Synthetic Studies Towards Oligonucleotide Derivatives and Conjugates

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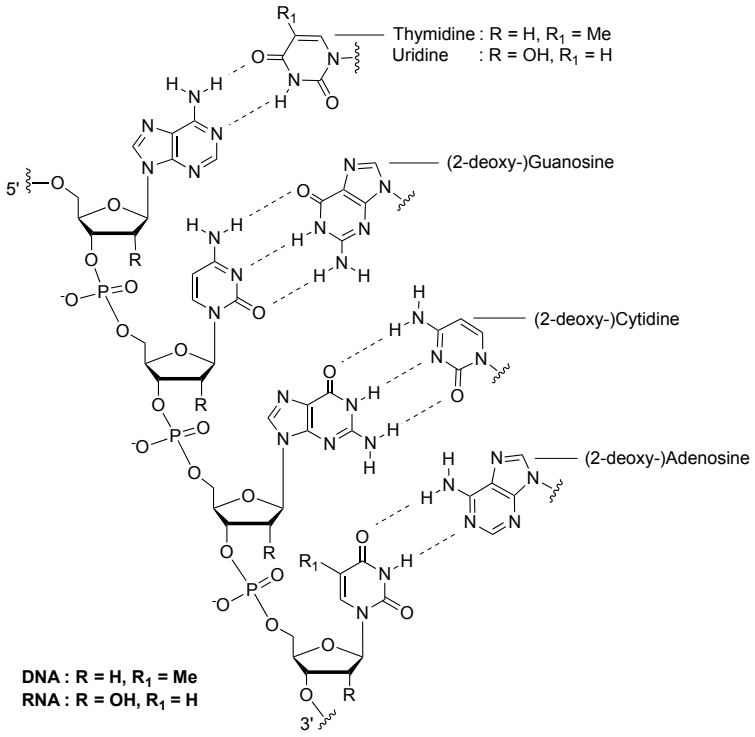
1 General introduction

1.1 Introduction

Nucleic acids form one of nature's three major types of biopolymers. Two structurally similar classes of nucleic acids, DNA and RNA, have distinct functions important for all forms of life. DNA, the carrier of all hereditary information, was first isolated by Miescher in 1871 from nuclei of white blood cells.[1] RNA was discovered in the early 1900s.[2] and proved to have a number of functional roles, that is, the messenger of genetic information (mRNA), a part of the protein synthesis machinery (tRNA and rRNA) and, as recently discovered, a regulatory element (siRNA, microRNA). Over time, the importance of nucleic acids has become evident and new insights on nucleic acid function and involvement in cellular processes are emerging at present.

Figure 1.1: DNA and RNA primary structure (left strand). Through Watson and Crick hydrogen bonding a secondary structure can be obtained (DNA and RNA hetero or homo duplexes).



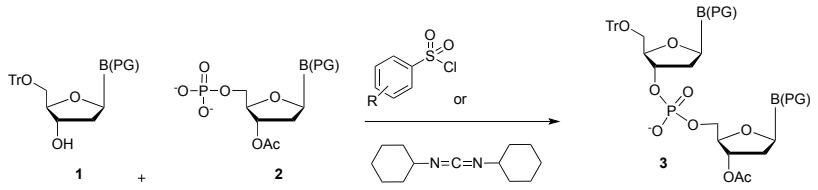
In contrast to the numerous distinct carbohydrate building blocks occurring in polysaccharides and the 21 amino acids of proteins, both DNA and RNA polymers are built up from only four different building blocks. These are connected by a phosphodiester bond and form a linear strand as depicted in figure 1.1. However, it is the fascinating property of both the DNA and RNA oligonucleotides to form pairs by hydrogen bonding creating distinct secondary structures. As has been discovered by Watson and Crick[3] in a DNA-duplex dA opposes T and dC base pairs with dG. The nucleic acid duplex is essential for DNA replication and furthermore it secures the genome integrity, as damage on one strand can be repaired guided by the opposing strand.[4] RNA polymers are able to form base pairs in the same fashion (A – U and C – G) however RNA secondary structure is generally more complex[5] aiding its larger functional diversity compared to DNA. Additionally, as depicted in figure 1.1, DNA and RNA are also complementary to each other, this is especially important for the transfer of DNA's information to the messenger RNA.

Much research towards the development of chemical methods for synthesis of oligonucleotides has been driven by the importance of nucleic acids. Throughout the past decades several of such methods have been successfully applied to synthesis of both DNA and RNA fragments. The availability of such well defined fragments have led to major breakthroughs in our understanding of nucleic acid biology. For instance with the aid of synthetic oligonucleotides the genetic code was resolved.[6,7] Additionally the function of the Lac-operon was clarified and the chemical synthesis of the gene encoding for insulin allowed its expression in bacteria and subsequent isolation of the resultant protein could be used for treatment in diabetics.[8] Currently our understanding and the use of (oligo)nucleic acids is still emerging which is greatly aided by the availability of oligonucleic acids from synthetic source.
The first part of this chapter describes the main synthetic strategies towards the synthesis of oligonucleotides including modern solid phase synthesis techniques with an emphasis on the construction of the phosphodiester bond. The second part gives an overview of chemistry that gives access to oligonucleotides functionalized with other (bio)molecular entities for use as research tools focusing on the recently developed "reagent-free" approaches.

1.2 Synthetic strategies towards oligonucleotides: phosphodiester bond formation

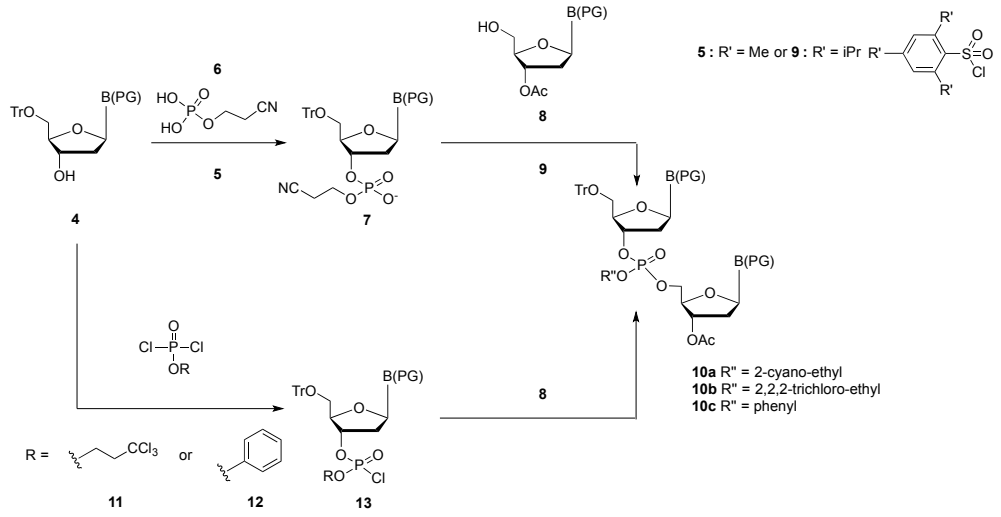
The first synthesis of a dinucleotide was achieved by Michelson and Todd[9], who reported the reaction of a 5'-protected thymidine 3'-benzyl phosphochloridate with the 5'-hydroxyl of the second thymidine nucleoside yielding benzyl protected phosphotriester. Major advances towards the synthesis of longer nucleic acid fragments was accomplished by Khorana and co-workers.[10] As depicted in scheme 1.2 condensing agents, such as DCC or tosyl chloride, react with nucleobase protected phosphomonoester (**2**) yielding the corresponding activated intermediates.

Scheme 1.2: Phosphodiester approach towards oligonucleic acids.



Subsequently, these reactive intermediates react with the free hydroxyl of a partially protected nucleoside (**1**), leading to dinucleotide phosphodiester (**3**). This method is commonly known as the phosphodiester method. Although successfully applied to the synthesis of several oligonucleotides, this pioneering work suffered from arduous anion-exchange purification procedures and low yields, especially when block couplings were used.[11] This loss in yield could be prevented by protection of the phosphodiester to an uncharged phosphotriester. The isolation of the latter is much more practical and yields are generally better. This approach, known as phosphotriester method is depicted in scheme 1.3.

