strides in this research area.

Table 1. FDA and/or EMA approved oligonucleotide therapeutics.[1, 3]

Drug Name	Tissue/organ, administration	Chemistry (Modality)	Disease	Approval date, agency	Developer
Fomivirsen* (Vitravene)	Eye, Intravitreal injection	21-mer PS DNA (first-generation ASO)	CMV retinitis	1998 FDA 1999 EMA	Ionis Pharmaceuticals Novartis Opthalmics
Mipomersen (Kynamro)	Liver, subcutaneous injection	20-mer PS 2′-MOE (gapmer ASO)	HoFH	2013, FDA	Ionis Pharmaceuticals Genzyme
Eteplirsen (Exondys 51)	Skeletal muscle, IV infusion	30-mer PMO (steric block ASO)	DMD	2016, FDA	Sarepta Therapeutics
Defibrotide (Defitelio)	Liver, IV infusion	Mixture of PO ssDNA and dsDNA	VOD	2016, FDA 2016, EMA	Jazz Pharmaceuticals
Nusinersen (Spinraza)	Spinal cord, intrathecal injection	18-mer PS 2′-MOE (steric block ASO)	SMA	2016, FDA 2017, EMA	Ionis Pharmaceuticals
Patisiran (Onpattro)	Liver, IV infusion	19 + 2-mer 2′-OMe modified (siRNA LNP formulation)	ATTR	2018, FDA 2018, EMA	Alnylam
Volanesorsen (Waylivra)	Liver, subcutaneous injection	20-mer PS 2′-MOE (gapmer ASO)	FCS	2019, EMA	Akcea Therapeutics
Inotersen (Tegsedi)	Liver, subcutaneous injection	20-mer PS 2′-MOE (gapmer ASO)	ATTR	2018, FDA 2018, EMA	Ionis Pharmaceuticals Akcea Therapeutics PTC Therapeutics
Givosiran (Givlaari)	Liver, subcutaneous injection	21/23-mer Dicer substrate siRNA (GalNAc conjugate)	AHP	2019, FDA 2020, EMA	Alnylam
Golodirsen (Vyvondys 53)	Skeletal muscle, IV infusion	25-mer PMO (steric block ASO)	DMD	2019, FDA	Sarepta Therapeutics
Viltolarsen (Viltepso)	Skeletal muscle, IV infusion	21-mer PMO (steric block ASO)	DMD	2020, FDA	NS Pharma
Oxlumo (Lumasiran)	Liver, subcutaneous injection	21/21-mer partial PS 2′-F, 2′-OMe modified siRNA (GalNAc conjugate)	PH1	2020, EMA	Alnylam
Casimersen (Amondys 45)	Skeletal muscle, IV infusion	22-mer PMO (steric block ASO)	DMD	2021, FDA	Sarepta Therapeutics
Inclisiran (Leqvio)	Liver subcutaneous injection	21/23-mer partial PS 2′-F, 2′-OMe modified siRNA(GalNAc conjugate)	ASCVD HeFH	2020, EMA 2021, FDA	Novartis Europharm Limited

**Withdrawn from the market due to reduced clinical need*[1, 3]

Although there have been successful ON delivery examples in targeting specific organs, a systemic ON administration has mainly been hampered by poor cell and tissue internalization.[1-5]

Relatively small, hydrophobic, and/or uncharged ONs, such as, for example, PS (phosphorothioate) ASOs, can enter the cells and escape endosomes without a delivery/transfection agent. In such case a relatively high dose of the PS-modified ON is needed, while in most other cases the therapeutic ONs are simply too large to enter the cells without additional assistance.[3]

To overcome the above-mentioned hurdles, a wide range of synthetic ON improvements, such as monomer and backbone modifications, conjugation with various entities, as well as carrier-assisted delivery systems are being investigated. The focus of this thesis is the development and preparation of ON conjugates as well as larger scale synthesis of 2′ -modified nucleoside monomers.

1.2 SOLID-PHASE OLIGONUCLEOTIDE SYNTHESIS

The first attempts to form internucleosidic linkages emerged in the early 1950s.[6, 7] In 1955 this goal was achieved by Todd and Michelson using a method, which later was called the phosphotriester approach.[8] Since that time, several other significant approaches (e.g. H-phosphonate,[9, 10] phosphodiester,[11, 12] phosphoramidite[13]) were developed. However, today the most widely accepted method for the synthesis of ONs is the phosphoramidite approach which was introduced by Beaucage and Caruthers[13] in the early 1980s.[7] There are several reasons for the phosphoramidite approach to dominate the field. First, it was adopted and enhanced by Matteucci and Caruthers to automated solid-phase synthesis.[14] Second, the coupling yields, using phosphoramidite monomers in the presence of tetrazole can reach above 99%. In addition, the coupling reaction is very rapid, many side reactions can be avoided and 50-100-mers, or even longer ONs can be successfully prepared. And third, solid-phase synthesis facilitates the purification as most of the reagents are washed out during each synthesis cycle.[7, 15] Furthermore, since phosphoramidite nucleosides became commercially available and their cost progressively decreased, the search and development of alternative synthetic methodologies became less important.[7]

Solid-phase ON synthesis generally is performed on controlled-pore glass beads[16] or on crosslinked polystyrene.[17] Selected monomers are repeatedly added one at a time, extending the nucleoside or oligonucleotide bound to the solid support. Contrary to natural DNA bio-synthesis, the solid-phase ON synthesis is performed from 3′ to 5′ direction, i.e. as opposed to the natural from 5′ to 3′ direction.[7] The fully assembled sequence is released from the solid support using basic conditions (e.g. aq. ammonia solution) and then purified. The automated phosphoramidite-based ON synthesis cycle consists of three-four steps:[15] detritylation (step 1), activation and coupling (step 2), oxidation (step 3) capping (step 4), (Figure 1).

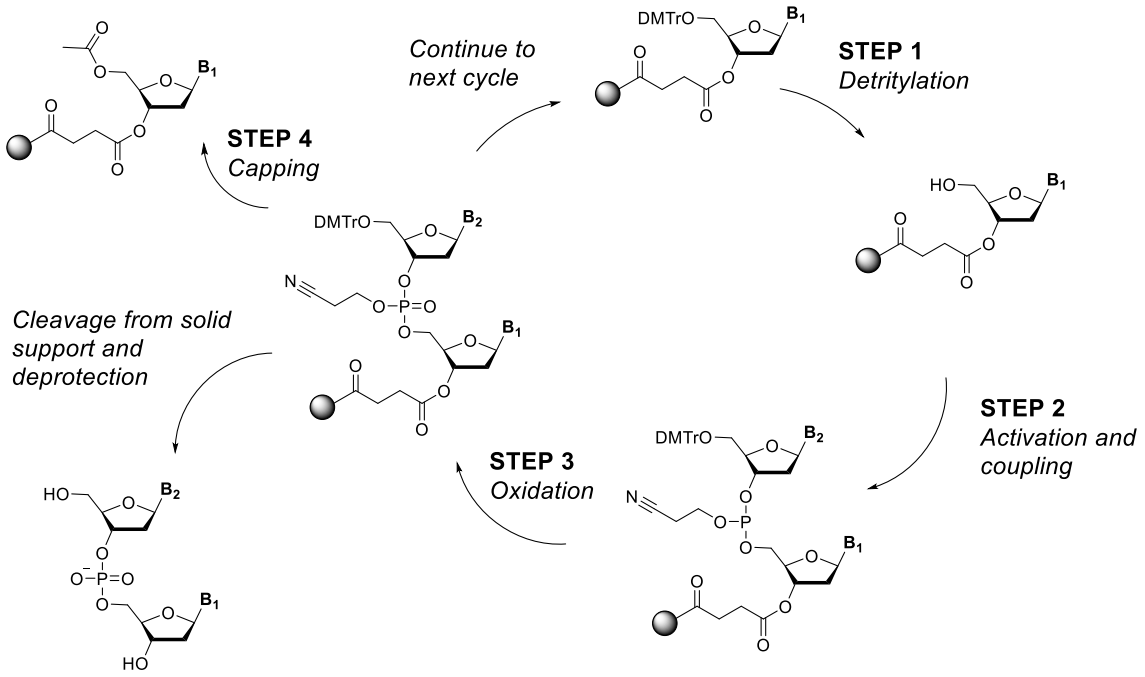


Figure 1. Oligonucleotide synthesis cycle using phosphoramidite approach.

The cycle begins with the removal of DMTr protecting group using acidic conditions, e.g. 3% trichloroacetic acid in dichloromethane or 3% dichloroacetic acid in toluene (step 1). The next monomer is then coupled to the solid-bound nucleoside (or a universal linker) to give a phosphite (III) triester (step 2). Tetrazole or a tetrazole based derivative is usually used as an activator for the coupling reaction. Then the oxidation follows (step 3): The unstable phosphite (III) triester has to be oxidized to a stable phosphate (V).[15] If the final goal is to obtain phosphate linkages, iodine in pyridine is used for this step. For phosphorothioate linkages, a reagent which can provide a sulfur atom is necessary, such as, for example, xanthan hydride.[18] The final step (step 4) is capping: unreacted 5-hydroxyl groups are blocked with acetic anhydride. Capping is performed to prevent the formation of incorrect sequences (deletion mutation).

1.3 OLIGONUCLEOTIDE MODIFICATION SITES

As mentioned above, chemical modification of ONs can improve stability, cellular uptake and delivery limitations. Figure 2 illustrates potential ON modification sites and the properties these modifications provide to ON.[19]

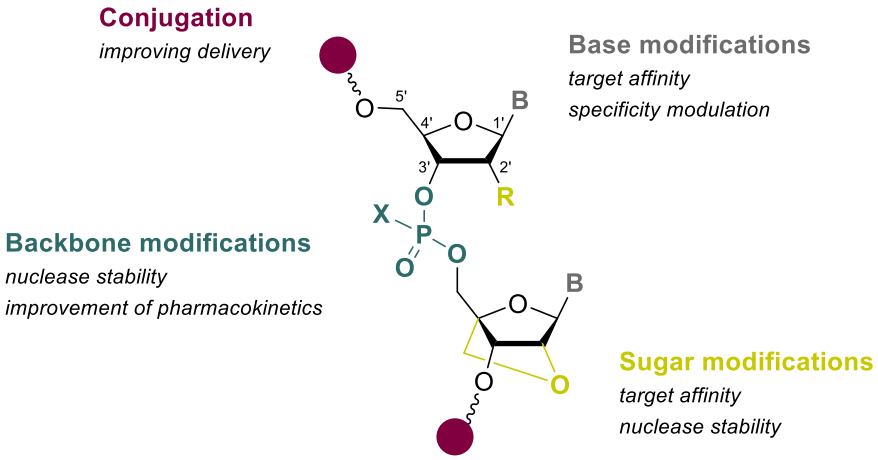


Figure 2. Representative oligonucleotide modification sites and their potential effects.

There are two main strategies to chemically improve the ON: bioconjugation and chemical modification of the ON chain. In the case of bioconjugation, ON is coupled with various specific ligands to improve delivery limitations. Chemical modifications on base, sugar and backbone mainly address stability, target affinity and overall improvement of pharmacokinetics of the ON.[1-5, 19]

1.4 BIOCONJUGATED OLIGONUCLEOTIDES

Bioconjugated ONs are a rapidly developing subclass of ON-based therapeutics.[1, 3, 5] Generally, the ligands used for ON conjugation can be grouped into passively targeting and actively targeting subsets.[1] For instance, cell-penetrating peptides (CPPs), lipids and polymers are used for passive targeting while antibodies and receptor ligands are used for active targeting. In addition, ligands can be used for multiple conjugation,[5] when either multiple identical ligands or combinations of different ligands are conjugated to a single ON sequence. Multiple conjugation can improve and/or provide tailored properties for a given ON conjugate. Approaches towards the preparations of mono- and multi functionalized ONs exploit a variety of suitable and available chemistries.

1.4.1 Oligonucleotide Conjugation Strategies

Broadly, the first step in selecting a strategy for ON-conjugation involves the choice between solid-phase or solution-phase conjugation (Figure 3).[20-24] In addition, in some instances, a hybrid approach can also be applied.

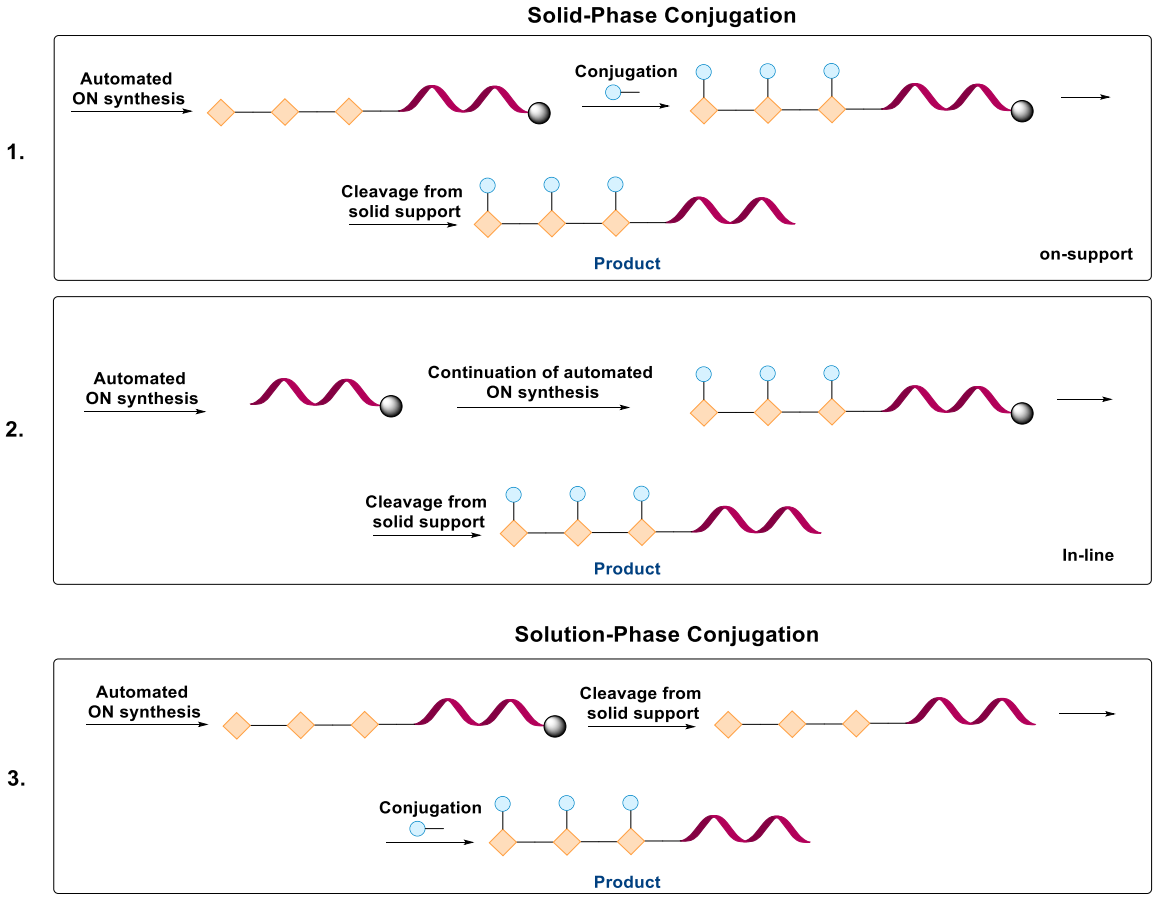


Figure 3. Schematic representation of conjugation strategies: on-support conjugation (1), in-line conjugation (2), solution- phase conjugation (3).

