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Studies on phosphate ester cleavage and development of oligonucleotide based artificial nucleases (OBAN's)

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ABSTRACT

Five different Oligonucleotide Based Artificial Nuclease (OBAN) systems have been synthesized. OBAN's may be regarded as a development of traditional antisense methodology where inhibition of gene expression can be achieved by hybridizing a synthetic oligonucleotide to natural mRNA and thus inhibiting further translation into protein products. The OBAN's have a hydrolytic transesterification agent covalently attached via a linker to the oligonucleotide scaffold. In contrast to antisense methodology cleavage of substrate mRNA can then be obtained without the assistance of cellular enzymes like RNase H. Efficient synthetic methodologies for the synthesis of the OBAN's are presented. A highly convergent approach was used for the synthesis of the OBAN's where the catalytic neocuproine unit was introduced in aqueous buffer to the fully deprotected oligonucleotide. Isolated yields for the conjugation step were as high as 80% after purification on RP-HPLC. The substrates for the five Zn (II) dependent 11 or 12-mer OBANs were RNA sequences that upon association with the OBAN's formed bulged RNA structures. By changing the number of nucleosides in the bulged out region, structures having bulges varying from 0-5 nucleosides in the bulge were obtained. Degradation of these structures were studied to allow direct comparisons between different append points, directionalities and length of the linkers. The proximity factor is one of the most important design factors in construction of an efficient system. This was shown both in comparisons of the different OBAN's and in the preference for certain bulge sizes for each OBAN. The top-performing OBAN, called OBAN 1 in this thesis, was shown to degrade a 4-nt bulge in a catalytic fashion. Cleavage was also demonstrated at Zn (II) concentrations that are present in human serum.

The acidity of the secondary hydroxyls of ATP, deoxy ATP and three 2' modified analogues of ATP was determined in aqueous buffers. In addition, the pKa values for secondary hydroxyls of adenosine, 2'- and 3'-*O*-methyl adenosine in water, methanol and DMSO were determined. The results suggested that a hydrogen bond between the 2'-hydroxyl and a 3'-oxyanion, if present, is virtually energy neutral. These studies provide data useful in mechanistic studies of phosphate esters and for design of catalysts for cleavage of phosphate esters.

Phosphate ester cleavage was also studied in RNA model compounds. Several Furanosides 5-(diphenylphosphate)'s, including a 2-deoxy-2-aminoderivative, were synthesized and the transesterification where the oxygen displaces a phenyl group was studied in presence of several different metal ions.

LIST OF PUBLICATIONS

- I. **H. Åström** and R. Strömberg "A Method for Synthesis of an Artificial Ribonuclease" *Nucleosides, Nucleotides and Nucleic Acids*, 2001, 20(4-7), 1385-1381

- II. **H. Åström**, N. H. Williams and R. Strömberg "Oligonucleotide Based Artificial Nuclease (OBAN) Systems. Bulge Size Dependence and Positioning of Catalytic Group in Cleavage of RNA-Bulges" *Organic and Biomolecular Chemistry*, 2003, 1, 1461-1465

- III. **H. Åström** and R. Strömberg "Oligonucleotide based artificial nuclease (OBAN) systems, Part 2. Synthesis of New OBAN's and Further Studies on Positioning of Catalytic Group". *Accepted for publication in Organic and Biomolecular Chemistry*, 2004, DOI: 10.1039/B403652B, (*In Press*)

- IV. **H. Åström**, E. Limén and R. Strömberg "The Acidity of Secondary Hydroxyls in ATP and Adenosine Analogues and the Question of a 2',3' Hydrogen Bond in Ribonucleosides". *Submitted to Journal of the American Chemical Society*, 2004

- V. E. Limén, **H. Åström** and R. Strömberg "Metal Ion Promoted Intramolecular Phosphate Transesterification. Enhancement of Hydroxyl Nucleophilicity in Furanoside 5-Diphenylphosphate Derivatives". *In Manuscript*

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LIST OF ABBREVIATIONS

A	Adenosine
ATP	Adenosine triphosphate
C	Cytidine
DNA	Deoxy ribonucleic acid
G	Guanosine
HEPES	N-2-Hydroxy ethyl piperazine N'-2-ethane sulphonic acid
HIV	Human imuno deficiency virus
hnRNA	Heterogenous nuclear RNA
HPLC	High performance liquid chromatography
Intron	Intervening RNA sequence
LNA	Locked nucleic acid
Me	Methyl
MMT	Mono methoxy trityl
mRNA	Messenger RNA
MS	Mass spectroscopy
NMR	Nuclear magnetic resonance
OBAN	Oligonucleotide based artificial nuclease
Ph	Phenyl
Piv-Cl	Pivaloyl chloride
PNA	Peptide nucleic acid
RNA	Ribonucleic acid
RNAi	RNA interference
RNase	Ribonuclease
rRNA	Ribosomal RNA
T	Thymidine
TBDMS	<i>tert</i> -Butyldimethylsilyl
Tf	Trifluoromethane sulfonyl
THP	Tetrahydropyran-2-yl
T _M	Thermal melting temperature
tRNA	Transfer RNA
U	Uridine