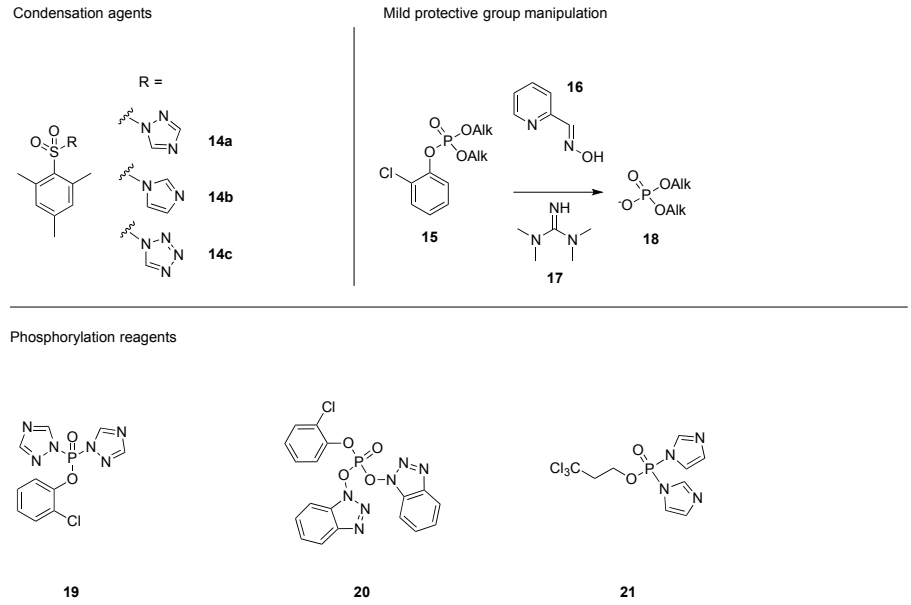
Scheme 1.3: Outline of the phosphotriester method.



For instance, phosphodiester **6** can be activated using sulfonyl chloride **5** to form a mixed anhydride, which was then reacted with from 5'-*O*-trityl protected **4** obtaining 2-cyanoethyl phosphodiester **7** as described by Letsinger.[12,13] In a similar arylsulfonyl chloride mediated coupling, **7** was coupled to nucleoside **8** using **9** as the activating agent obtaining protected **10a**. Eckstein and Rizk[14,15] made use of trichloroethyl dichloridate (**11**) to obtain **13,** which was in situ converted to fully protected dinucleotide **10b**. In an analogous approach Reese[16] used the more reactive phenyl dichloridate (**12**) of which the resulting protected phosphotriester **10c** was deprotected by aqueous sodium hydroxide. Compared to the phosphodiester method, this strategy significantly improves yields and allows the purification of the intermediate protected oligonucleotides by normal phase silica gel chromatography. Because of these advantages, phosphotriester approach was extensively used and over the years many modifications of phosphorylation procedures, coupling agents and protective

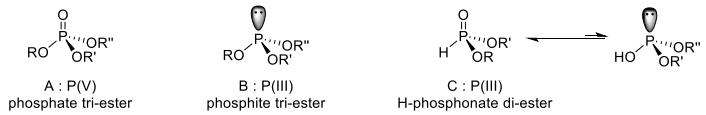
groups have been reported. Scheme 1.4 lists some of the alterations made to the phosphotriester method. For instance, arylsulfonyl azoles were found to be more efficient coupling agents in the phosphotriester bond formation (**14a**[17], **b**[18], **c**[19]). In addition, the use of oximes in combination with organic base (**15** → **18**) for the deprotection of 2-chlorophenyl protective groups was found to increase the yield of oligonucleotides by reducing the concomitant unwanted cleavage of the internucleotide phosphate linkages.[20] Finally, several new phosphorylating agents were introduced (**19** – **21**). As depicted in scheme 1.3, so far two consecutive steps had to be applied in the synthesis of the phosphotriester linked oligonucleotides however reagents **19**[21] and **20**[22] allow for a one pot two step strategy. Additionally, **21**[23] was coupled at the 3' of a nucleoside followed by coupling with *p*-chlorophenol obtaining an orthogonal protected 3' phosphotriester. Subsequent elongation at the 5' end, followed by selective removal of the cyanoethyl protective group allowed block couplings in the synthesis of longer oligonucleotide fragments.

Scheme 1.4: Various improvements on the phosphotriester method.



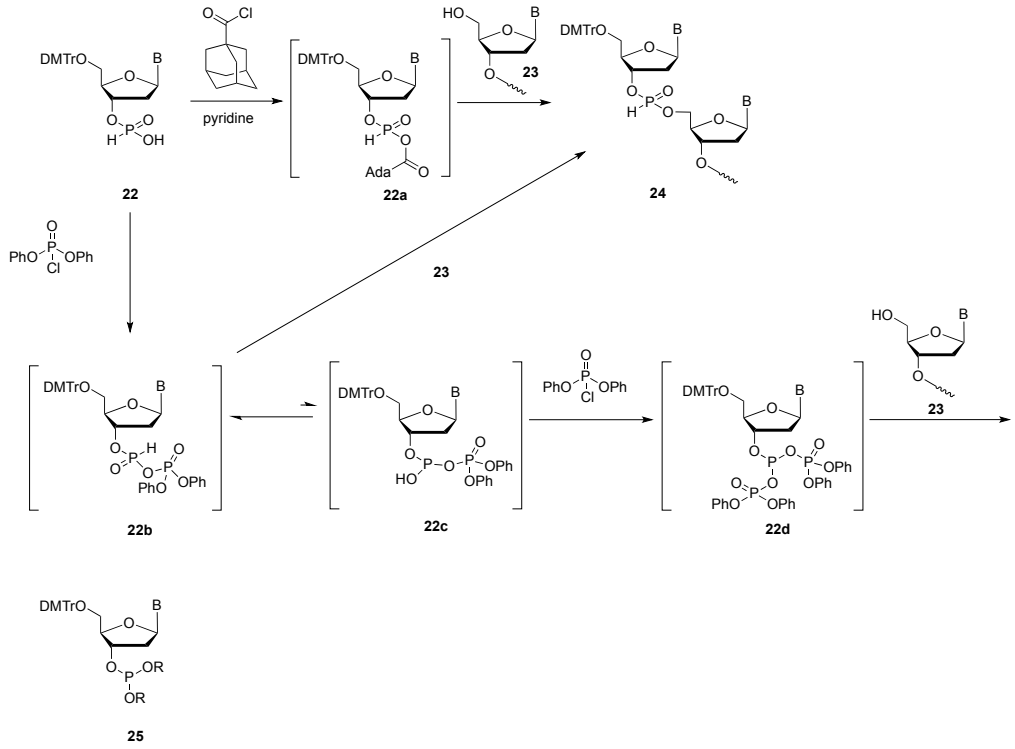
Guided by the success of the solid phase oligopeptide synthesis a lot of effort was taken to transfer established solution phase oligonucleotide synthesis procedures to a solid phase protocol. In this framework the H-phosphonate, originally introduced by the group of Todd[24], was re-examined for application on solid phase[25]. In contrast to the phosphodi- and tri-ester method, H-phosphonate chemistry uses the tetravalent P(III) species depicted in Figure 1.2 (C). Such P(III) species are more reactive than the P(V) (A) intermediates used in the phosphodi- and tri-ester method thus making fast condensation rates possible.

Figure 1.2: Commonly used phosphorous species in oligonucleotide synthesis.



The synthesis of a dinucleotide using H-phosphonate methodology is shown in Scheme 1.4. Activation of H-phosphonate ester **22** with a suitable activating agent, usually a bulky acid chloride, forms reactive intermediate **22a** that is subsequently attacked by the hydroxyl group of partially protected nucleoside **23** resulting in H-phosphonate diester **24**. The relatively stable H-phosphonate diester **24** withstands the subsequent coupling cycles and can be readily converted into phosphodiester in the final oxidation step. Besides the superior phosphorylation kinetics, another advantage of the H-phosphonate approach is the ease of converting the H-phosphonate diesters into modified phosphate diesters such as, phosphorthioates, phosphoramidates and C-phosphonates.[26,27]

Scheme 1.4 : Phosphodiester synthesis by means of H-phosphonate chemistry.