In the case of solid-phase approach, conjugation is performed on an ON which is still bound to the solid support. In addition, solid-phase conjugation can be further split into two

subtypes: in-line or step-wise solid-phase conjugation (Figure 3.2) and on-support fragment conjugation Figure 3.1).[23] Solution-phase conjugation (Figure 3.3) is achieved using an ON after it is cleaved off from the solid-support.

1.4.2 Solid-phase (On-support) Oligonucleotide Conjugation

This thesis focuses on the ‘on-support’-type fragment conjugate synthesis. During on-support fragment conjugate synthesis a reactive functional moiety (a chemical 'handle') is introduced to the ON sequence during the automated ON synthesis.[23] Chemical handles can be incorporated into the ON chain either at the terminal ends and/or at any other position inside the sequences using different approaches, for example, phosphoramidite or H-phosphonate chemistries. Similarly, branched linking molecules can be incorporated into the ON sequence as well. Depending on the chemical properties and the reactivity of the functional moiety, it can either contain a protecting group (e.g. Fmoc-protected amine functionality) or can be left unprotected (e.g. terminal alkyne).[25] If a functional moiety is protected, the protecting group (stable during ON synthesis) is removed prior to the conjugation and the ON remains bound to the solid support for the reaction with e.g. a biologically active moiety. The final conjugate is obtained by cleaving it off from the solid support.

In-line solid-phase conjugation slightly differs from the on-support fragment approach as the final conjugate is obtained directly during automated ON synthesis: e.g. a biologically active moiety (e.g. reporter molecule) and the ON are assembled on the same solid support and no post synthetic manipulations are needed.

The main advantages of bioconjugate preparation on solid support include a simplified purification as well as the commercial availability of some of the required modified building blocks and solid supports in a 'ready-to-use' fashion. However, for more specific applications and needs, modified phosphoramidites or supports have to be synthesized in the laboratory. In addition, chemistries of the building blocks must be compatible and orthogonal, especially when in-line conjugation is performed. The prepared conjugate should remain stable under the cleavage and deprotection conditions[23] which usually are rather harsh (e.g. aq. ammonia solution).

1.4.3 Solution-phase Oligonucleotide Conjugation

In the solution-phase conjugation approach (Figure 3.3), complementary reactive functional moieties are first incorporated on both the ON and the biologically active ligand respectively to ensure conjugation. In contrast to solid-phase conjugation, solution-phase

approach allows for the conjugation of molecules with incompatible chemistries. For example, while the direct synthesis of peptide-oligonucleotide conjugates is difficult on solid support due to different protection-deprotection strategies and different pH ranges in which these molecules operate, these types of conjugates can nevertheless still be made in solution. However, conjugates prepared in solution, as a general rule, may require exhaustive purification depending on the conversion and the type of modality to be conjugated, which may lead to lower overall yields. Another important limitation associated with conjugation in solution is the solubility of the ON, the other modality and the final conjugate. Since the vast majority of ON-conjugates necessitate aqueous solutions, it can prove problematic to dissolve some of the reporter molecules.[23]

1.4.4 Chemistries for Conjugation

The chemistry of chemical handles and linkages is very important for the success of the underlying conjugation techniques. Apart from a necessary modification to introduce or transform a functional group into a suitable handle, the location of the handle needs to be pre-determined to have a minimal impact on the expected activity. Suitable chemistry can enable selective conjugation to one or more ligands of interest and a suitable choice of chemical linkage is invoked with regard to its biological properties.[23]

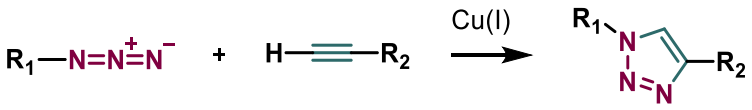
Click chemistry is a powerful tool for conjugation of various types of molecules. The azide-alkyne cycloaddition takes the leading role in reactions for this area of research. The concept of 'click' chemistry was first introduced by Sharpless in 1999 to characterize a group of reactions which result in few easily removable or no byproducts, are high yielding, extensive in scope, selective, stereospecific and easy to perform.[26] To date, four types of reactions conform to these standards: nucleophilic substitutions, in particular, ring openings of strained heterocyclic electrophiles (e.g. epoxides), additions to C-C multiple bonds (e.g. Michael addition), 'non-aldol' type carbonyl chemistry (e.g. urea formation) and cycloadditions (e.g. Huisgen's cycloaddition, Diels-Alder reaction).[26, 27]

Among traditional conjugation strategies such as thiol-maleimide, amide, sulfide and disulfide bond formations,[23] copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) (Scheme 1) and strain-promoted azide-alkyne cycloaddition (SPAAC) (Scheme 2) stand out as versatile biorthogonal high-yield methods.[27, 28]

1.4.4.1 Copper(I)-Catalyzed Azide-Alkyne Cycloaddition

Copper(I)-catalyzed azide-alkyne cycloaddition, or copper(I)-promoted ‘click’ reaction (Scheme 1), is a variation of Huisgen 1,3-dipolar cycloaddition.[29] Copper catalyzed

variation was independently introduced by Sharpless[30] and Meldal[31] in 2002. Since that time this reaction was gaining popularity among several different disciplines because of its versatility. Furthermore, many comprehensive reviews were published to this date regarding this reaction.[27, 32, 33]



Scheme 1. Schematic representation of copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC).

The CuAAC proceeds via the 1,3-dipolar cycloaddition of organic azides with alkyne functional moieties to form 1,2,3-triazole linkages in the presence of copper (Scheme 1). Numerous metals and metal complexes have been explored for this reaction as well,[34-37] but copper and ruthenium appear to be dominating in the nucleic acid-related research.[27]

Azide and alkyne functional moieties can easily be placed in many types of small and large biologically relevant molecules. For example, our group has developed triple bond donors such as p-(*N*-propynoylamino)toluic acid (PATA)[38, 39] or 4-((2-(prop-2-yn-1-yloxy)acetamido)-methyl) benzoic acid (PAMBA)[40] (Figure 4), which can be used as efficient chemical handles in copper(I)-catalyzed 'click' reactions.

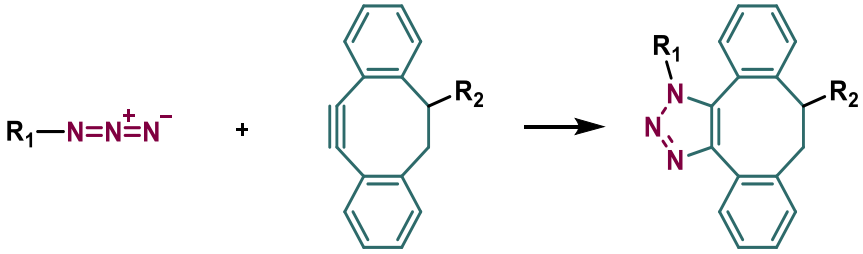
Figure 4. Triple bond donors p-(N-propynoylamino) toluic acid (PATA) and 4-((2-(prop-2-yn-1-yloxy)acetamido)-methyl) benzoic acid (PAMBA)for copper(I)-promoted 'click' conjugation.

Since azides and alkynes are inert towards most biological substrates and other functional groups, they are considered to be bioorthogonal and biocompatible. However, copper is cytotoxic[41, 42] and can also cause several undesirable side-effects such as strand degradation (mostly on DNA)[43, 44] and protein denaturation.[45] In addition, the purification of the product and removal of copper might be problematic, especially if the reaction is performed in solution-phase.[46-48]

1.4.4.2 Strain-Promoted Azide-Alkyne Cycloaddition

Strain-promoted azide-alkyne cycloaddition (SPAAC) is a reaction between an organic azide and a strained cyclic derivative, e.g., cyclooctyne. Since, as mentioned above, copper(I) 'click' reaction causes undesirable side-effects and is toxic, SPAAC provides a

metal-free alternative and is the most broadly utilized type of metal-free conjugation today.[49] SPAAC was discovered by Blomquist et al. in the early 1950s.[50] Approximately 50 years later Bertozzi et al. reintroduced this type of reaction in living systems[51] which led to its outstanding popularity to date.



Scheme 2. Schematic representation of strain-promoted azide-alkyne cycloaddition (SPAAC), other isomers may apply.

Typically, SPAAC is performed on strained 8-membered rings (Scheme 2): smaller ring systems are too reactive/chemically unstable and larger rings are typically not reactive enough.[52, 53] Cyclooctynes can react to form triazoles without a catalyst at ambient conditions. However, the reaction rate of unmodified cyclooctyne can be rather slow. Therefore, more reactive cyclooctyne systems were developed and have since been successfully applied for SPAAC reactions (Figure 5). It is known that the presence of electron-withdrawing functional groups such as halogen atoms or additional cyclic structures on the cyclooctyne ring can increase the reaction rates.[53] For example, addition of the two fluorine atoms at the C3 position of a difluorinated cyclooctyne (DIFO) (Figure 5) augment the rate of a cycloaddition by 60-fold compared to the plain cyclooctyne.[54, 55] Dibenzofused cyclooctyne (DIBO),[56, 57] (DBCO)[58-60] and non-benzoannulated bicyclo[6.1.0]nonyne (BCN)[61] have also been investigated (Figure 5). Notably, the use of SPAAC is dominated by above-mentioned DIBO, DBCO and BCN[53] as well as various types of their non-nucleosidic phosphoramidite derivatives, likely because they are readily available from commercial sources.

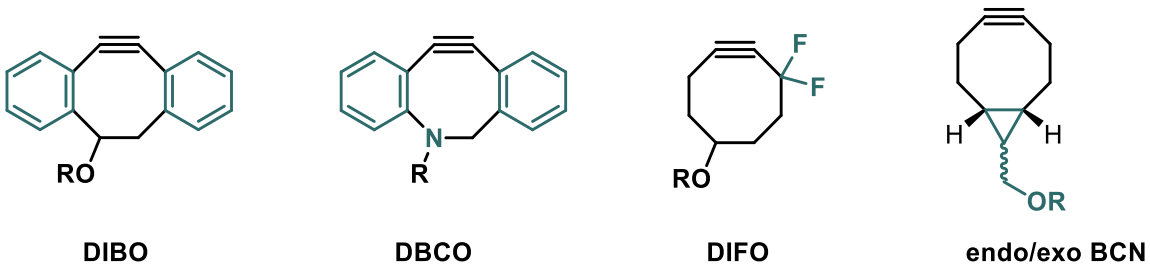


Figure 5. Selected examples of cyclooctynes for strain-promoted azide-alkyne cycloaddition.

DBCO was first introduced by van Delft et al.[58] and rapidly followed by two other research groups[59, 60] using different synthetic approaches. In addition, the synthesis of DBCO was

optimized by Adronov et al.[62] It is worth noting that the molecule was given different names by each group: DIBAC by van Delf et al.,[58] ADIBO by Popik et al.[59] and *aza*-DBCO by Feringa et al.[60]

Despite that dibenzofused systems display great reactivity properties,[53] they are asymmetrical with respect to the position of the triple bond moiety.[27] This leads to formation of mixtures of stereoisomeric products which is often undesirable. An alternative to these systems, BCN, was also introduced by van Delft et al.[61] An extensive number of studies was done by the same group to enable BCN for biorthogonal labeling.[58, 61, 63-65] Notably, BCN exists in two diastereomeric forms: *endo* and *exo.* These isomers can be separated via normal-phase chromatography on silica gel.[61] The reactivities of *endo* and *exo* isomers are comparable to other cyclooctyne systems.[54, 66] When both BCN isomers were reacted with benzyl azide in MeCN/water mixture (1:2), calculated second-order rate constants were 0.29 $M^{-1}s^{-1}$ and 0.19 $M^{-1}s^{-1}$ for *endo* and *exo* isomers respectively.[61] Comparing to dibenzofused systems, BCN has two advantages. First, it contains a plane of symmetry which prevents the formation of mixtures of stereoisomeric products. Second, BCN provides lower lipophilicity which is typically more beneficial when reaction is performed in aqueous solutions. However, the use of BCN brings constraints in terms of stability under standard automated ON synthesis conditions, which limits the versatility of this moiety.[63]

1.4.5 Examples of ON Conjugates

1.4.5.1 Peptide-ON Conjugates

Peptides are an appealing class of modalities for bioconjugation. They may improve tissue/cell-targeting, provide cell-penetrating properties and/or facilitate endosomal escape[1, 3, 5] as well as improve binding affinity.[67]

For example, cell penetrating peptides (CPPs) are a class of drug delivery vectors which have been studied for delivering numerous types of macromolecular therapeutics into cells.[68, 69] CPPs usually consist of no more than 30 amino acids and are able to cross the cell membrane and can also provide enhanced endosomal escape properties.[1, 3, 5, 68, 70] CPPs can either be mixed with ONs in formulations or can be directly conjugated to an ON sequence.[1] In addition, conjugation of multiple peptides to the ON may provide additional properties such as improved sensitivity of a bioconjugate to its receptor.[5]

Another extensively investigated group of peptides for ON conjugation are linear tripeptide RGD (Arginylglycylaspartic acid) and its cyclic-RGD analogues. As these peptides bind to

αvβ3 integrins, which are overexpressed in several types of cancers,[71-73] they are attractive for their potential in targeting and delivery, albeit positive results *in vivo* are not yet confirmed.[5]

1.4.5.2 ON Conjugation to Hydrophobic Ligands

Conjugation to hydrophobic ligands such as cholesterol, fatty acids or α-tocopherol improves ON delivery. Such systems may augment delivery to the liver and peripheral tissues, e.g. muscle.[74] For example, conjugation of cholesterol to ONs can promote endosomal escape, prolong the plasma half-life and promote ON accumulation in the liver.[75] Moreover, Nagata et al.[76] demonstrated that conjugation of cholesterol or α-tocopherol at the 5′-end of RNA strand in DNA/RNA heteroduplex systems helps them reach the central nervous system (CNS) after an intravenous or a subcutaneous administration in mice and rats. In addition, it was determined by Wang et al. that PS-ASO conjugates with hydrophobic ligands such as cholesterol, α-tocopherol, and palmitic acid, have improved protein binding and increased intracellular uptake.[77] Furthermore, it was demonstrated that ON-fatty acid conjugates show high potential for myelofibrosis treatment.[78-80] Conjugation of siRNA to α-tocopherol was reported to have an effect on apolipoprotein B (ApoB) levels in miceliver.[81]

1.4.5.3 GalNAc-ON Conjugates

To date, *N*-acetylgalactosamine (GalNAc) is possibly the most successful tissue targeting ligand.[82-84] Trimeric GalNAc ligand was first introduced by Lee et al.[85] and Nair et al.[86]

GalNAc increases cellular internalization in the liver as it binds to the abundantly expressed asialoglycoprotein receptor (AGPR).[87] When GalNAC was conjugated to siRNAs and ONs, it provided a highly specific delivery to hepatocytes.[88-90]

In addition, there are three GalNAc-conjugated ONs already approved by the FDA (givosiran[91] and lumasiran[92]) or the EMA (inclisiran[93]) (Table 1) as well as many drug-candidates which currently are being evaluated in preclinical and clinical trials.[83, 94, 95]

1.4.5.4 Multiple Labeling of Oligonucleotides

As mentioned above, the effects of several modalities can be combined by the multiple functionalization of the ONs.