1 INTRODUCTION

1.1 GENERAL INTRODUCTION

Nucleic acids have a wide variety of functions in living cells. The two main types of nucleic acids are deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) they are both oligomeric polymers of nucleotides which in turn consists of a phosphate, a heterocyclic aromatic base and a sugar moiety. In the case of DNA the sugar is 2-deoxy-D-ribose and in the case of RNA, D-ribose. The heterocyclic bases in DNA are adenine, guanine, thymidine and cytosine. RNA shares the same bases with the

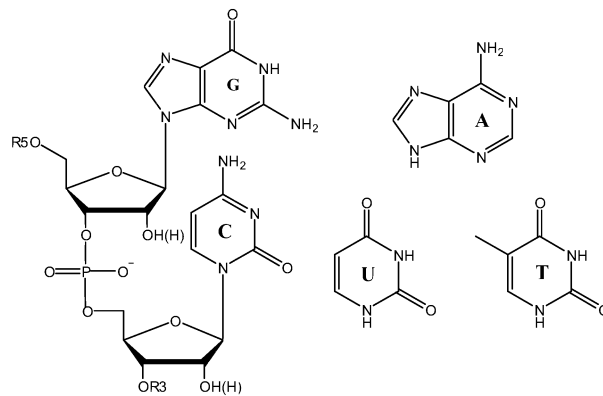


Figure 1: The structures of DNA and RNA. Nucleosides linked together via 3',5' phosphodiester bonds. The sugars are 2'-deoxy in the case of DNA and 2'-OH in the case of RNA.

exception that uracil replaces thymidine. The inter-nucleosidic phosphate function bridges the sugar moieties together with 3',5' phosphodiester bonds (Figure 1), rendering the oligomers poly-anionic. The charged nature of the back-bone of nucleic acids has many important implications, e.g. electrostatic protection against incoming nucleophiles and inhibition of diffusion out from the cell through the lipophilic cell membrane¹.

DNA in eukaryotes is located in the nucleus of the cell and stores the genetic information. One major function of RNA is to act as a link between the DNA and protein synthesis. The presence of a 2'-hydroxyl group makes RNA much more labile than DNA. The 2'-hydroxyl group readily performs an intramolecular attack on the adjacent phosphate and cleaves the chain under basic conditions. If the pH is instead acidic, the RNA is subject to migration of the phosphate function from the 3'-position to the 2'-hydroxyl group. The difference in reactivity between DNA and RNA is crucial both for keeping the integrity of the genetic information intact (DNA) and for degrading the RNA when its function e.g. of transferring information is accomplished.

The secondary structure of DNA is in most cases the familiar double helix defined by Watson and Crick², two anti-parallel strands coiled around a common axis and held together by hydrogen bonds, three bonds between G and C and two between A and T. A right-handed B-helix resembling a twisted ladder with 10 base-pairs per turn is formed. The hydrophilic phosphate backbone of the two strands is on the outside of the

duplex and the hydrophobic heterocyclic bases are located on the inside. Other types of DNA (e.g. Z-helix or A-helix) duplexes are known but they are less common. RNA in most cases exists either as single stranded or as a right handed A-form duplex. The structure of single-stranded RNA can however be rather complicated due for instance to internal base pairing creating various structural motifs including hairpins, bulges etc. Complex 3-D structures can be formed and these also involve hydrogen bonding with the 2'-hydroxyl functions.

The four letter alphabet defined by organizing the nucleotides of DNA in a specific sequence constitutes the complete map of genetic information needed for a specific organism. However, the information stored in DNA needs to be copied into RNA before any information may be utilized. The process of copying is called transcription and it makes use of one strand of the DNA double helix as a template on which a complementary RNA strand can be assembled. The transcribed RNA is called messenger RNA (mRNA) and after being synthesized, it is released from the nuclei of the cell into the cytoplasm where it in turn is used as a template for protein synthesis at the ribosomes. In this process called translation, segments of three nucleotides on the mRNA, codons, codes for specific amino acids which are assembled via amide bonds under the influence of tRNA, rRNA and enzymes to form proteins. The 20 common amino acids are combined in a fashion decided by the sequence of the mRNA to form proteins.