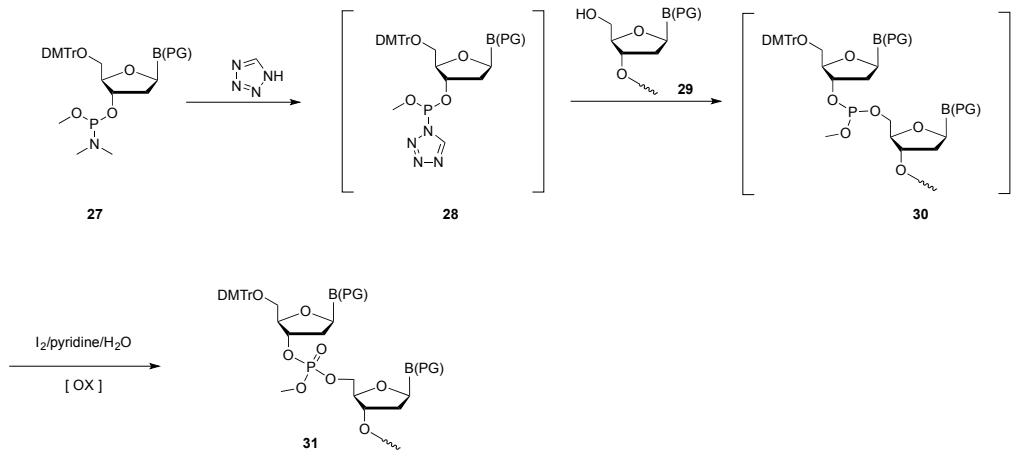


The high reactivity of H-phosphonates in condensation reactions with alcohols is likely to be a result of the inability of the hydrogen substituent in for instance **22b** to donate electrons to the electrophilic phosphorus. This is in contrast to phenoxy substituents of activated species in

phosphotriester method where the oxygen donates the unshared electrons into the vacant orbitals of the phosphorous and makes it less electrophilic.[27] The H-phosphonate method is versatile but, unfortunately, it is also prone to side reactions. For instance, as depicted in Scheme 1.4, anhydride **22b** is in equilibrium with its trivalent tautomer **22c**. This phosphite diester-like species can undergo a second phosphorylation with diphenylphosphoryl chloride, leading to bifunctional **22d**. Upon alcoholysis with **23** a phosphite triester is obtained in contrast to the target H-phosphonate diester **24**.[28]

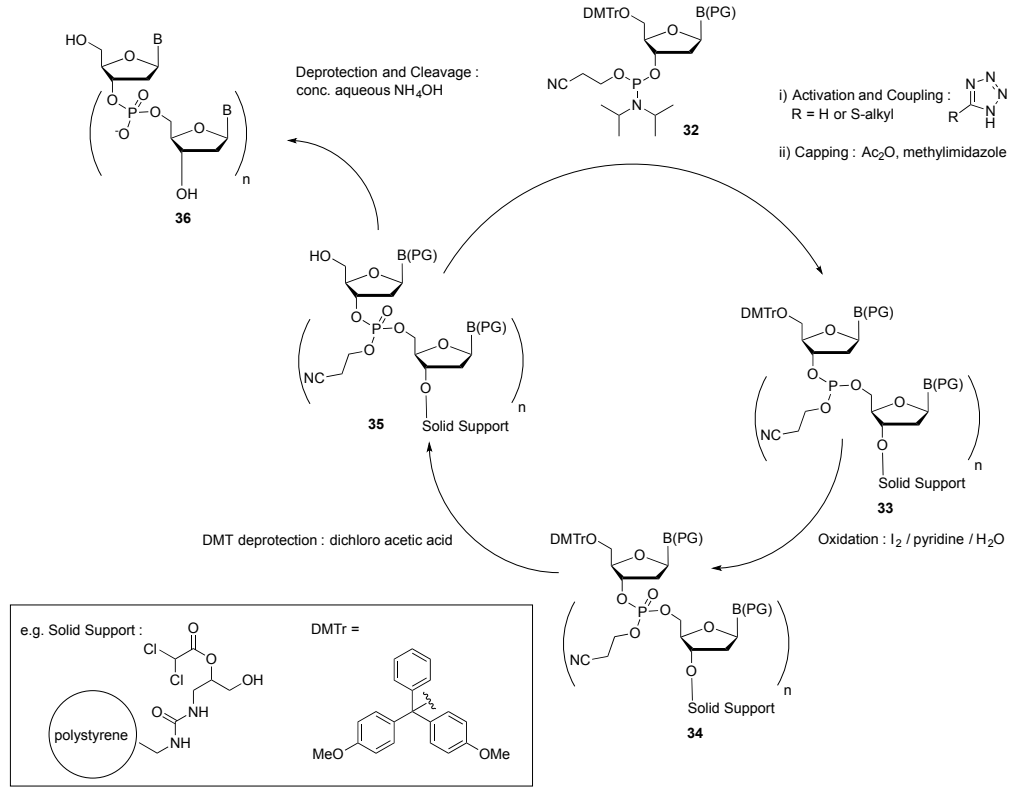
A breakthrough in the field of P(III) chemistry was brought about by Beaucage and Caruthers[29,30]. They introduced phosphoramidites in combination with azoles as activators in oligonucleotide synthesis. As illustrated in Scheme 1.5 upon addition of at least 2 equivalents of the activator tetrazole, a reactive tetrazolide **28** is formed as intermediate. The formation of **28** is believed to be initiated by protonation of the basic P(III) compound at the phosphorus followed by a rearrangement and proton transfer to the nitrogen atom of the phosphoramidite. The exact mechanism of this step (**27**→ **28**) is, however, still subject of debate.[31] Substitution of the tetrazole by nucleophilic attack of the added alcohol yields phosphite intermediate **30**. As mentioned earlier, phosphites are fairly unstable and have to be immediately oxidized to obtain a stable phosphotriester (**31**).

Scheme 1.5 : Representation of the phosphoramidite approach based on initial work by Beaucage and Caruthers.



The initial work[29,30] instigated a rapid development of the phosphoramidite method leading to adaptations and improvements that culminated in a general approach for the synthesis of oligonucleotides on the solid support.[32] Current protocols of oligonucleotide synthesis benefit from, readily available shelf stable phosphoramidite building blocks, mild coupling conditions without loss of reactivity, high coupling efficiency, usually exceeding 99% in 5 – 15 minute coupling cycles (DNA or RNA) and paucity of side reactions. These features have made the phosphoramidite approach (Scheme 1.6) the method of choice for the preparation of synthetic nucleic acid fragments.

Scheme 1.6 : Phosphoramidite based solid phase oligonucleotide synthesis.



Phosphoramidite based automated solid phase oligonucleotide synthesis usually starts from a preloaded resin (**34**, n = 0), of which the 5'-hydroxyl is first deprotected using a 2-3% solution of dichloroacetic acid an apolar solvent. The immobilized nucleoside **35** is subsequently coupled to the second nucleoside by flushing the resin with a solution containing phosphoramidite **32** and a weakly acidic azole activator (*e.g.* dicyanoimidazole, tetrazole or a thioalkyl derivatized tetrazole (pKa's 5.2, 4.9 and 4.3)). Next, a capping step is performed to avoid chain elongation on any uncoupled residues minimizing the formation of unwanted sequences of the length comparable to that of the desired oligonucleotide. The formed phosphite triester **33** is then oxidized using I_2/pyridine/H_2O yielding stable phosphotriester **34**. Acid treatment deprotects the 5'-hydroxyl (**35**, n = 1) and subsequent elongation can be performed. This cycle is repeated until the desired oligonucleic acid sequence is obtained. At this point the protected, immobilized, oligonucleotide is treated with aqueous concentrated (25%) ammonia leading to cleavage of the oligonucleotide from the solid support and the removal of the phosphate and nucleobase protective groups yielding final product **36**.

Essential for the success of the phosphoramidite approach to oligonucleic acids is the elegant protective group strategy applied. The use of the acid labile 4,4'-dimethoxytrityl to protect the 5'-hydroxyl group, introduced by Khorana,[33] is stable enough under the acid promoted coupling of the phosphoramidites yet labile enough to be cleaved using 2% dichloroacetic acid. Additionally, the cyanoethyl[34] protection of the phosphates, nucleobase acyl protective

groups and linkage to the solid support are perfectly stable under the mild basic capping and oxidation steps, but are effectively cleaved in the final step of the synthesis using aqueous ammonia.