Meyer et al. achieved the synthesis of bis-ON conjugates with a combination of mono- or poly-thiol Michael-type additions and CuAAC chemistry.[96] CuAAC was also employed to prepare ON conjugates with different fluorescent labels,[97] ON-GalNAc dendrimer

conjugates[98] and site-specific hetero bis-labeling of long RNAs.[99] Our research group published an efficient method to prepare ON multiconjugates using 'click cycles' [40] and a versatile method to prepare multi-labeled ON-conjugates, enabling CuAAC in combination with an amide bond formation.[25]

Wada et al.[100] described a cholesterol-GalNAc conjugate (Figure 6) with an ASO. This conjugate represents an effective approach to reduce ASOs toxicity in the kidneys, while maintaining gene-silencing in the liver. The conjugate was prepared using phosphoramidite chemistry and the synthesis of a GalNAc phosphoramidite was described earlier by the same group.[101]

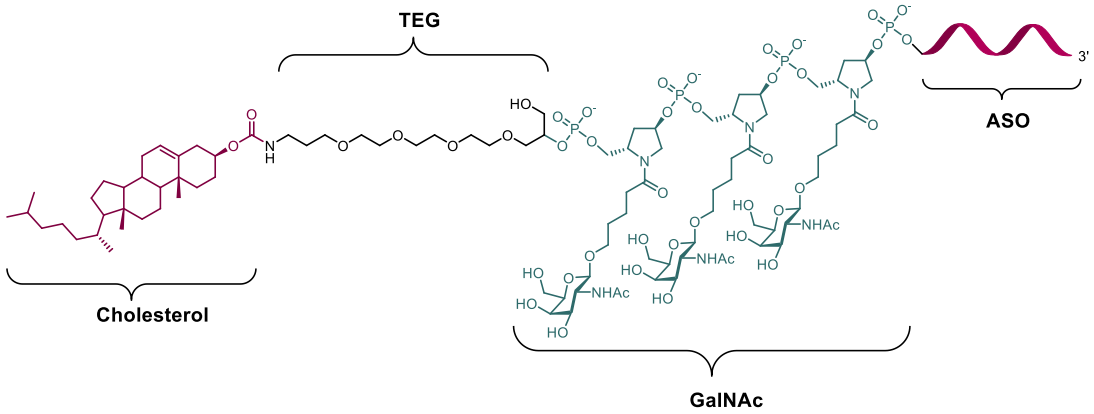


Figure 6. An example of an ON-multiconjugate developed by Wada et al.;[100] ASO: antisense oligonucleotide; GalNAc: N-acetylgalactosamine; TEG: triethylene glycol.

Tajik-Ahmadabad et al.[102] synthesized a bis- self-assembling myristic acid and a CPP-modified ASO. In this approach, conjugation was achieved via thiol-maleimide reaction: a peptide, containing C-terminal cysteine, was coupled with ASO which was functionalized with 3-maleimidopropionic acid. Attachment of myristic acid to the peptide N-terminus led to the spontaneous self-assembly of the ASO. In addition, bis-conjugated ASO exhibited a 4-fold rise in effectiveness compared to the CPP-ASO monoconjugate.[102]

1.5 OLIGONUCLEOTIDE MODIFICATIONS

ONs exhibit low stability both *in vitro* and *in vivo* due to enzymatic degradation. For instance, after being injected into simian models, phosphodiester ONs have a reported half-life of only 5 min.[103]

Whereas the above described ON-bioconjugates address delivery limitations, chemical modifications are introduced to the ON sequence to improve its stability. The most common ON modifications are summarized in Figure 7.[1-4]

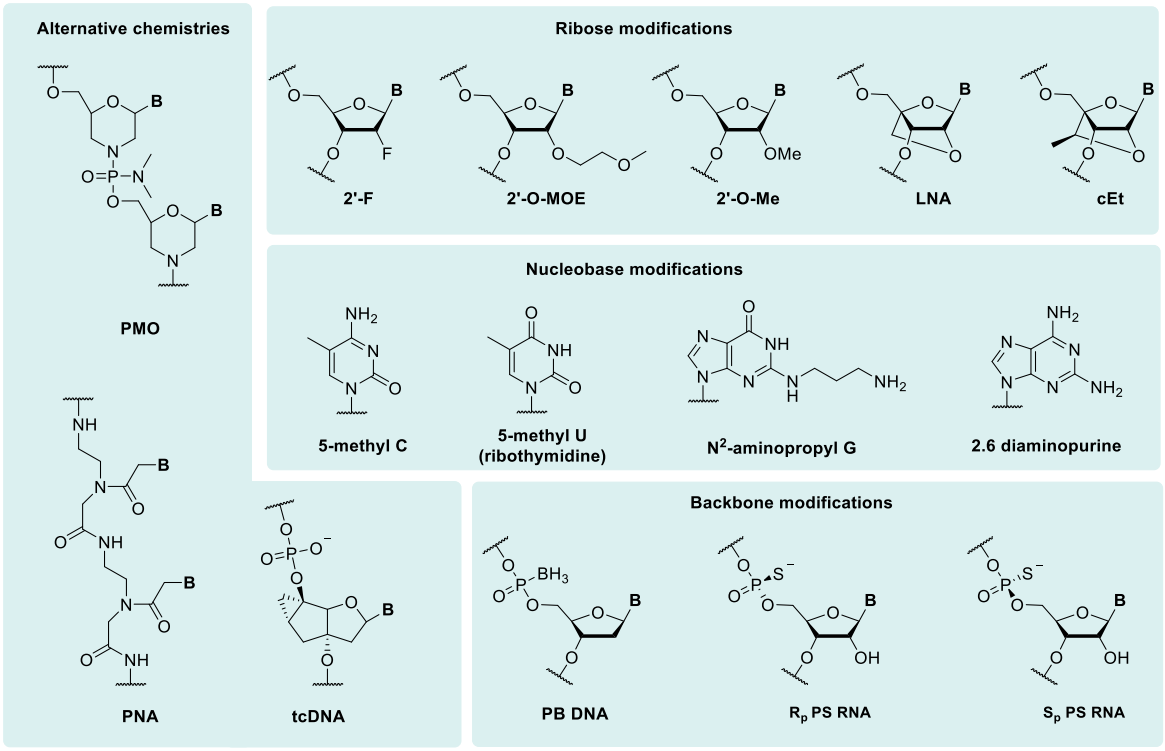


Figure 7. Selected examples of oligonucleotide structure modifications. On the left: phosphorodiamidate morpholino oligomer (PMO), peptide nucleic acid (PNA), tricyclo-DNA (tcDNA). From above: ribose modifications: 2'-fluoro (2'-F), 2'-O-methoxy-ethyl (2'-O-MOE), 2'-O-Methyl (2'-O-Me), locked nucleic acid (LNA), 2',4'-constrained ethyl bridged nucleic acid (cEt); Nucleobase modifications: 5-methyl cytidine (5-methyl C), 5-methyl uridine (5-methyl U), N^2-aminopropylguanine (N^2-aminopropyl G), 2.6 diaminopurine; backbone modifications: boranophosphate DNA (PB DNA), phosphorothioate (PS) RNA in two isomer forms: R_p and S_p.

Extensive chemical modification of the ON backbone can suffice to enable the delivery of ONs to a wide range of tissues without any additional delivery vehicle. For example, most of the FDA and/or EMA approved ON therapies (Table 1) do not contain any additional delivery agent.

1.5.1 Backbone Modifications

Although there are several types of backbone modifications (e.g. boranophosphate[104]), the most widely used example is a phosphorothioate (PS) linkage[105] (Figure 7), which provides resistance to endonucleases as well as promotes bioavailability.[19, 105] However, the disadvantage of this backbone modification is that it reduces the affinity for the target RNA. This limitation can be compensated by incorporating other types of modifications into the ON sequence.[1, 3]

Another interesting aspect in PS modification is that the introduction of an additional sulfur atom generates a chiral center with two possible stereoisomers (S_p and R_p) (Figure 7). This means that a fully-PS-modified 20-mer in theory can produce a diastereomeric mixture of 2^{19} possible isomers - in principle, this corresponds to a combinatorial space of over half a

million different molecules.[3] Each stereocenter can display different properties in terms of nuclease resistance, lipophilicity/ionic character, RNase H activity and target affinity.[106] For instance, a triple 3′-$S_pS_pR_p$-5′ 'stereochemical code' in 'gap' region of a gapmer ASO proved to be especially effective in promoting target RNA cleavage by RNase H1.[106] On the other hand, it has been suggested that a stereo-random mixture of PS isomers is needed to balance the activity and silencing-stability.[107]

1.5.2 Nucleobase Modifications

Nucleobase modifications (Figure 7) enhance target binding while retaining the base pairing together with an unchanged conformation of the double helix.[1-3, 19]

Pyrimidine modification examples include 5-methyl, 5-propynyl, 5-thiazolyl, 5-bromo- and 5-iodo- modifications, which have a positive effect on duplex stability.[19] The 5-methyl modification has been among the most investigated modifications to date and it has not only shown significant improvements towards duplex stability, but also a net-positive effect on reducing the immune response.[108-111]

Modifications on both adenine and guanine can also give rise to thermal stability. For example, 2,6-diaminopurine has an additional hydrogen bond to its T and U counterparts. The N-alkylated guanines, in particular N^2-imidazolylpropyl- and N^2-aminopropylguanine can enable additional electrostatic interactions with the phosphate backbone.[19] Moreover, abasic nucleotides (nucleotides that do not contain a nucleobase) have been used to cancel miRNA-like silencing.[112]

1.5.3 Ribose Modifications

Ribose modifications (Figure 7) have provided substantial improvements in enhancing drug-like properties of ONs.[1-3] 2′-*O*-Methyl (2′-*O*-Me), 2′-*O*-methoxy-ethyl (2′-*O*-MOE) and 2′-fluoro (2′-F) modifications are the most widely used types of modifications, which have also been utilized in many clinically approved drugs (Table 1). 2′-*O* and 2′-modifications further improve resistance to nucleases and increase the binding affinity to RNA. Conformationally constrained DNA analogues such as the tricyclo-DNA (tcDNA), locked nucleic acid (LNA) or 2′,4′ – constrained ethyl bridged nucleic acid (cEt) (Figure 7) provide an even greater binding affinity to target sequences as well as stability enhancements against nucleases.[1-3] However, bridged nucleic acids are incompatible with RNase H-mediated cleavage and therefore are not used in the DNA gap region.[3] In terms of structural diversity, LNA has a methylene bridge between the ribose 2′-*O* and 4′-carbon[113,

[114] and tcDNA contains an ethylene bridge with a cyclopropane ring between 3′- and 5′-carbon positions of the ribose ring (Figure 7).[115]

1.5.4 2′-O-(N-(Aminoethyl)carbamoyl)methyl Modification

Whereas most 2′-modified oligonucleotides usually do not improve cellular uptake, 2′-*O*-(N-(aminoethyl)carbamoyl)methyl (2′-*O*-AECM) modified ONs (Figure *8*) seem to possess cell penetrating properties.[116-118]

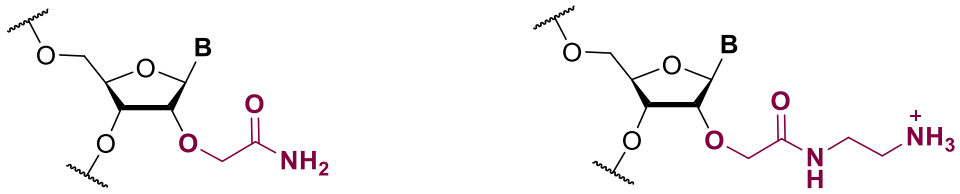


Figure 8. On the left: 2′-O-carbamoylmethyl (2′-O-CM) modified ON. On the right: 2′-O-(N-(aminoethyl)carbamoyl)methyl (2′-O-AECM) modified ON.

The first, rather similar analogues to 2′-*O*-AECM modification, a 2′-*O*-carbamoylmethyl (2′-*O*-CM) modified nucleosides (Figure 8), were introduced by Grøtli et al.[119] It was demonstrated that the CM modification is highly resistant to enzymatic degradation[120] and also provides a substantial stabilization of duplexes.[119] Moreover, as CM can be further functionalized at the amide nitrogen, these possibilities have been since investigated by several research groups.[121-127] In addition, Ozaki et al.[124] investigated a post-synthetic ON modification with 2′-*O*-AECM (Figure 8).