For a successful synthesis of oligoribonucleotides protection of the 2'-hydroxyl is necessary. To this end the *tert*-butyldimethylsilyl (TBDMS) group is commonly used and seems most suitable given its stability under both acidic and basic conditions applied and the mild fluoride mediated cleavage conditions. Stability of TBDMS-group is important to avoid base-induced cleavage of the phosphodiester bond under the basic deprotection conditions, used to unmask the phosphodiesters and the exocyclic amino groups of the nucleobases. The steric bulk of the TBDMS protective group however demands in comparison with the synthesis of DNA fragments elongated coupling cycles and requires the use of more acidic activating agents. In order to improve the coupling efficiency other protective groups have been reported such as the triisopropylsilyloxymethyl[35] (TOM) group and other base or acid labile protective groups.[36] However these are only applied to a limited extend.

1.2 Oligonucleotide conjugates: applications and synthetic methodologies

1.2.1 Examples of synthetic oligonucleotide conjugates

The availability of oligonucleotides from synthetic source has stimulated the development of new diagnostics, research tools and therapeutics. In the search toward a new class of therapeutics directed to nucleic acids instead of proteins, strategies known as antigene, antisense and RNA interference (RNAi) are pursued.[37]
In the antigene approach oligonucleotides target the DNA duplex forming triple helices thereby obstructing gene translation to specific mRNAs.[38] The antisense[39] strategy makes use of small DNA fragments, which form a DNA-RNA duplex with the mRNA of interest. DNA-RNA hetero duplexes are substrates for RNAse H, which degrades the mRNA to prevent translation in a specific protein. As the latter, RNA interference is a naturally abundant phenomenon involved in endogenous regulation of gene expression.[40,41] When applied in therapeutics the RNAi approach makes use of small dsRNA's from synthetic source. These small dsRNA's (siRNA) form a complex known as RNA Induced Silencing Complex (RISC) with a protein of the Argonaute family. The fate of the bound target mRNA to the RISC is dependent on the Argonaut protein family member and varies. Of therapeutic interest is mainly the mammalian AGO2 protein, capable of degrading the target RNA (*slicing*) thereby inhibiting protein synthesis.[42]
Although up to now only few drugs based on oligonucleotide have entered clinic, the potential of nucleic acids as therapeutics is enormous. The endogenous origin of the DNA and RNA make these relatively safe in terms of toxicity while the target specificity of nucleic acid is high. On the other hand several shortcomings must be overcome. Examples of these problems are; the process of enzymatic degradation, which significantly lowers the half-life and thus effectiveness of oligonucleotide drugs and the fact cells poorly take up the negatively charged oligonucleotides. By the development of several strategies, including nanoparticles such as liposomes[43], the poor cellular uptake of oligonucleotides is currently being addressed by the design and synthesis of conjugates with molecules that promote uptake. For instance, conjugates of nucleic acids and amphiphilic oligopeptides, rich in arginine and lysine residues enhance uptake.[44,45,46] To take advantage of cell surface receptors, conjugates with for instance anandimide,[47] cholesterol[48] and other lipophilic entities[46] have been investigated and it has

been found that these enhance the uptake and improve the gene-silencing activity of the oligonucleotide based drug leads. Furthermore carbohydrates[49] such as galactose[50] proved to be useful as olignucleotide transporters.

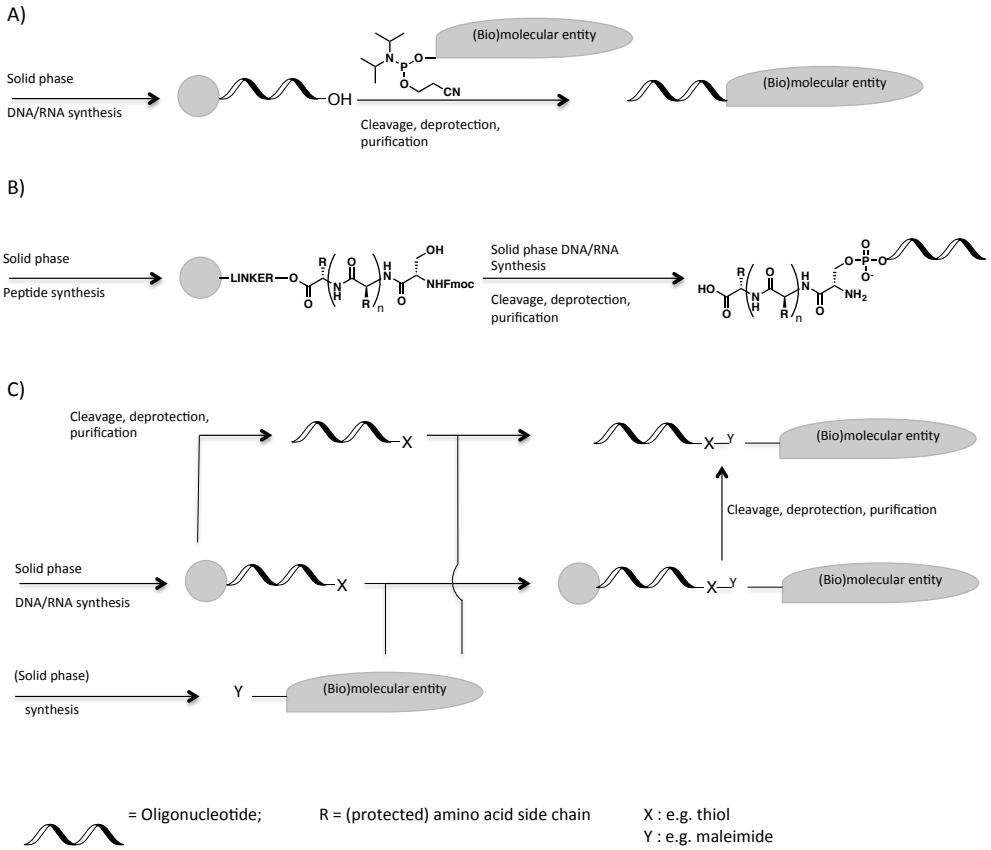
Another potentially useful oligonucleotide based therapeutic is so called CpG DNA. Bacterial genomic material is known to contain unmethylated CpG dinucleotides which are recognized by the Toll-like receptor 9 (TLR-9) of dendritic cells stimulating the immune system.[51] The immunostimulatory properties of such nucleic acid fragments makes them suitable as adjuvants in vaccinations.[52] For instance, antigenic oligopeptides connected to a specific CpG containing oligonucleotide with phosphorothioate backbone have been demonstrated to induce dendritic cell activation, maturation and presentation of the peptide antigens.[53] Conjugates of this type might thereby be interesting tools to improve our understanding of the immune system and eventually lead to synthetic vaccines.

Oligonucleotides as diagnostic tools are commonly found as molecular beacons and as probes in fluorescent *in-situ* hybridization (FISH). Both molecular beacons and FISH-probes are fluorescently labeled oligonucleotides which are used to detect specific mRNA's or genes. FISH is applied in genome analysis by fixation of chromosomes during metaphase. Oligonucleotides, fluorescently labeled and complementary to a gene of interest is then used in a hybridization assay using fluorescent microscopy allowing the detection of specific genes and mutations thereof in various diseases.[54] Molecular beacons consist of a hairpin forming oligonucleotide containing a fluorescent label and a quencher at the opposite terminus. Unbound to the target and in the hairpin conformation the molecular beacon exhibits virtually no fluorescence due to the close proximity of the fluorophore and the quencher. However upon binding to the complementary target the quencher and fluorescent label are separated and fluorescence is observed. The applications of molecular beacons are many and extend from diagnostics to monitoring and quantifying the polymerase chain reaction, in the latter case the fluorescence is the measure of amplification.[55]

1.2.2 Synthetic strategies and methodologies in oligonucleotide conjugate synthesis

As described in the previous section the use and versatility of synthetic oligonucleotides as therapeutics, diagnostics and research tools are greatly aided by the connection of these nucleic acid fragments to other molecular entities that range from small fluorescent labels or monosaccharides to larger oligopeptides. For the construction of such conjugates different strategies have been pursued which can be subdivided in three general classes (Scheme 1.7). The linear approach (**A**) entails incorporation of the modifications of interest as phosphoramidite building blocks. This strategy is attractive for small molecules such as cholesterol or fluorescent labels, which can be converted into a phosphoramidite.

Scheme 1.7: Approaches towards oligonucleic acid conjugates: A) linear approach using small phosphoramidite building blocks. B) sequential linear approach combining different solid phase synthesis techniques C) convergent approach in which each molecular entity is separately synthesized followed by 1 conjugation reaction.

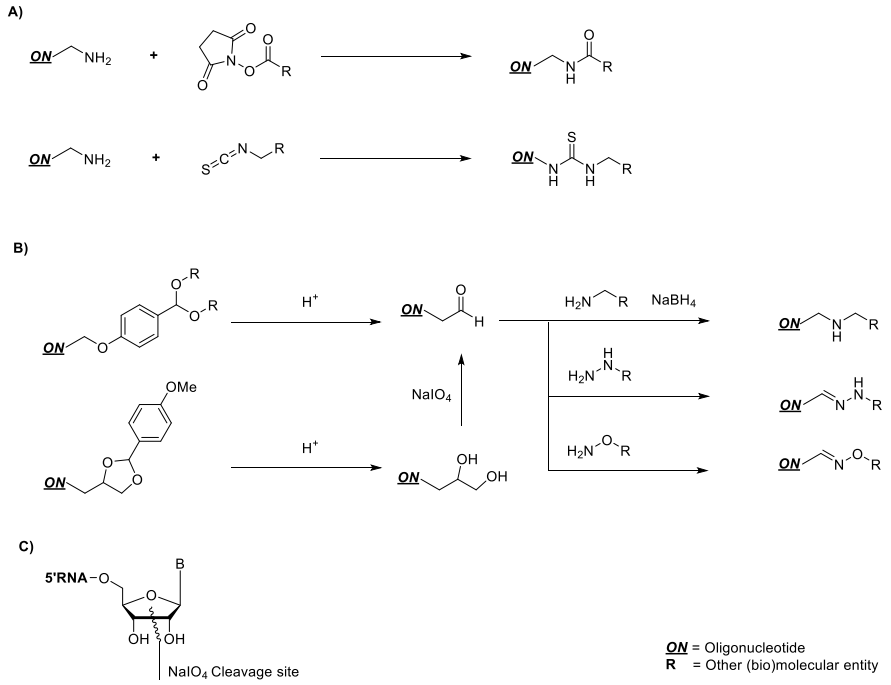


The second approach (**B**) combines stepwise synthesis of a peptide and an oligonucleotide sequentially on the same solid support. In the example depicted in Scheme 1.7 the peptide is synthesized first followed by the assembly of oligopeptide chain on the same solid support using the serine side chain. The order in which the oligonucleotide and the peptide syntheses are performed can also be reversed. Although, recent reviews[56,57] discuss some successful applications of this strategy to obtain oligonucleotide-peptide hybrids, there are limitations. For instance, the acid mediated deprotection of the peptide side-chain residues using TFA is not compatible with nucleic acids. Additionally, the basic treatment neccesary to cleave the Fmoc protective groups of the peptide might also cleave some of the base labile protected nucleic acid residues prior to the completion of the synthesis leading to side reactions.

Hence, to obtain large and more complex constructs the third, convergent approach is perhaps most suitable. This pathway (**C**) makes use of attaching conjugation handles (scheme 1.7, **X** and **Y**) on two separately synthesized and purified molecules. These functional groups are chosen to react in an orthogonal fashion, selectively coupling the two entities together. For

example (**A** in scheme 1.8) an 5'-amine functionalized oligonucleotide (ON) is reacted with the activated *O*-succinyl ester of another biomolecule (R).
Such "post-synthetic" conjugation (**C**, scheme 1.7) can be carried out in solution or alternatively the conjugation reaction might be carried out on the nucleic acid solid support. The latter requires stability of the conjugated entity towards concentrated ammonia and in case of oligoribonucleic acids to a fluoride treatment.
The convergent approach is superior when large and structurally diverse conjugates are desired. However, smaller molecular entities might also be conjugated in this fashion. Chemistry does not have to be adjusted for the different parts the conjugate is build up from and final conjugation generally is the only step to be optimized. This has led to the development of a large variety of conjugation reactions suitable for this approach with an even larger variety of molecules as conjugation partners such as oligopeptides, oligosaccharides, fluorescent labels, lipids, large molecular weight polyethylene glycol and transition metal complexes. The majority of the studies on the nucleic acid conjugates has been covered by excellent review articles.[56,58–61]

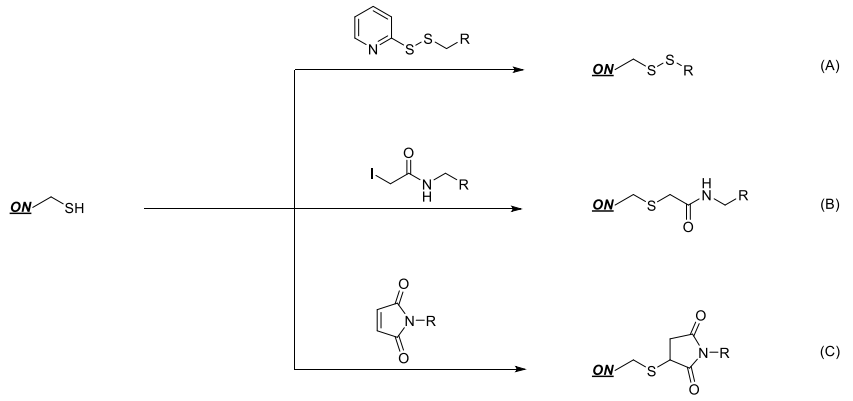
Scheme 1.8: Examples of oligonucleotide conjugation reactions.



For instance, a primary amine appended to an oligonucleotide (scheme 1.8, **A**) is coupled with an activated molecular entity such as a succinyl ester or isothiocyanate yielding amides or thioureas, respectively. Unfortunately these methods might suffer from competing hydrolysis of the activated carbonyls in the often, aqueous conjugation solutions. Entry 1.8 **B** shows the use of oligonucleotide aldehydes. These can be obtained by incorporating an acetal protected benzaldehyde followed by mild acidic treatment or 1,2-diols which can be cleaved by sodium periodate to provide aldehydes. Additionally, when RNA conjugates are desired, the 3'-

terminal residue (1.8 **C**) can also be used to form aldehydes when treated with $NaIO_4$. These aldehydes can then form secondary amines by the addition of a primary amine and sodium borohydride. Alternatively, avoiding the necessity of added sodium borohydride the aldehydes can be reacted with hydrazides or oxy-amines forming hydrazones and oximates respectively.

Scheme 1.9: Oligonucleotide conjugates from thiol functionalized oligonucleotides.

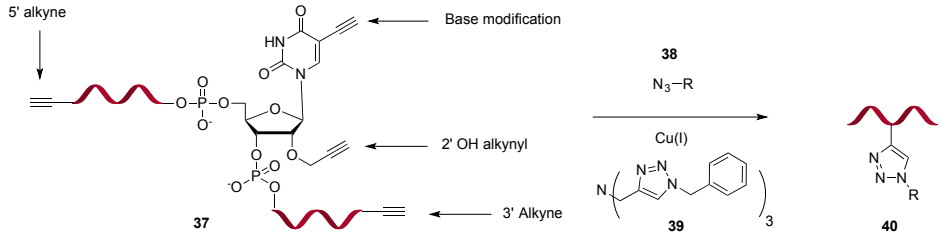


ON = Oligonucleotide
R = Other (bio)molecular entity

Other attractive methods use oligonucleotides functionalized with a thiol (Scheme 1.9). Both 3'- and 5'- thiols can easily be introduced and are reactive towards activated disulfides (A) yielding disulfides. Iodoacetamides (B) and maleimides (C) are also used in thiol conjugation reactions both yielding thioethers. Reactions are generally fast and selective and the thiols on oligonucleotides are fairly stable towards oxidation.

To maximize the efficiency of the conjugation between oligonucleotides and other molecules, both containing a variety of functional groups, much research has been carried out towards conjugation reactions in which the reacting functional groups are (generally) absent in bio-molecules. For instance the Diels-Alder cycloaddition has been successfully applied by using 1,3-diene functionalized oligonucleotides and maleimide containing oligopeptides.[62] Another example is the application of the copper catalyzed[63,64] [2+3] dipolar cycloaddition of azides to alkynes, also referred to as the click reaction. Both the azide and alkyne are unnatural and have almost no reactivity towards biomolecules. The reaction under the influence of the copper catalyst is fast, virtually quantitative and the resulting triazole moiety is small and stable. As depicted in scheme 1.10, the alkyne moiety is most often incorporated into oligonucleotides due to its compatibility with phosphoramidite chemistry however the azide moiety can also be introduced on the oligonucleotide. The alkyne moiety can either be introduced at the termini or at different positions in oligonucleotide sequence such as the nucleobase[65,66,] and the 2'-OH of the ribose sugar[67].