Our research group has focused extensively on the synthesis of 2′-O-AECM-modified nucleosides (and biological studies when incorporated into ON) over the last couple of years.[116-118, 120, 128] The syntheses of 2′-*O*-AECM modified nucleosides (A, C, G, U)[116] as well as their methylated analogues (5-MeC, 5-MeU) were reported.[118, 129] Notably, the methylated 2′-*O*-AECM modified nucleosides were also prepared in larger scale.[129] In addition, it was demonstrated that this modification provides a unique combination of resistance towards enzymatic degradation and an improvement of cellular uptake properties for the ON.[117, 118]

1.5.5 Alternative Chemistries

Although most of modified ONs are derived from standard RNA or DNA, chemistries that substantially differ from natural analogues have also been explored to a varying degree.[1-3, 19] Phosphoramidate morpholino oligomers (PMOs)[130] and peptide nucleic acids (PNAs)[131, 132 133] probably are the best-known examples in this case. Sometimes above mentioned

tcDNA is also attributed to this group of modifications. In PMOs, standard nucleobases are retained, but the ribose is replaced with a 6-membered morpholino ring and phosphorodiamidate linkages (Figure 7). The nucleobases of PNAs are linked by a pseudo-peptide backbone (Figure 7). Both PMO and PNA macromolecules are inherently uncharged. They display enhanced resistance to nucleases as well as a more variable affinity towards target sequences.[130, 134] The main disadvantage of PMOs and PNAs is that they both minimally interact with plasma proteins. This means that these ON analogues are rapidly cleared via urinary excretion.[3] To date, four PMO products for the treatment of Duchenne Muscular Dystrophy (DMD) have been approved by FDA (Table 1): eterplirsen which targets exon 51, golodirsen and viltolarsen target exon 53 and casimersen targets exons 43, 44, 45 of the dystrophin mRNA.[3]

2 RESEARCH AIMS

This thesis aims to expand the scope of oligonucleotide multiorthogonal-conjugation techniques and to develop convenient methods to synthesize 2′-*O*-AECM modified nucleosides in larger scale.

The first chapter (papers I and II) presents a strategic approach to prepare multi-conjugates which is based on the development of linker molecules bearing suitable chemical handles for orthogonal on-support conjugation.

In the second chapter (paper III) upscaling and optimization strategies for the synthesis of 2′-*O*-AECM modified methyl-uridine and methyl-cytidine nucleosides are explored. Separate synthesis steps are screened for the best conditions and reagents as well as best work-up and purification strategies are optimized for a larger (up to 100 g) scale approach.

3 RESULTS AND DISCUSSION

3.1 CHAPTER I. DEVELOPMENT OF ORTHOGONAL LINKERS AND OLIGONUCLEOTIDE CONJUGATES (PAPERS I AND II)

3.1.1 Design of Linkers for Multiple Conjugation of Oligonucleotides

Oligonucleotide (ON) modifications can improve several parameters essential for efficient oligonucleotide therapy. Typically, oligonucleotide conjugates are designed to address the delivery-specific challenges.[1, 5, 27] For example, ON properties such as tissue and/or cell targeting together with cellular internalization can be improved by conjugation of ONs with various modalities. Moreover, multi-labeling of oligonucleotides can further improve the desired properties as well as provide some additional and tailored properties.

The aim of the studies summarized in this chapter are related to general methodology development for direct incorporation of linkers into oligonucleotide sequences, specifically - during solid-phase automated ON synthesis. This approach serves as a versatile platform for incorporation of multiple suitable/desired moieties, permitting each moiety at any chosen position of the ON sequence.

Achieving the synthesis of an ON sequence modified with several different chemical moieties requires the incorporation of several different chemical handles. For an effective multi-conjugation strategy, a set of three novel linkers was contrived with these two essential features in mind: 1) orthogonal functionalities, 2) ability of easy transformation to phosphoramidites (Figure 9), since the phosphoramidite moiety allows for the direct incorporation of a handle into an ON sequence during the automated ON synthesis. In addition, the possibility to prepare these linkers from the same scaffold was seen as an advantage in terms of the required synthetic effort.

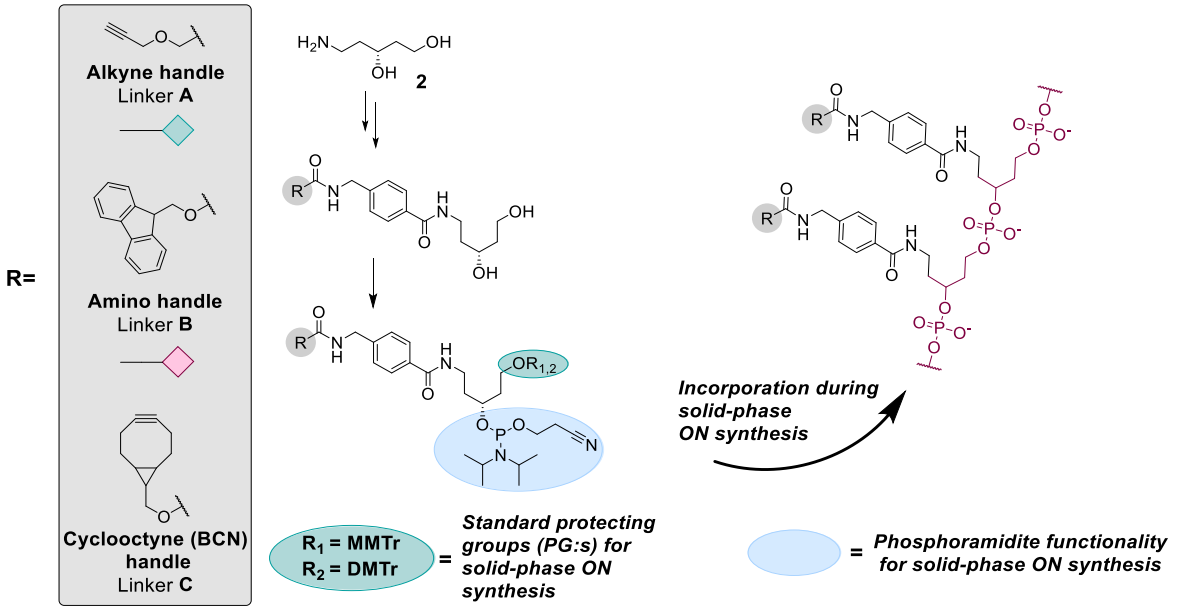


*Figure 9. Newly developed orthogonal linkers compatible with automated ON synthesis. The grey-highlighted parts (**R**) indicate the chemical handles for orthogonal conjugation. **A** - terminal alkyne-bearing linker for copper(I)-promoted ´click´ conjugation; **B** – Fmoc-protected amino linker for amide bond formation; **C** - cyclic alkyne-bearing linker (BCN) for strain-promoted 'click' conjugation.*

The first handle - Linker **A** (Figure 9. A), carries a terminal triple bond, which is sufficiently active for conjugation via a copper(I)-promoted 'click' reaction (CuAAC). Linker **B** (Figure 9. B) enables orthogonality by amide bond formation. Linker **C** (Figure 9. C) provides a handle for strain-promoted copper-free 'click' reaction (SPAAC). All three linkers are synthetically accessible from a common aminodiol intermediate **2**. Linkers **A** and **B** were utilized in the preparation of ON conjugates whereas the utilization of Linker **C** is under continued investigation in our group.

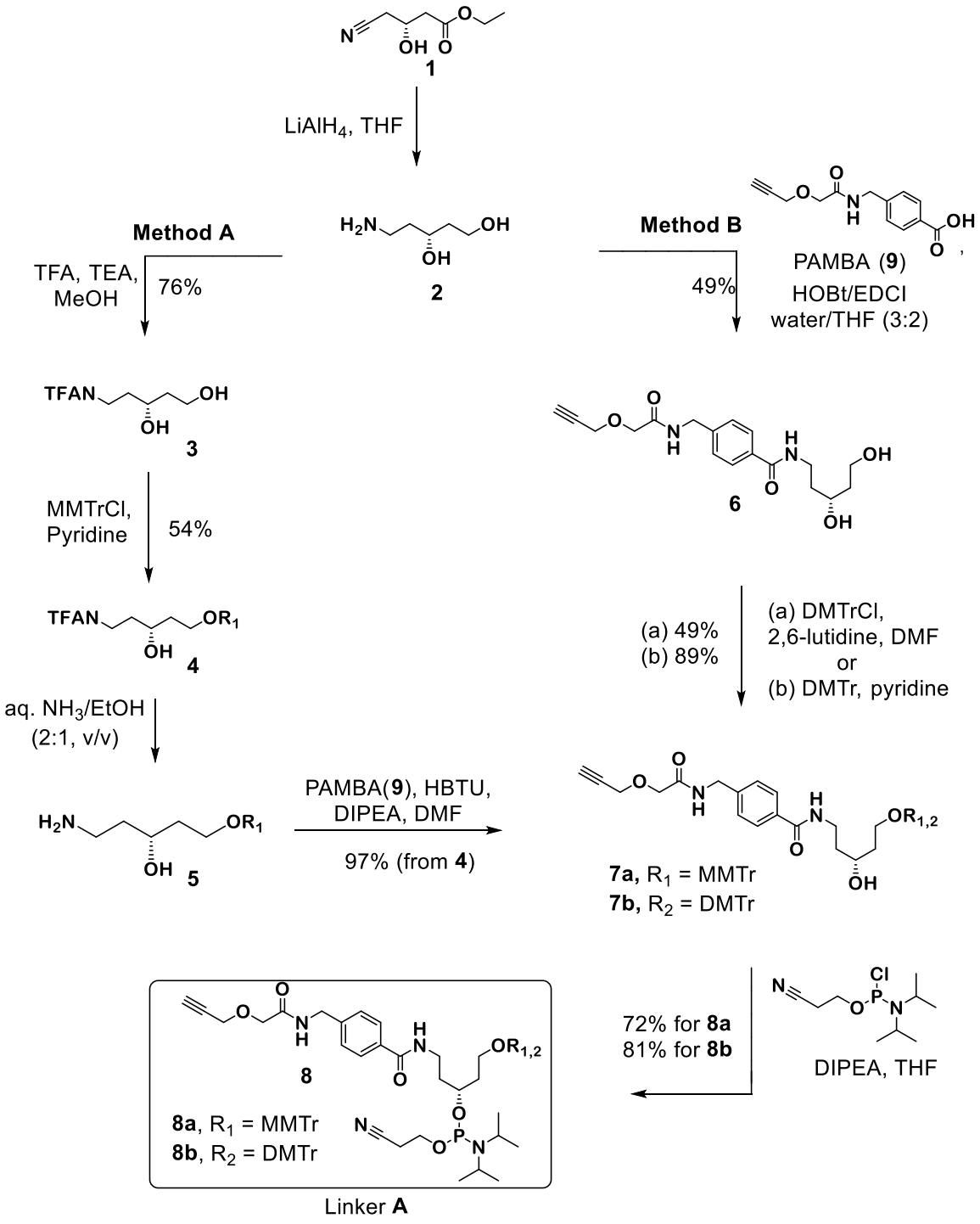
3.1.2 Synthesis of Orthogonal Alkyne and Amino Linkers for Multiple Conjugation

3.1.2.1 Synthesis of Alkyne Linker (Linker A)

Two methods (Scheme 3) for the synthesis of two variations of the Linker **A** with either 4-methoxytrityl (MMTr) or 4,4′-dimethoxytrityl (DMTr) protecting group at 5′-OH were developed.

The syntheses of both variations of Linker A started with the preparation of aminodiol (**2**) according to a previously published procedure.[135] The synthesis of MMTr-protected linker (Method A, Scheme 3) was continued with the protection of aminodiol **2** with trifluoroacetyl (TFA) protecting group using ethyl trifluoroacetate, which proved to be advantageous for purification purposes. The primary hydroxy group was then protected with MMTr in the presence of pyridine following the removal of TFA protecting group

with aq. ammonia-ethanol solution (2:1, v/v) to give compound **5**. Compound **5** was then coupled with 4-((2-(prop-2-yn-1-yloxy)acetamido)methyl) benzoic acid (PAMBA[40]) to yield the compound **7a**, which was then converted to phosphoramidite derivative **8a** (72 % yield).

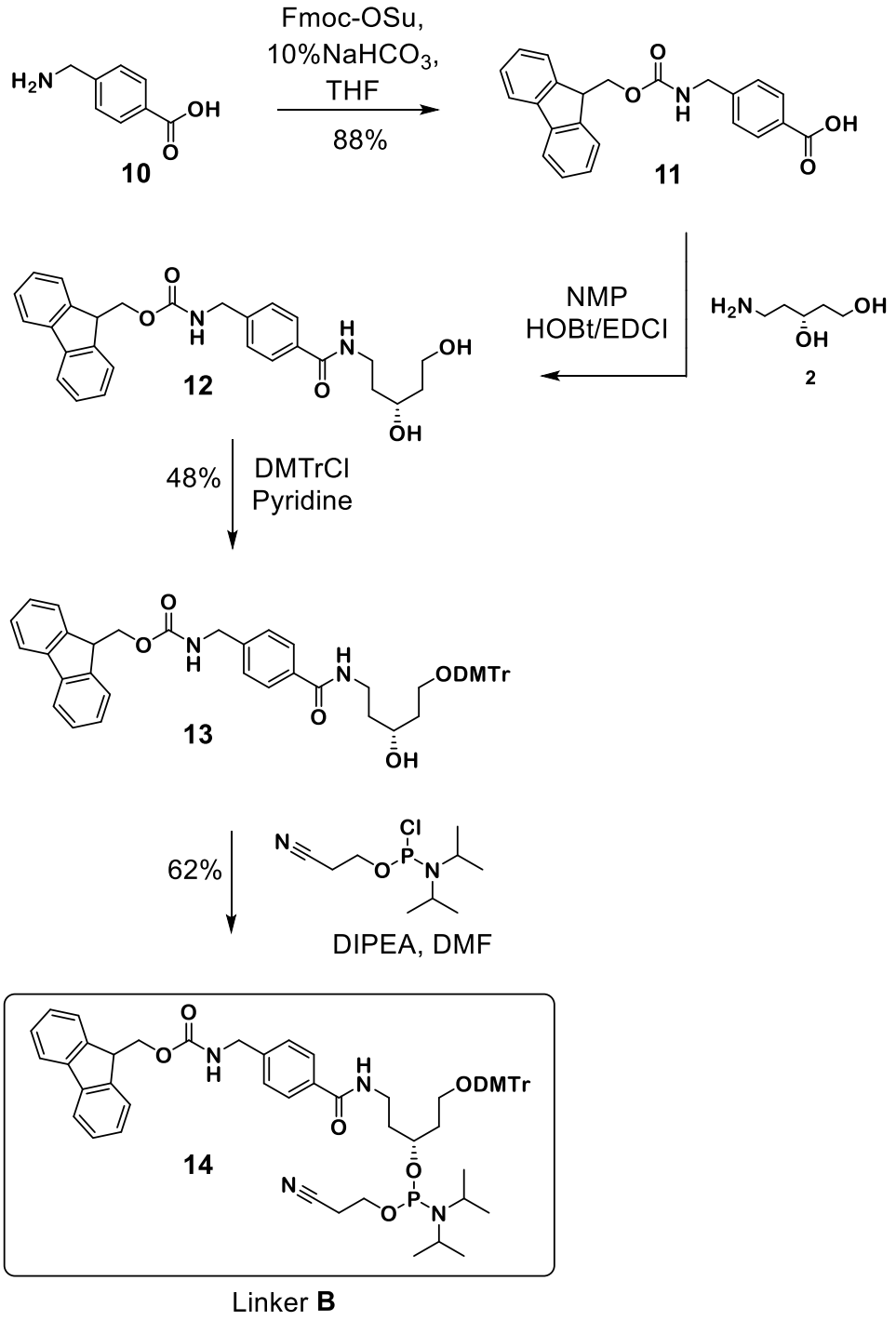


*Scheme 3. The complete synthesis of Linker **A** starting from commercially available ethyl (3R)-4-cyano-3-hydroxybutanoate. Method A yields the desired Linker A phosphoramidite in a total of six steps and 72% yield for the final step. The modified Method B requires a total of four steps for Linker **A** phosphoramidite synthesis and yielded 82% in the final step. The protection of primary hydroxy group with DMTr or MMTr and conversion of secondary hydroxy group to phosphoramidite allows direct incorporation of Linker **A** to the ON sequence.*