Scheme 1.10: Application of Cu(I) catalyzed alkyne azide [2+3] dipolar cycloadditions in DNA conjugation reactions.

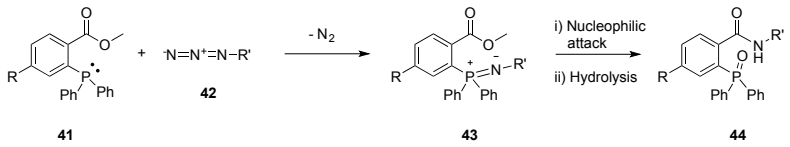


The beneficial characteristics of the copper catalyzed click reaction make this chemistry useful for the synthesis of oligonucleotide conjugates.[68,69,70] It is important to note, however, that copper can degrade the DNA sugar phosphate backbone and even more so that of RNA. Additionally, the Cu(I) catalyst is subject to oxidation. Both side reactions can be circumvented when a stabilizing ligand[71] (Scheme 1.10) is used and exposure of the reaction to air is avoided. Finally, the toxicity of the copper demands a thorough purification prior to the use of the conjugates *in vivo*. Nonetheless, the copper catalyzed click reaction is perhaps the most powerful and widely used conjugation method currently at hand and therefore novel methods to improve the application of click chemistry to nucleic acid conjugates are constantly being developed.[72,73]

1.3 Copper free click chemistry: exploring novel conjugation methodologies

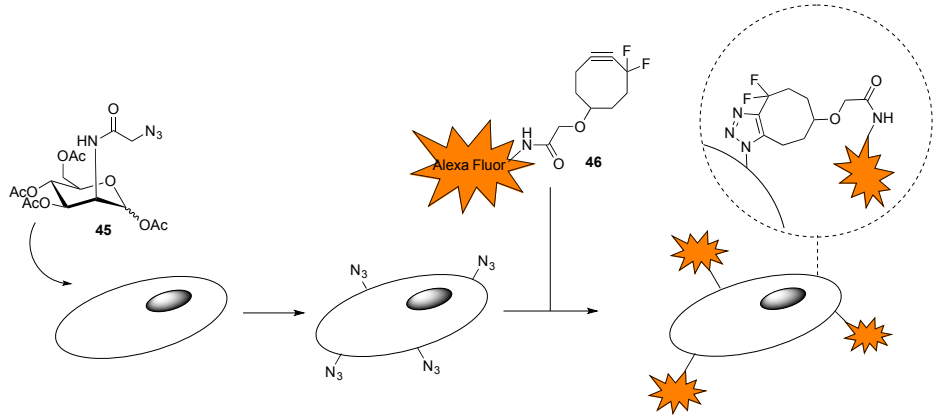
In the emerging field of chemical biology, chemical tools for the connection of two molecular entities *in vivo, ex vivo* or in complex biological samples such as cell lysates are essential. The chemistry used for this purpose has to be bio-orthogonal and sufficiently reactive due to the dilute conditions of working in biological matrices. To this end, the previously mentioned "click" reaction has been widely used in cell lysates. However due to its toxicity, the addition of a copper catalyst for the reaction to proceed at acceptable reaction rates has been of limited value for use *ex* and *in vivo*. A more successful alternative approach towards the coupling of two molecular entities in live systems was published by Bertozzi and Saxon[74] where they employed Staudinger ligation for the labeling of cell surface glycans. As depicted in Scheme 1.11 triphenylphosphine derivative **41** containing a label (i.e R = biotin) at the *para* position and an *o*-methyl ester is reacted with an azide functionalized carbohydrate residue (**42**, R = 2-deoxy-2-azidoacetyl-galactose). The phosphine serves as the reducing agent forming an intermediate aza-ylide **43**. The negatively charged nitrogen attacks the methyl ester and upon hydrolysis an amide bond is formed yielding **44**.

Scheme 1.11: Bertozzi's Staudinger ligation.



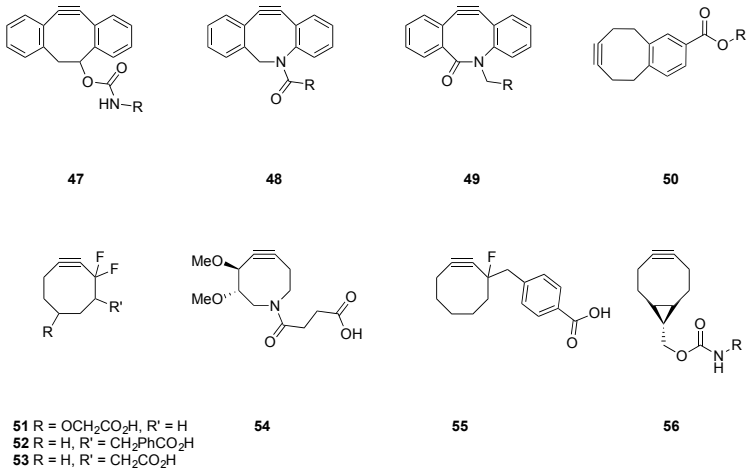
A major leap forward in the development of bio-orthogonal reactions was the introduction of the strain promoted alkyne azide [3+2] dipolar cycloaddition.[75] In this reaction a cyclooctyne and azide react rapidly forming a triazole without the need for a copper catalyst. Cyclooctynes posses ring strain due to a distortion imposed on the "native" 180° bond angle of the alkyne, this strain is relieved upon reaction of the alkyne with a 1,3-dipole such as an azide generating an alkene. To further enhance the reactivity; the alkyne was polarized by the introduction of two fluorines adjacent to the alkyne.[76] Cells were treated with azide labeled carbohydrates to be incorporated in larger oligosaccharides anchored in the cell's membrane. Next, an Alexa-fluor fluorescent dye or biotin, functionalized with a difluorocyclooctyne were added to the cells thereby successfully labeling the cell surface glycans by means of strain promoted alkyne-azide [2+3] dipolar Huisgen cycloaddition that is now commonly referred to as the *"copper-free click reaction"*.

Figure 1.11 : Strain promoted or copper-free click labeling of cell surface glycans.



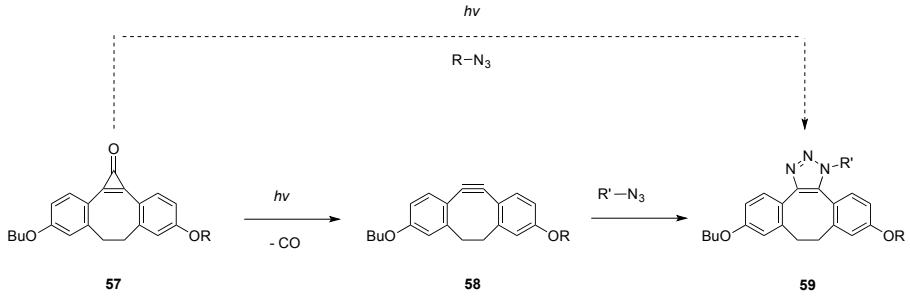
The success of this approach has led to the rapid development of other strained cyclooctynes focusing mainly on increased reactivity and lowered hydrophobicity. To this end difluorocyclooctynes with different substitution patterns (**51**[76], **52**, **53**[77]) and mono fluorinated variants have been synthesized (**55**)[78]. Other means of increasing reactivity was achieved by the synthesis of cyclooctynes **47** – **50**. In these di- and monobenzocyclooctynes, the sp^2 orbitals adjacent to the alkyne flatten the cyclooctyne structure thereby leading to increased reactivity.[79,80,81,82] Compound **49** is the most reactive of this series due to the additional carbonyl in the 8-membered ring.

Figure 1.12: Strained cyclooctynes developed for application in copper-free click ligations.



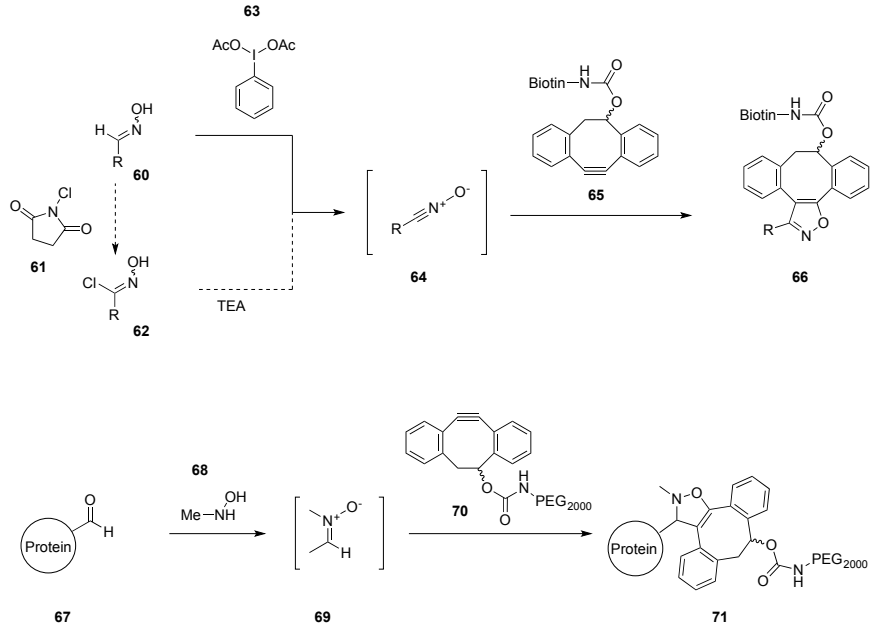
However, the aromatic substituents lead to increased lipophilicity, an undesirable property when working in an aqueous environment. In order to reduce the lipophilicity of the cyclooctyne ligation handle aza-dimethoxycyclooctyne **54**[83] was introduced, which is significantly less lipophilic but unfortunately also less reactive than the difluorinated- and dibenzocyclooctynes.[84] Recently the group of Rutjes and van Delft have developed bicyclononyne **56**.[85] This moiety has attractive characteristics as the reactivity is high and hydrophobicity is relatively low. Additionally, the compound is easily accessible from cheap cycloocta-1,4-diene in a limited number of steps. Another interesting derivative was published by Boons *et al*[86]; they demonstrated that in a similar experiment as described in scheme 1.11 but by using cyclopropene **57** (Scheme 1.13) and *in situ* UV radiation, cyclooctyne **58** is formed which subsequently reacted with azides on cell surface. Control experiments carried out in the absence of light did not lead to labeling. This photo-generated cyclooctyne can therefore be used in dual labeling strategies[87] making this entity an useful addition to the "tool-box" of the bio-orthogonal chemistry.

Scheme 1.13 : UV-light induced release of strained cyclooctynes in copper-free click chemistry (R = biotin, R' = *N*-azido-cetylmannosamine).



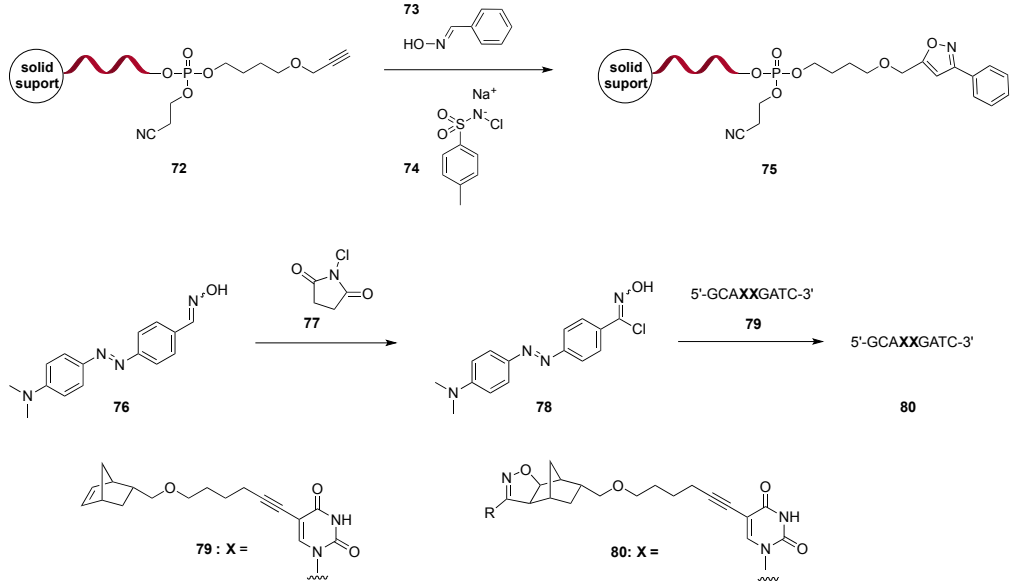
The reaction of nitrile oxides and nitrones as 1,3-dipoles with cyclooctynes have also been investigated and have been found to be superior in terms of the reaction rates to that of azides. The reactive dipoles are commonly generated *in situ*, for instance by oxidation of oximes (**60**) using hypervalent iodine species (**63**), basic elimination of alpha-chloro oximes (**62**) or in the case of nitrones (**68**) from methyl hydroxylamine and an aldehyde (Scheme 1.14). This makes it a less appealing strategy for application *in vivo* compared to the azide based strain promoted cycloadditions. Nonetheless, nitrones have successfully been applied for the labeling of proteins[88] while nitrile oxides were used in the synthesis of carbohydrate conjugates[89] (**66,** R = lactose, using **63** to form intermediate **64**).

Scheme 1.14 : Nitrile oxides and nitrones in strain promoted cycloadditions.



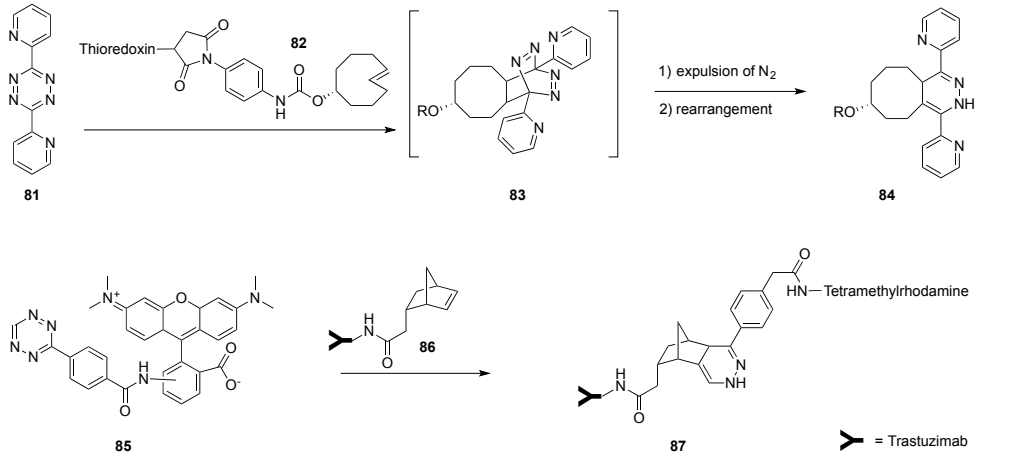
In addition to the reactivity towards cyclooctynes, 1,3-dipoles also rapidly react with norbornene and terminal alkynes. In their independent studies Heaney[90] and Carell[91] have shown the applicability of the nitrile oxide [2+3] dipolar cycloadditions in the synthesis of nucleic acid conjugates applying oligonucleotide **72** that contained 5'-terminal alkyne and norbornene derivatized thymidine residues **79** respectively (Scheme 1.15). Although representative examples are depicted, both studies demonstrate the use of this methodology in solution and on the solid support.

Scheme 1.15: Application of *in situ* generated nitrile oxides in [2+3] dipolar cycloadditions on oligonucleic acids.



Another major advance in the field of bio-orthogonal labeling strategies was made with the adoption of the inverse electron-demand Diels-Alder reaction as another biocompatible transformation. The reactivity of a variety of electron-rich dienophiles towards electron-poor tetrazines for the use as a bio-orthogonal labeling strategy was recently introduced by Fox *et al*.[92] The cycloaddition of tetrazine **81** (scheme 1.16) with *trans*-cyclooctene **82** proved to be exceptionally fast; well exceeding those observed in the strain promoted alkyne-to-azide cycloaddition for example.

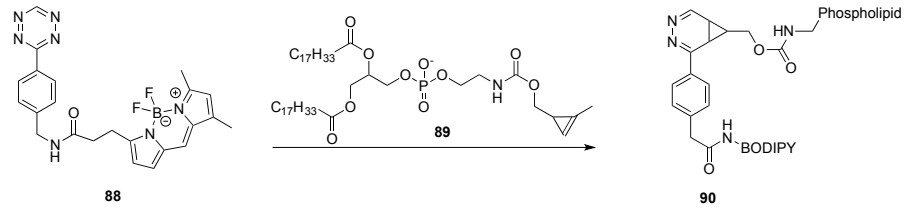
Scheme 1.16: Inversed electron demand Diels-Alder reactions with tetrazine dienes.