For the preparation of DMTr-protected Linker **A** (Method B, Scheme 3), crude aminodiol **2** was coupled with PAMBA **9** using 1-ethyl-3-(3-dimethylaminoaminopropyl)carbodiimide (EDCI) and 1-hydroxybenzotriazole (HOBt) as condensing agents, to give compound **6** in 49% yield. DMTrCl or MMTrCl were then used to protect the primary hydroxy group. The obtained compound **7b** was then converted to phosphoramidite **8b** in 81% yield.

3.1.2.2 Synthesis of Amino Linker (Linker B)

Fluorenylmethoxycarbonyl (Fmoc)-protected Linker **B** (**14**) (Scheme 4) was prepared using the same aminodiol (**2**) scaffold.



*Scheme 4. The complete synthesis of Linker **B** starting with the protection of amino group of p-(aminomethyl)-benzoic acid with Fmoc-protecting group. The Fmoc derivative was then coupled with aminodiol, the primary hydroxyl group was then protected with DMTr and 4,4'-dimetoxytritylated compound was converted into phosphoramidite 14.*

First, *p*-(aminomethyl)-benzoic acid **10** was protected with Fmoc-protecting group. The obtained derivative **11** was then coupled with aminodiol **2** using EDCI/HOBt in *N*-methylpyrrolidone (NMP). The primary hydroxyl group was subsequently protected with DMTr and resulting intermediate **13** was converted to phosphoramidite **14** in 62 % yield.

3.1.3 Preparation of Oligonucleotide Conjugates

Several ON conjugates (Figure 10, Figure 11, Figure 12) were prepared using Linkers **A** and **B.** The incorporation efficiency for these linkers was also evaluated during automated ON synthesis. Oligonucleotide sequences used for conjugations are described in Table 2.

Table 2. Sequences of oligonucleotides used for conjugations.

ON	Sequence 5' → 3'
ON1	$((L^{alkyne})_2 – (L^{alkyne})_2)$-doubler-GCGTTGATGCAATTTCTATGC
ON2	(L^{alkyne})-*G*G*C*C*A*A*A*C*C*U*C*G*G*C*U*U*A*C*C*U*
ON3	(L^{alkyne})- (L^{alkyne})-*G*G*C*C*A*A*A*C*C*U*C*G*G*C*U*U*A*C*C*U*
ON4	(L^{alkyne})- (L^{alkyne})- (L^{alkyne})-*G*G*C*C*A*A*A*C*C*U*C*G*G*C*U*U*A*C*C*U*
ON5	TCAAGGAAG-(L^{alkyne})-ATGGCATTTCT
ON6	(L^{alkyne})- (L^{NHFmoc})-(L^{alkyne})- TCAAGGAAG ATGGCATTTCT

L = linker, * = PS, in *italic* - 2′-*O*Me modified monomers, rest - standard DNA, doubler - see Fig 10.

The alkyne Linker **A** was utilized in the synthesis of several ON conjugates (Figure 10, Figure 11, Figure 12). In the first example (Figure 10), the ON conjugate carried four (2x2) benzylguanine (BG) units (ON1). BG is a valuable tool for SNAP-display technology.[136, 137] The synthesis of the construct began with a post-synthetic functionalization using a commercially available ‘doubler modifier’ at the terminal 5′-position of solid-supported ON. A total of four units of Linker **A** were attached sequentially during automated ON synthesis. RP-HPLC analysis of the intermediate **15** showed that the coupling efficiency was rather moderate, however we deemed it sufficient to continue with conjugation experiments using BG units. Copper-promoted ‘click’ reaction was used for the synthesis of the final construct. Solid-supported intermediate **15** was activated with copper(I) iodide and reacted with azido-functionalized BG (**BG**-N_3). The final conjugate was cleaved off from the solid support and its identity was confirmed via mass spectrometry.

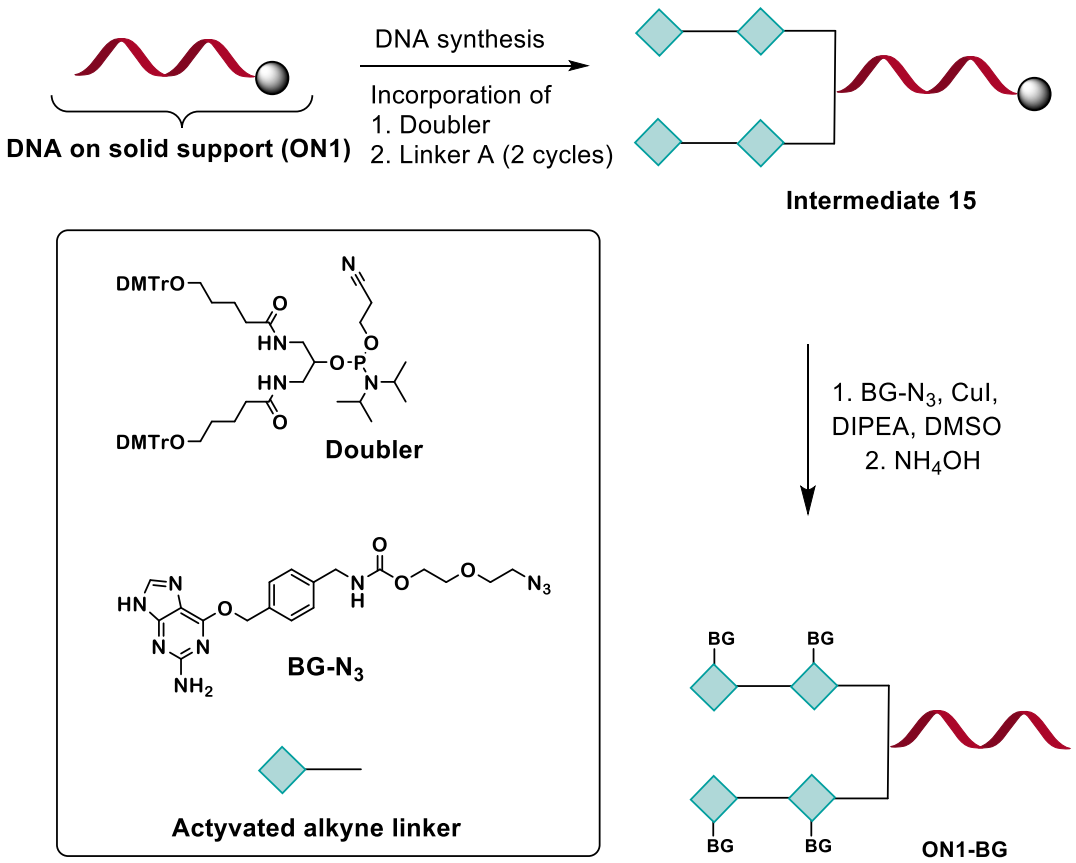


*Figure 10. Schematic representation of the synthesis of oligonucleotide conjugate carrying four benzylguanine units (**ON1-BG**).*

Next, the Linker **A** was used to prepare three different peptide derivatives containing phosphorothioate (PS) backbones (Figure 11), ON2-4. A muscle-homing P4 peptide (N_3-P4 (Ac-K(N_3)LGAQSNF-NH_2))[138] was used in the conjugation. The prepared conjugates contained one, two or three P4 peptide units (Figure 11) which were conjugated via corresponding terminal alkyne handles on Linker **A** units using CuAAC and CuBr × Me_2S as a copper(I) source. The identity of the obtained constructs was confirmed by mass spectrometry.

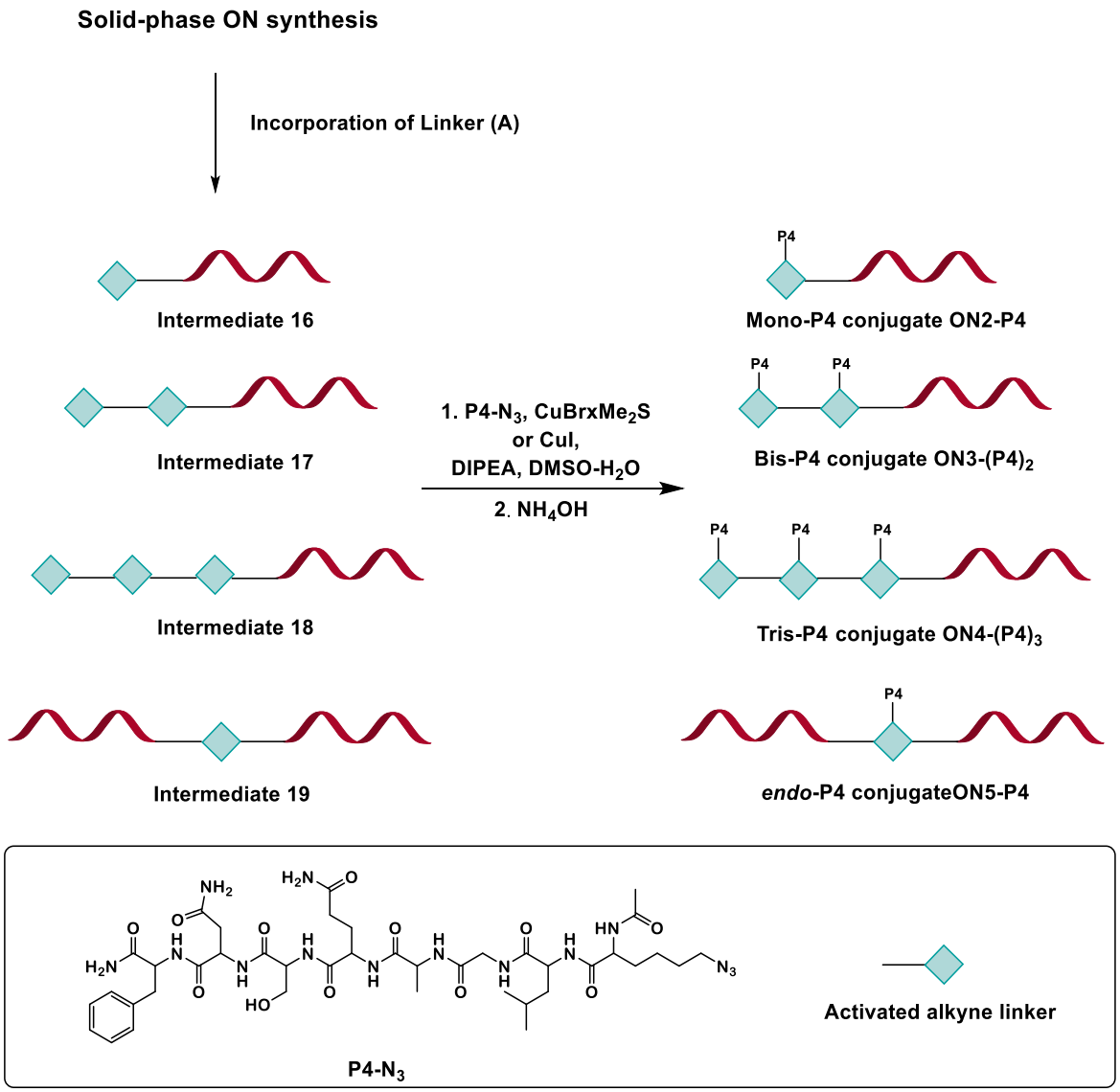


*Figure 11. Schematic representation of synthesis of mono- (**ON3-P4**), bis- (**ON3-(P4)**$_2$), tris- (**ON3-(P4)**$_3$) and endo-labeled (**ON5-P4**) ON conjugates.*

In addition, Linker **A** was incorporated internally into oligonucleotide sequence (Figure 11) during automated oligonucleotide synthesis. Solid-supported intermediate **19** was conjugated with P4 peptide via CuAAC using copper(I) iodide as catalyst. *Endo*-P4-labeled DNA (**ON5-P4**) was then cleaved off from the solid support and its identity was confirmed by mass spectrometry.