The formed tricyclic intermediate rapidly expels N_2 followed by a rearrangement towards the product **84**. In addition, Weissleder and Hildebrand also elaborated on the inverse demand Diels-Alder reaction with tetrazines.[93,94] They have reported the use of norbornene derivatives (such as **86**) as electron rich alkenes, which also undergo inverse electron demand Diels-Alder reactions. The latter approach benefits from the commercial availability of norbornene starting materials whereas the synthesis of *trans*-cyclooctenes requires specialized photo-isomerization procedures.[95]

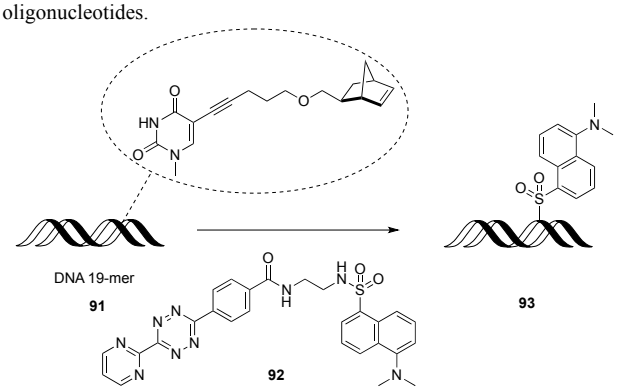
In addition, cyclopropenes have also been reported to undergo rapid inverse-electron demand Diels-Alder reaction with tetrazines. In a recent example the cyclopropene entity was connected to phospholipids and added to human breast cancer cells followed by the addition of a tetrazine functionalized BODIPY-fluorophore allowing live cell phospholipid labeling and visualization (scheme 1.17, **89**).[96] Cyclopropenes as bio-orthogonal conjugation handles are attractive because of the small size compared to the bulkier norbornene and *trans*-cyclooctene and synthetic accessibility.

Scheme 1.17: cyclopropenes as ligation handles in the inverse electron demand Diels-Alder reaction with tetrazines.



The speed of the inverse electron-demand Diels-Alder reaction makes this an attractive strategy both for bio-orthogonal labeling in complex biological matrices and for the synthesis of bioconjugates "in test tube". A recent example of the latter chemistry is depicted in Scheme 1.18. Thus, norbornene modified thymine was incorporated 1, 2 or 3 times in DNA 19-mer duplexes and reacted with tetrazine functionalized dansyl or biotin labels (scheme 1.18).[97]

Scheme 1.18: representative example of inverse electron demand Diels-Alder approach towards labeled oligonucleotides.



It must be noted however that tetrazines are less stabile than other ligation handles and the synthesis towards these heterocycles can be laborious in comparison with, for instance, the the introduction of an azide.

Summary

The importance of nucleic acids, as they fulfill a plethora of functions in every form of life, is well established. This had led to the development of different types of chemistry to obtain the well-defined synthetic fragments of these biopolymers. At present, the phosphoramidite based automated solid phase synthesis is widely used for this purpose and allows the synthesis of nucleic acid fragments up to a hundred nucleotides in length. In addition, the development of therapeutics and diagnostics is greatly aided by the availability of synthetic oligonucleotide conjugates with added functionality. As discussed, fluorescently or biotin labeled oligonucleotides can be used in diagnostics and nucleic acid fragments conjugated to oligopeptide, carbohydrate and fatty acid have found their application in the field of therapeutics. To accomplish this, chemical methods have been developed based on amide, oxime and thio-ether bond formation. More recently the copper catalyzed [2+3] dipolar cycloaddition was used for this purpose. While most of these methods have proven their applicability, the field of chemical biology has recently expanded the bio-orthogonal chemistry toolbox offering additional attractive conjugation reactions. Amongst these are the strain promoted cycloaddition of cyclooctynes to azides and the inverse electron-demand Diels-Alder reaction of tetrazines with electron rich dienophiles, such as norbornene. Some of these reactions such as the inverse electron-demand Diels-Alder reaction and nitrile oxide based [2+3] dipolar cycloaddition have already been applied in the field of oligonucleotide conjugation. However, other examples of these new bio-orthogonal reactions yet need to be evaluated as candidates for their use in nucleic acid chemistry. For example, the strain induced alkyne-to-azide [2+3] dipolar cycloaddition might also prove to be a useful method provided that the conjugation handles (e.g. cyclooctynes) are stable under solid phase oligonucleotide synthesis conditions

Aim and Outline of this Thesis

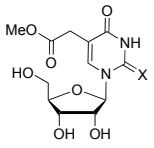
The work described in this Thesis primarily focuses on the application of the strain-promoted alkyne-azide [2+3] Huisgen dipolar cycloaddition in the synthesis of oligonucleotide conjugates.

Chapter 2 describes the automated oligonucleotide synthesis of short (5-mer) DNA and intermediate (16-mer) RNA fragments functionalized at the 5'-end with the dibenzocyclooctyne (scheme 1.12, **47**) described by Boons *et al*.[79] This was accomplished by the design and synthesis of a phosphoramidite derivative. The functionalized oligonucleotides were subsequently conjugated with an azide functionalized amphiphilic oligopeptide, a zwitterionic tetra-saccharide and a derivative of BODIPY fluorescent dye.

Chapter 3 describes the exploration of the general applicability of the copper-free click reaction in nucleic acid conjugation. Two cyclooctyne containing building blocks for 3'-end functionalization and a building block for in-strand modification are synthesized and incorporated in short DNA fragments (5- or 6-mers) at the 3'-end. Furthermore, the compatibility of cyclooctynes with Beaucage's sulphurisation reagent to obtain functionalized phosphorthioate nucleic acid analogues is explored.

With the aim of obtaining antibodies against two naturally occurring tRNA modifications (Figure 1.19) **Chapter 4** demonstrates the application of the methodology, described in chapter 2 and 3, to conjugation of hyper modified mono- and ribooligonucleotides with bovine serum albumine.

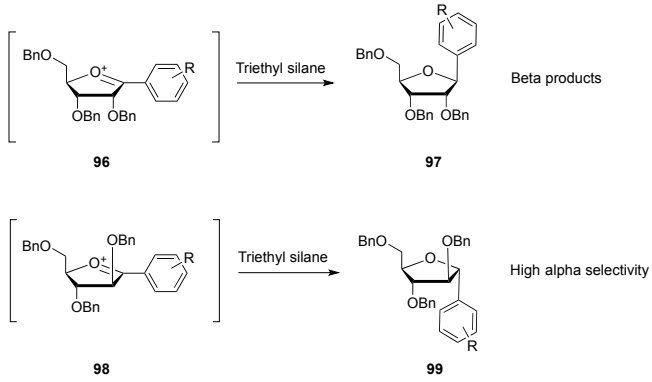
Figure 1.19: two naturally abundant tRNA modifications.



94 : X = O, mcm^5U
95 : X = S, mcm^5S^2U

In an effort to obtain the naturally occurring RNA modification pseudouridine by a method commonly used for other *C*-nucleosides the mechanistic details of the involved reduction were investigated (scheme 1.20). **Chapter 5** describes a study on the elucidation of the stereochemical outcome of this reaction by the synthesis of a variety of ribose and arabinose *C*-glycosides and support these findings with quantum mechanical calculations.

Scheme 1.20: Reduction of ribose and arabinose hemiketals, selectivity in *C*-nucleoside synthesis.



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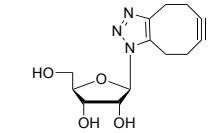
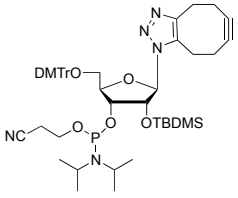
Chapter 6 reports on the design and synthesis of a cyclooctyne based nucleotide analogue. The synthesis of a ribosyltriazole annulated cyclooctyne building block (RITRICO, **100**, figure 1.21), its conversion into a phosphoramidite (**101**) and incorporation into an RNA 13-mer is described. Cycloadditions of a 3-azido-7-hydroxy-coumarine derivative to the RITRICO-modified single-stranded RNA and RNA-DNA heteroduplex were performed yielding fluorescently labeled RNA oligomers and duplexes respectively.

Figure 1.21.



Ribose-Triaza-Cyclooctyne RITRICO

100



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