The orthogonality of alkyne (**A**) and amine (**B**) linkers was evaluated by the synthesis of multiconjugate ON construct, containing different entities at the 5′-end of the ON (Figure 12). Two units of **MIF-1** peptides and one palmitoyl unit were used for the preparation of a *tris*-construct. **MIF-1** is an endogenous brain peptide which delivers a variety of pharmacological effects on the central nervous system.[139] The conjugation of long chain fatty residues is known to modulate the lipophilicity of the modified ON[140] and, as

demonstrated in the case of palmitic acid, the affinity to albumin as well as the uptake into cardiac and skeletal muscles.[74]

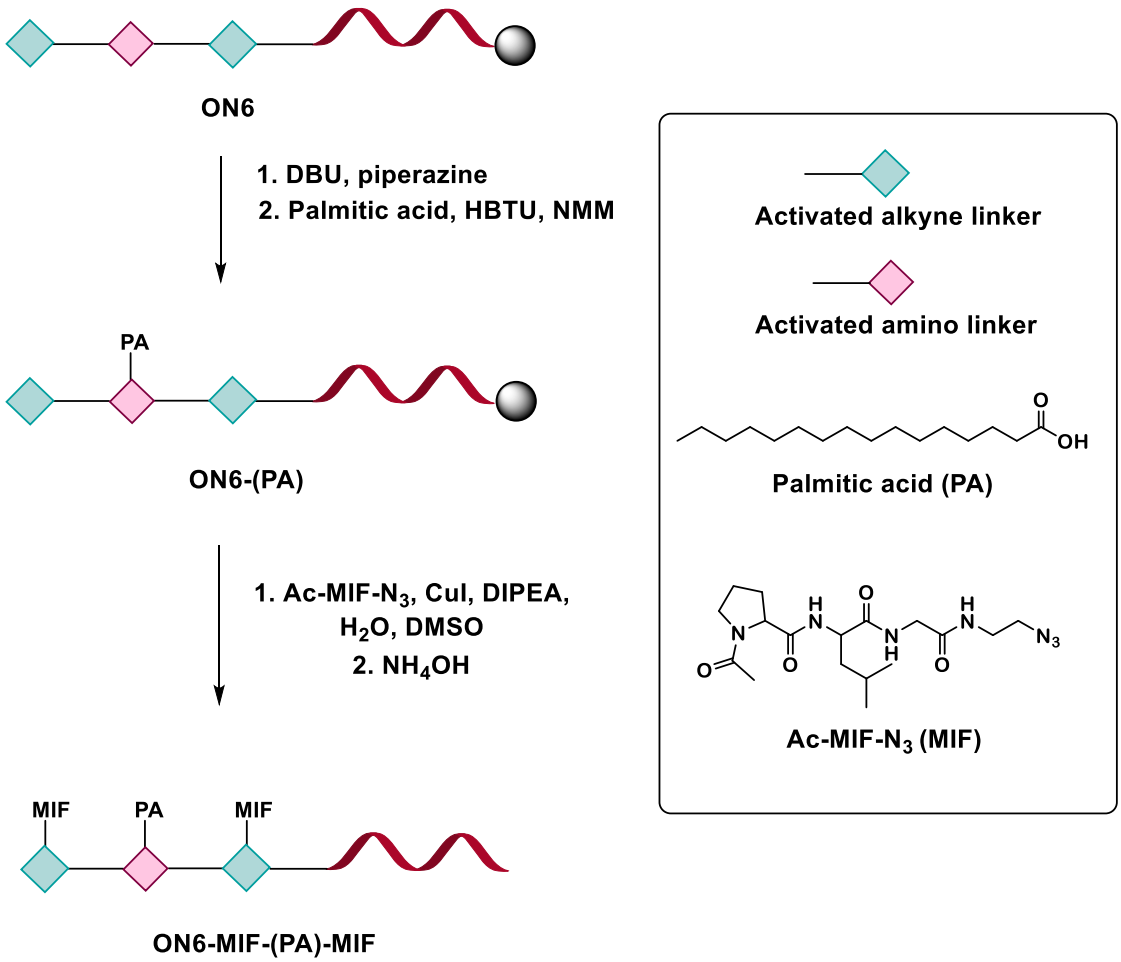


*Figure 12. Schematic representation of the synthesis of tris conjugate **ON6-MIF-(PA)-MIF**.*

The synthesis of *tris*-conjugate **ON6**-**MIF**-(**PA**)-**MIF** began with the sequential addition of Linker **A** and Linker **B** and then Linker **A** again at the 5′-end of the ON during the automated synthesis. Fmoc protecting group of Linker **B** was removed using a mixture of 2% 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) and 5% piperidine in DMF [141]. Palmitic acid was then conjugated to amino group on the solid support using HBTU and *N*-methylmorpholine (NMM). The obtained intermediate **ON6**-(**PA**) was then coupled with azide-funtionalized MIF peptide (Ac-**MIF**-N_3, Ac-**PLG**-N_3) using CuAAC and copper(I) iodide as activator. The obtained *tris*-construct **ON6**-**MIF**-(**PA**)-**MIF** was removed from the solid support and identified by mass spectrometry.

3.1.4 Synthesis of BCN-functionalized Linker (Linker C)

To achieve the synthesis of Linker **C**, BCN alcohol (both *endo*- and *exo*- isomers (Figure 13)) was prepared from readily available starting materials using published procedures.[142, 143]

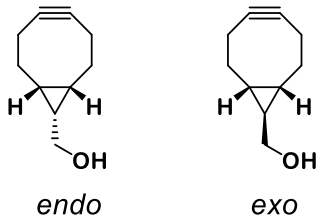
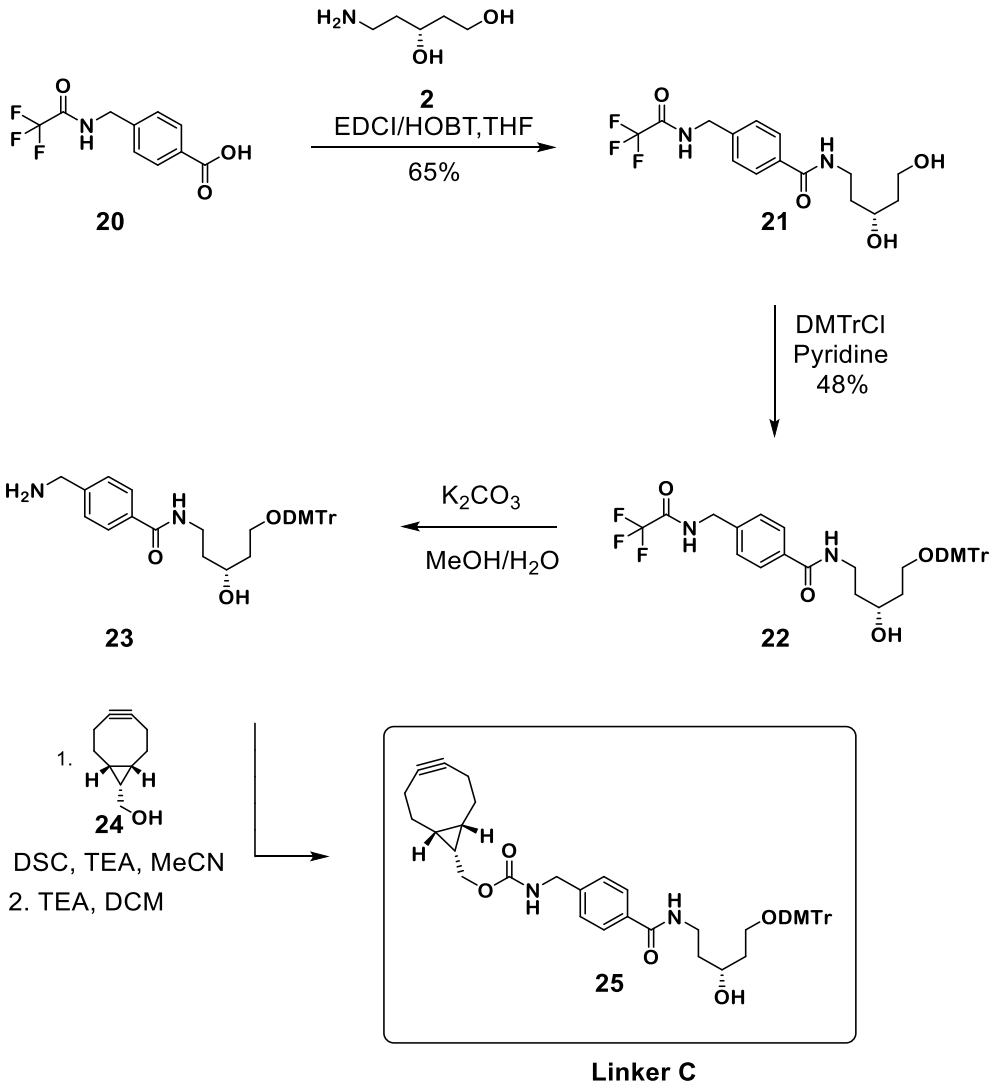


Figure 13. Two possible isomers of BCN alcohol.

The synthesis of Linker **C** (Scheme 5) was started with the protection of p-(aminomethyl)benzoic acid with TFA following a reported method.[144] Obtained intermediate **20** was then coupled with aminodiol **2** using EDCI/HOBt as activating agents. Primary hydroxyl group of the obtained derivative **21** was then protected with DMTr followed by removal of TFA protecting group under basic conditions to give compound **23**.



Scheme 5. Synthesis of Linker C. Linker C was prepared using the same common scaffold (2) as for the syntheses of Linker A and B. Aminodiol 2 was coupled with TFA protected p-(aminomethyl)benzoic acid. The obtained intermediate 21 was then protected with DMTr (on primary hydroxyl group) followed by TFA deprotection and coupling with BCN alcohol to give Linker C.

Endo-BCN (**24**) was activated with *N,N′*-disuccinimidyl carbonate according to a published procedure[65] and reacted with compound **23** to give the derivative **25** in 24% yield. The subsequent phosphoramidite formation and its utility for ON synthesis is part of an ongoing investigation in our group.

3.1.5 BCN Stability Studies

3.1.5.1 Studies of Degradation Patterns of BCN

The data on the stability of the BCN in the literature is limited. Reported data[63, 145] suggests that BCN is not stable under acidic conditions. It is therefore problematic to use BCN with automated ON synthesis since acidic conditions are employed to remove the DMTr/MMTr protecting groups prior to the coupling steps. Moreover, while handling and performing experiments on the prepared BCN alcohols (Figure 13), we have noticed that solubility

properties of the crystalline BCN alcohol change over time. The previously apparent solubility changed despite keeping the compound in the freezer (approx. -10 °C) and in the dark. These observations raised a suspicion that the issues are caused by the triple bond in the bicyclic cyclooctyne structure. The changes in the crystalline structure of BCN alcohol were further evaluated using X-ray powder diffraction (XRPD) and differential scanning calorimetry (DSC).

Both *endo*- and *exo*-BCN isomers (Figure 13) were freshly prepared and immediately subjected to XRPD and calorimetry measurements. For comparison, a sample of aged *exo*-BCN (Figure 13) alcohol (kept in -10 °C for 3 years) was included in the measurement. When samples of *exo*- and *endo*- isomers were left in open air for two weeks and remeasured, an increase of amorphous content became apparent. The change was especially prevalent in the *exo*- isomer. The aged sample also displayed an increase of amorphous content. NMR of the two-week-old sample indicated formation of decomposition products, but whether the collapse of an unstable solid-state is the cause of this decomposition, or if the decomposition results in changes in the solid-state remains ambiguous and would require further investigation.

In addition, it was noted that despite the fact that the acidic groups in the silica gel used during purifications (flash chromatography) were consistently pretreated with 0.1-1% TEA, the yields of both BCN alcohols were lower than anticipated and reported in the literature.[143] These observations indicated that silica which was used for purification might be the cause of low yields and degradation.

The stability of *exo*-BCN was therefore further tested via deposition and incubation on a TLC plate (Figure 14), which revealed degradation of the compound on silica under ambient (rt) conditions over time.

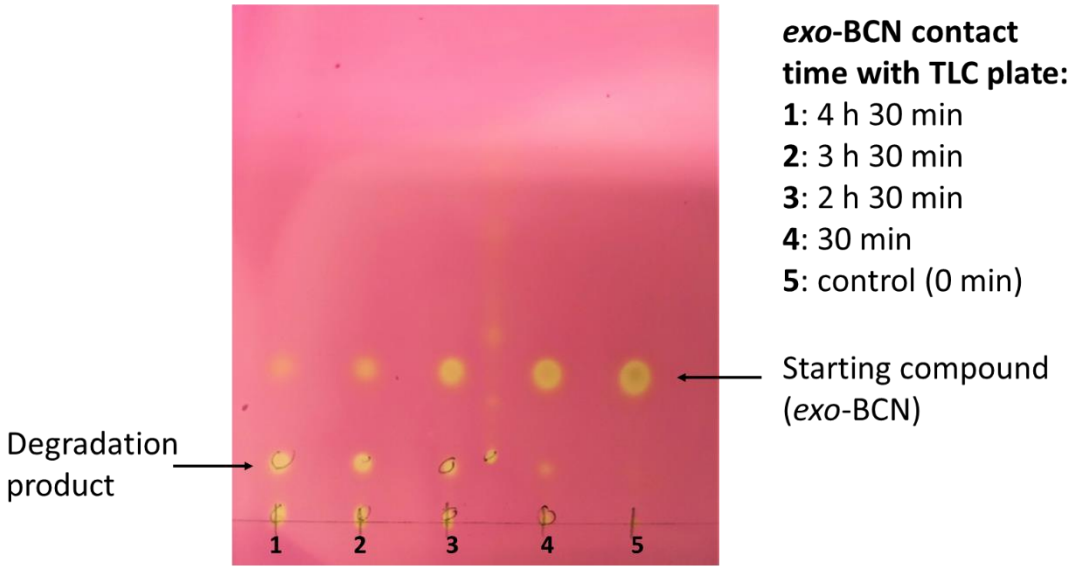


Figure 14. exo-BCN stability on TLC plate over time.

Some reports[63, 145] suggest that hydrolysis of the triple bond into ketone (via vinyl alcohol) in the presence of acid occurs. To test these reports, the degradation product of BCN-silica interaction was isolated and analyzed by NMR. The NMR data indicates, however, that both hydrolysis as well as oxidation reactions can be attributed to degradation on silica (Figure 15).

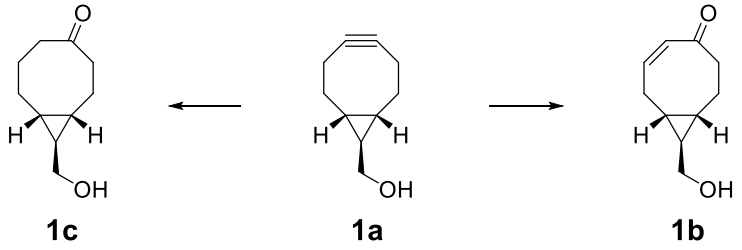


Figure 15. Probable degradation pathways of exo-BCN alcohol (1a); acid induced hydrolysis and oxidation product (1b); acid induced hydrolysis product (1c).

Oxidative degradation is not reported in the literature to the best of our knowledge. Also, from our experience, no degradation occurred in solution, therefore the silica seems to have a detrimental effect to BCN stability.

3.1.5.2 Kinetic Experiments

Van Delft et al. suggested that BCN is not compatible with automated oligonucleotide synthesis,[63] however only the solution of TCA (trichloroacetic acid) in DCM was reported. Since DCA in toluene is another standard solution for DMTr or MMTr deprotection during ON synthesis, and it is mentioned in the literature that DCA (dichloroacetic acid) should be used instead of TCA,[27] the evaluation of the sensitivity of the triple bond in BCN alcohol using different acidic solutions and concentrations seemed worth to investigate.

Four mixtures of different acid-solvent systems were prepared and investigated: 1.4 mg/ml *exo*-BCN alcohol was dissolved in 3% (w/v) TCA or DCA solution in either DCM or toluene. Change of the *exo*-BCN alcohol concentration was monitored using GC. Samples were analyzed after 5, 10, 15, 20 and 30 min. The BCN amount was estimated from the chromatograms and plotted to give the kinetic curves (Figure 16).

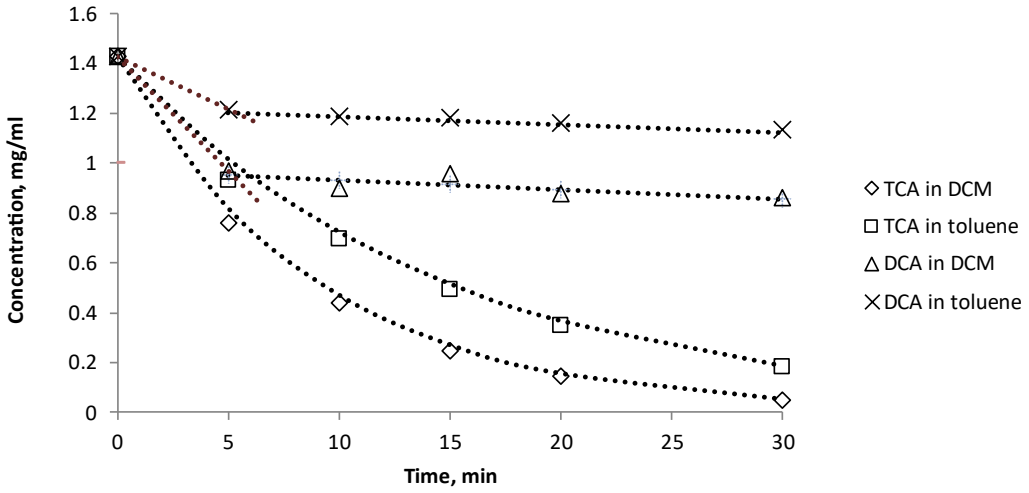


Figure 16. Kinetic study of exo-BCN alcohol degradation in the presence of TCA or DCA solutions in either DCM or toluene.

The results show that TCA in either toluene or DCM proved to be the worst choice in terms of stability. Although BCN was slightly more stable in toluene, with 13% of initial compound remaining compared to 3.5 % in DCM after 30 min, the results revealed that DCA could be a better choice for BCN: 60% of starting compound remained intact in DCM and 79% in toluene (Figure 16) after 30 min.

Since this preliminary investigation revealed a rather significant difference in stability comparing TCA and DCA, the investigation of BCN stability in acidic conditions was expanded further: the conditions for chemoselective Linker **C** detritylation were investigated. In order to find suitable conditions for the removal of DMTr while keeping the triple bond of the BCN moiety intact, three acids - DCA (pKa=1.25), chloroacetic acid (MCA, pKa=2.9) and acetic acid (pKa=4.8) were tested. Based on the previous result (Figure 16), toluene was chosen as solvent for further investigations. Mixtures with 3% (w/v) DCA, MCA or acetic acid in toluene were prepared, containing 1 mg/ml of Linker **C** each. The reaction was monitored using LC-MS. Analyses of the unquenched samples were started after 4, 8, 18, 30 and 60 min.

As anticipated, the degradation of the Linker **C** was the slowest in the presence of acetic acid. However, the DMTr protecting group also remained intact even after 1 h of the

reaction. In comparison, DCA was able to remove DMTr immediately, albeit several unidentified products also formed. The detritylated product (r.t. 0.85 min) and an unidentified impurity is visible (r.t. 0.3 min) in the initial analysis after 4 min of reaction. A minor new peak (r.t. 1.08 min) appears after 8 min of the reaction and subsequently increases over time. The identity of the peak is not clear probably due to poor ionization during the MS analysis.

Finally, detritylation in the presence on MCA occurs almost immediately. However, after 1 h, approximately 2% of starting compound is still detectable. Furthermore, no clearly visible degradation products appear to be forming. These results suggest that MCA or a mixture of MCA/DCA could be further evaluated at different concentrations.

3.2 CHAPTER II. SYNTHESIS AND UPSCALING OF 2′-O-AECM-5-METHYL PYRIMIDINE PHOSPHORAMIDITES FOR SOLID-PHASE OLIGONUCLEOTIDE SYNTHESIS (PAPER III)

As mentioned in the introduction, sugar and/or base modifications in the ON can provide several advantages in terms of therapeutic properties. It was previously shown that a unique combination of enhanced cell penetration and resistance against enzymatic degradation can be provided by a 2′-*O*-(*N*-(aminoethyl)carbamoyl)methyl (2′-*O*-AECM) modification (Figure 17). [116-118, 120, 128]

Since 2′-*O*-AECM modification can provide valuable properties for the ON, there was a need to prepare monomers containing 2′-*O*-AECM modification in gram scales for further evaluation when incorporated into ONs. Therefore, as described in paper III, a process to prepare methyl-uridine (AECM-MeU) and methyl-cytidine (AECM-MeC) monomers ready for ON synthesis in larger (up to 100 g) scales was developed.

The study described in this chapter starts with evaluation of the potential synthetic and purification problems for the synthesis of AECM-MeU and AECM-MeC: Even though the corresponding analogous non-methylated AECM-modified cytidine and uridine nucleosides were synthesized before in milligram scales,[116, 118] several steps required further optimization due to solubility and/or purification problems as well as expensive reagents, to become suitable for larger scale synthesis. Moreover, to be able to adapt the synthesis for a larger scale, the development of better purification (less chromatography) or work-up strategies was necessary.

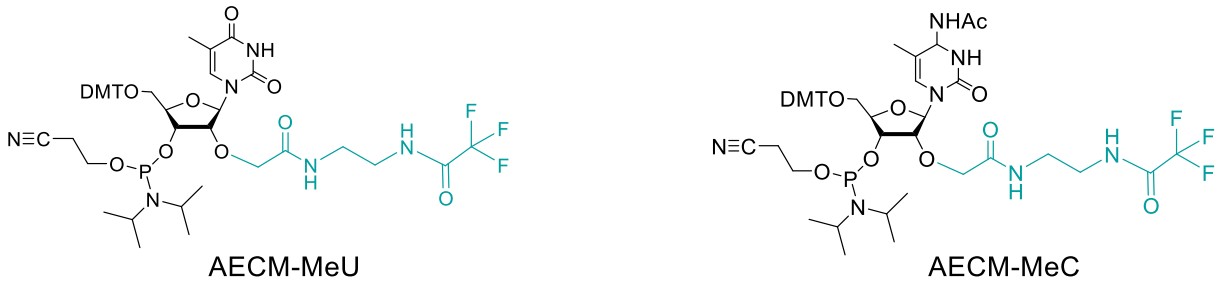


Figure 17. Left: 2′-O-(N-(Aminoethyl)carbamoyl)methyl (2′-O-AECM) modified methyl uridine (AECM-MeU); Right: 2′-O-(N-(aminoethyl)carbamoyl)methyl (2′-O-AECM) modified methyl cytidine (AECM-MeC).

3.2.1 Process Development and Upscaling of AECM-MeU Monomer

The study was initiated with the bench-scale (approx. 1 g) synthesis (Figure 17) of AECM-MeU monomer (**31**). Since the procedure for AECM-U nucleoside was described before,[116, 118] it was expected that a similar sequence could be applied for the synthesis of AECM-MeU monomer.

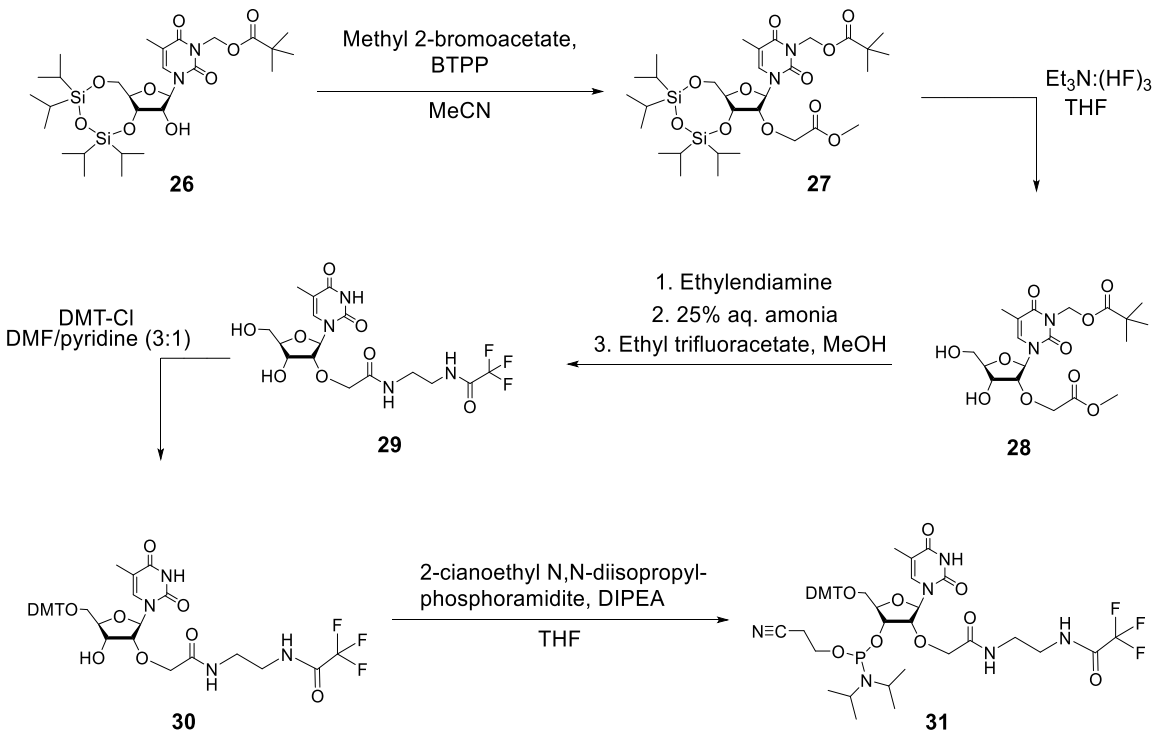


Figure 18. Synthesis route for AECM-MeU following the same pathway reported for AECM-U monomer.

After the complete synthesis, a yield of 0.54 g of the final amidite AECM-MeU **31** was successfully obtained in bench scale using the same synthetic pathway as in the procedure reported for AECM-U[116, 118] (Figure 18). However, after the bench scale synthesis was successfully reproduced for the modified monomer, it was clear that the synthesis path should be changed and optimized before going to larger scale due to purification difficulties and the need to use expensive reagents (e.g., BTPP (P_1-*t*-Bu-tris(tetramethylene)). Therefore, a new synthesis path was developed (Figure 19). All the intermediate steps, except the final amidite synthesis, starting with N^3-protection, were screened for optimized conditions in up to 1 g scale, before upscaling the reactions further.

3.2.2 Synthesis of AECM-MeU

The preparation of AECM-MeU monomer began with the synthesis of 3′,5′-*O*-[(1,1,3,3-tetraisopropyl-1,3-disiloxanediyl)]methyl-uridine (**32**)[146] in larger (100 g) scale. The protection of N^3 with pivaloxymethyl (POM) group using phase-transfer catalysis (PTC) was described before for unmethylated analogue.[147] However, optimization was needed for this synthesis step since more than 120 volumes of solvents were used in the published analogous N^3-alkylation. Large amounts of solvents generally are not satisfactory for any larger scale synthesis. The same rule applies to the amounts of reagents - any excessive quantities of reagents should also be avoided when moving to larger scales. Consequently, we also aimed to reduce the amount of chloromethyl pivalate needed for the N^3-protection.

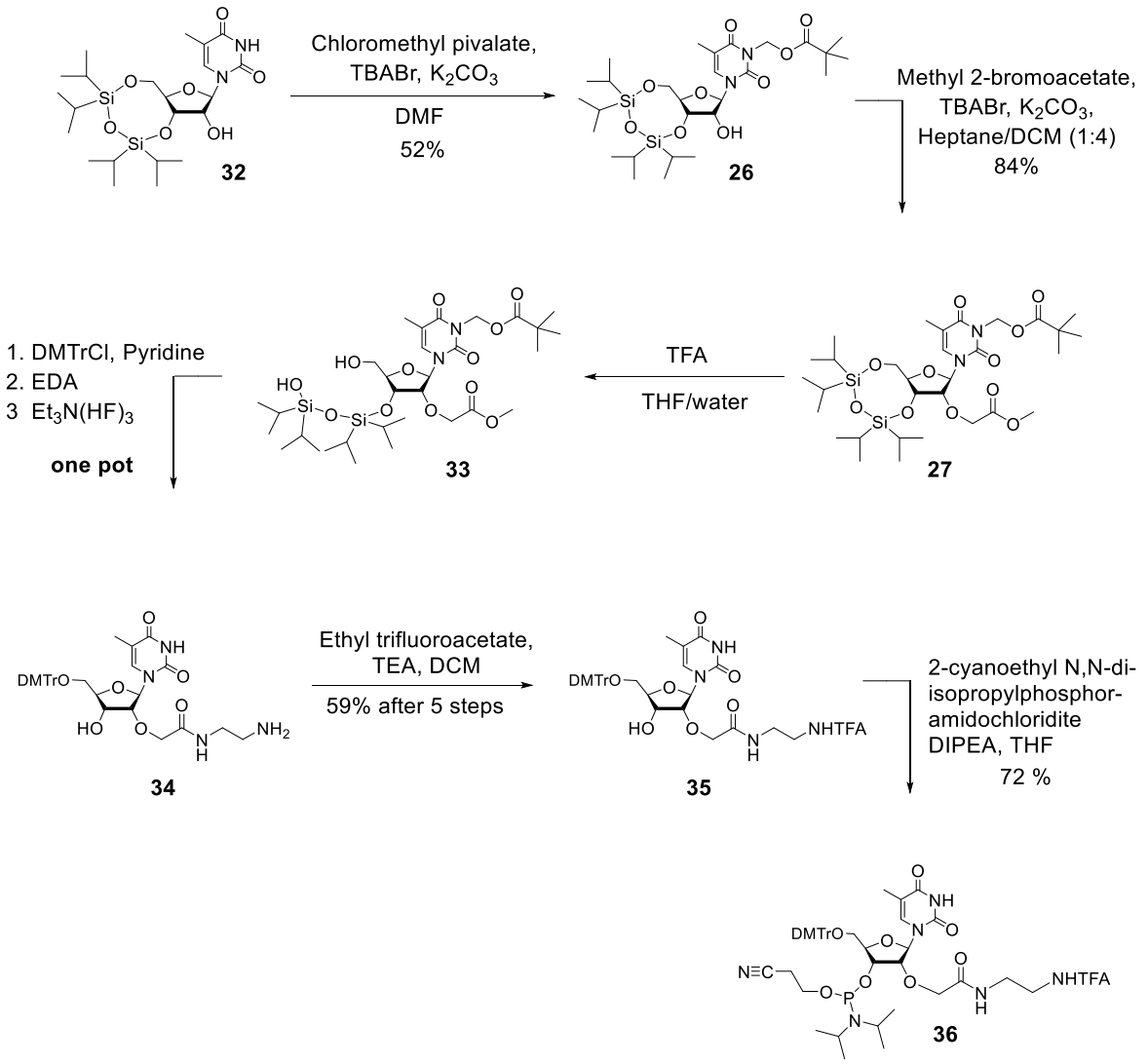


Figure 19. The newly developed synthetic route for the AECM-MeU building block.

Although we have decided to use the reported PTC approach for the analogous N^3-alkylation, the conditions for this step were further revised in order to achieve a more preferred outcome in terms of reagents, solvents and crude yield.

The study was started by screening different solvents (DMF, toluene, MeCN, THF, heptane, pyridine), bases (K_2CO_3, $KHCO_3$, Na_2CO_3, $NaHCO_3$) and equivalents of chloromethyl pivalate. Eventually, 2 equiv. of chloromethyl pivalate, 4 equiv. of K_2CO_3 and 0.2 equiv. of TBABr in DMF for the synthesis of compound **26** were chosen. HPLC analysis of the reaction progress revealed that using the above-mentioned conditions, the amount of side products significantly increases after 70-75% conversion. The possibility to decrease the amounts of both the solvent and chloromethyl pivalate was considered attractive enough and above-mentioned conditions were chosen for larger scale synthesis. The attempts to completely avoid chromatography in this step however were unsuccessful.

The final yield of the separated product **26** was 32.2 g (57.6%), starting from 50 g of compound **32**.

The synthesis of AECM-MeU was continued with 2′-alkylation of intermediate **26**. PTC was also used for this reaction step. Several solvents (heptane, MeCN, toluene, DCM, DMF), bases (K_2CO_3, K_3PO_4, NaOH) and phase transferring agents (Bu_4NBr (TBABr), Oct_4NBr, $MeNBu_3Cl$, $MeNOct_3Cl$) were screened. The combination of DCM/heptane mixture, 4 equiv. of K_2CO_3 and 0.05 equiv. of TBABr proved to be the most promising candidates for PTC reaction at milligram scales. Nonetheless, the scaling-up of PTC reactions can sometimes become challenging as the grinding effect of the magnet is more pronounced in smaller scale and fresh particle surface is made continuously available to a larger extent. As 2′-alkylation under PTC conditions was not complete after stirring at ambient temperature for over 66 h, additional amounts of K_2CO_3, TBABr and methyl bromoacetate facilitated the reaction to go to completion. After an acetonitrile-water (1:1 *v/v*) wash, the crude product was chromatographed to give 28.89 g (84.3%) of compound **27** as a colorless oil. Then, the 5′-position of compound **27** was selectively opened using trifluoracetic acid in THF:water (5:1 v/v) mixture. The retained silyl protecting group on the 3′-OH position reduced the risk of lactonization (2′-*O*-metoxyacetyl) and therefore was more advantageous for selective protection of the 5′-OH as well as subsequent steps in terms of purity and yield. The crude product **33** was subsequently treated with 4,4′-dimethoxytrityl chloride at ambient temperature for 2.5 h. The reaction was then quenched with methanol followed by addition of ethylenediamine. After stirring overnight at 60 °C, TEA:$(HF)_3$ was added to remove the remaining silyl groups. Then the crude product was *N*-trifluoroacylated using ethyl trifluoroacetate and purified by chromatography twice to afford 18.78 g of compound **35** (59 % after 6 steps starting from **27**, 95% in NMR assay). Compound **35** (5.1 g) was further phosphitylated at the 3′-position with 2-cyanoethyl *N*,*N*-diisopropyl-phosphoramidochloridite in the presence of DIPEA in THF to yield the AECM-MeU phosphoramidite (4.7 g, 72.5%).

3.2.3 Synthesis of AECM-MeC

The complete steps for the AECM-MeC monomer synthesis are shown in Figure 20. The procedure for the unmethylated analogue, AECM-C monomer was published earlier[116] and a similar synthetic pathway for AECM-MeC was considered as starting point. However, some additional changes were made before the upscaling of the procedure.

The synthesis of AECM-MeC was started with the protection of the 3′- and 5′-hydroxy groups on a ribose fragment. 3′,5′-*O*-[(1,1,3,3-tetraisopropyl-1,3-disiloxanediyl)]methyl-cytidine (**37**) was prepared using a previously published procedure[148] at 100 g scale. 50 grams of the obtained 3′,5′-protected compound **37** were used for the next step. A direct alkylation of 2′-position was problematic due to very poor solubility of compound **37**. It was however found that if the exocyclic amino group was converted into an amidine,[149] forming **38,** the solubility increased substantially. Next, PTC for 2′-alkylation of AECM-MeC monomer was employed. The optimization of this step was also started by screening different base-solvent combinations. The choice for the PTC agent (TBABr) remained the same as for AECM-MeU 2′-alkylation.

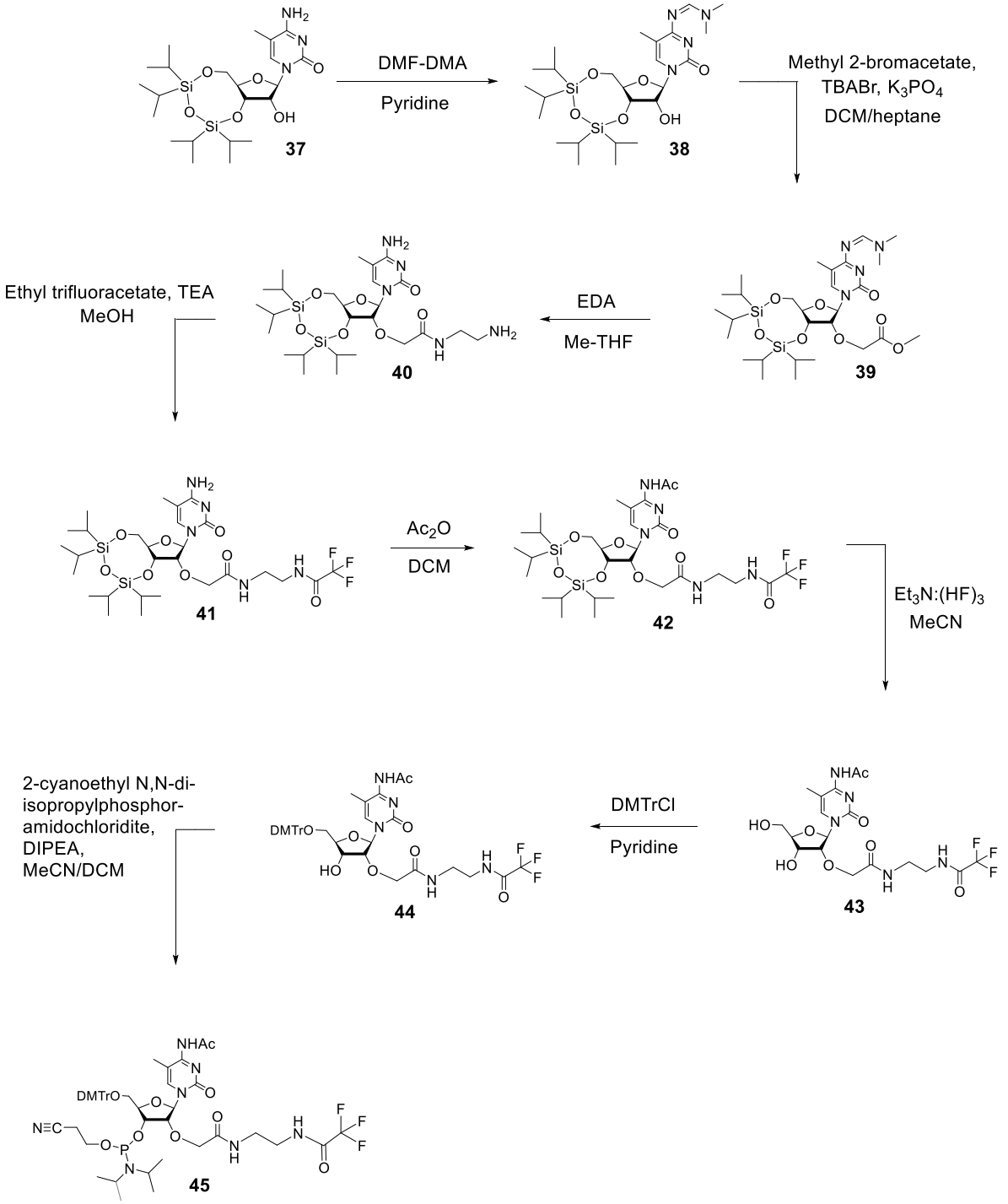


Figure 20. Synthetic route for the AECM-MeC building block.

The synthetic study for this building block was again started by screening K_2CO_3 and K_3PO_4 as potential bases and DCM, DMF, heptane, MeCN, toluene, iPrAc, MEK as potential solvents. The combination of K_3PO_4 in DCM/heptane mix proved to be the best choice. Despite that the temperature was increased to 40 °C in order to speed up the reaction, it still took 52.5 h for the reaction go to the completion as the grinding effect of the magnet was reduced at larger scale (58.6 g of starting compound **38** was used). The supplementation with additional reagents was also required. After the aqueous workup, the

crude alkylated compound **39** (74.2 g) was treated with ethylenediamine in methyl-THF to remove the exocyclic amine protecting group and to give the aminolysis product **40** (59 g). The excess ethylenediamine was removed by two sequential aq. NH_4Cl washes, this also transformed **40** into its HCl salt. The crude intermediate **40** was then reacted with ethyl trifluoroacetate and triethylamine in methanol to give compound **41** (74.2 g), followed by acetylation of the exocyclic amino group to give compound **42** (66.6 g). This step, however, required more attention than was initially anticipated from the earlier examples. We observed that 20% of bis-acetylated product was formed after following the analogous procedure.[116] To avoid bis-acetylation, the reaction was performed in the absence of base.[150] Instead of performing a selective opening at the 5′-postion using TFA/water, the crude product **42** was treated with triethylamine trihydrofluoride in acetonitrile to give crystalline compound **43** (17 g), thus greatly simplifying the overall route. Compund **43** was subsequently converted to 5′-*O*-4,4′-dimethoxytrityl derivative **44**, which was then purified via flash chromatography and re-slurried in heptane to give 20.1 g of the intermediate as a white powder. Tritylated compound **44** (5.0 g) was then phosphitylated at the 3′-position and purified by column chromatography to give the final AECM-MeC phosphoramidite product **45** (4.0 g, 62%).

4 CONCLUSIONS AND FUTURE PERSPECTIVES

This thesis aimed to broaden the scope of oligonucleotide multiorthogonal-conjugation techniques (chapter I) as well as to develop convenient methods to prepare 2′-*O*-AECM modified 5-methyl uridine and 5-methyl cytidine in larger scale (chapter II).

The study described in chapter I resulted in the preparation of three new orthogonal linkers. Two of these linkers were utilized for the synthesis of ON conjugates. The newly developed linkers are compatible with automated ON synthesis and can be prepared from a common and readily available scaffold. The newly developed methodology is suitable for both phosphate and phosphorothioate backbones. The incorporation of different linkers allows for attachment of different types of entities/modalities. In addition, series of stability experiments were performed to evaluate the potential of BCN as a handle for the preparation of ON conjugates. Some conditions have shown promising results, and more experiments are underway to find optimized conditions for the use of BCN. The prospect of introducing a BCN handle in a similar manner to the terminal alkyne and amino functionalities would expand the orthogonality perspectives and would open the possibilities for simultaneously attaching additional entities/modalities to ON constructs.

The study described in chapter II aimed to develop a process for preparation of AECM modification-bearing methyl-uridine and methyl-cytidine monomers in larger (up to 100 g) scales, which would be ready for ON synthesis. The objectives for fewer chromatographic purification steps by employing optimized work-up strategies and/or sequential one-pot reactions were met for both monomers. Several steps in the synthetic route for obtaining AECM-MeU were improved. First, PTC conditions employed for 2′-alkylation enabled the possibility to replace expensive reagents with cheaper and more sustainable alternatives. Second, the selective opening of 5′- position of intermediate **27** allowed for a one-pot synthesis (3 steps) resulting in four steps in total without the need for any chromatographic purifications.

Synthesis of AECM-MeC was also improved significantly. The introduction of an amidine protecting group improved the solubility of compound **39**. PTC conditions for 2′-alkylation improved the synthesis in a similar manner as for AECM-MeU since the use of an expensive BTPP base was omitted in this new procedure. In conclusion, 5′-methylated AECM-modified uridine and cytidine were reported for the first time. The possibility to prepare these AECM-modified monomers in larger amounts allows for the incorporation of these modifications into ON sequence designs for future experiments.

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