The primary transcript in eukaryotes is called the pre-mRNA or hnRNA (heterogenous nuclear RNA). This RNA, which is a linear copy of the gene to be expressed however goes through several maturation processes before it is translated into protein. In the case of eukaryotic mRNA the 5'-end of the mRNA is capped with 7-methyl guanosine attached backwards (5',5') through a triphosphate linkage. This modification facilitates the initiation of translation. In most cases, eukaryotic mRNA is poly adenylated at the 3'-end. A chain of 40-200 adenosines is added and this tag helps the mRNA to exit from the nucleus into the cytosol. In addition, the capping and poly adenylation serves to protect the RNA from degradation by 5'- and 3'-exonucleases. Another intriguing post-transcriptional event is the removal of introns. Introns or intervening sequences are stretches of transcribed DNA that do not code for a protein product. These fragments are post-transcriptionally spliced out before the mRNA is translated. The coding sequences, the exons, are ligated to form the mature mRNA. Eukaryotic RNA can contain up to 50 intervening sequences that will need to be removed before translation is carried out. In the beginning of the 1980's this process was found to take place without the need of protein catalysis³ which was a discovery of fundamental importance since proteins were believed to be necessary for biological catalysis. RNA molecules performing catalysis were named catalytic RNA or ribozymes. Furthermore, the fact that RNA both can store information and perform catalysis implies that RNA in an early phase of evolution may have existed without proteins and DNA in what has been named the "RNA world"⁴.

1.2 TRANSESTERIFICATION AND CLEAVAGE OF RNA IN BIOLOGICAL SYSTEMS

This thesis will be focused on reactions involving cleavage/transesterification of phosphate esters. It is then appropriate to shortly review some of the more common and for this thesis more relevant mechanisms by which phosphate esters in RNA are

processed in biological systems. The discussion will be focused on hydrolytic cleavage and no discussion regarding mechanisms working by oxidative degradation⁵ will be made. In addition, the effect of metal-ion catalysis in RNA cleavage will be discussed.

The presence of the 2'-OH has a large effect on the difference in stability of DNA compared to RNA. It has been estimated, based on studies done on model compounds, that RNA is cleaved 10⁹ times faster than DNA under hydroxide catalysis⁶. It is known that the 2'-OH accelerates the degradation by direct nucleophilic participation in the chemical step. It has also been suggested to stabilize the transition state in some reactions⁷. By studying the cleavage and transesterification of RNA at the level of RNA dimers or in RNA model compounds a lot of insight about the mechanistic pathways by which RNA is processed has been gathered. The pH rate profile for degradation of RNA dimers like UpU has a bell-shaped appearance with a minimum at pH ~5⁸. Cleavage is catalyzed both by specific acids and bases and to a lower extent by general acid and bases. Furthermore, isomerization of 3'-5' UpU to the 2'-5' isomer is more rapid than cleavage under neutral conditions⁹. The general results for UpU holds true also for oligomeric RNA although the susceptibility for acidic cleavage and isomerization is higher¹⁰. In fact, the sole presence of a terminal phosphate on ApAp accelerates the rate of reaction by a factor 2 compared to ApA¹¹. In addition, the rate of degradation of oligomeric RNA is clearly sequence dependent¹². In the cleavage step the 2'-OH performs an intramolecular attack on the adjacent phosphate ester. Figure 2 illustrates the mechanism of cleavage for UpU at pH >7¹³. The 2'-OH performs an intra

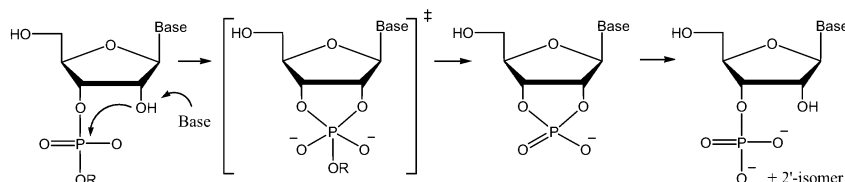


Figure 2: Based catalyzed cleavage of UpU.

molecular attack on the adjacent phosphate. The resulting phosphorane intermediate then collapses into a cyclic mono phosphate that subsequently is degraded to 2' and 3' mono phosphates¹³. If instead the attack of the internal 2'-OH is carried out when the phosphate is protonated the initially formed phosphorane may pseudorotate in agreement with Westheimers rules¹⁴ and in addition to the previous reaction also isomerize to 2'-5' UpU⁹. The structure of a penta co-ordinated phosphorane is usually that of a trigonal bi pyramid with two apical and three equatorial ligands. Displacement via a trigonal bi pyramid generally occur with the nucleophile and the leaving group in apical positions. Hence, the initial attack of the 2'-OH renders a phosphorane having the 2'-oxygen in an apical position. Furthermore, the 3'-oxygen must for energetic/steric reasons be in an equatorial position. Consequently, the phosphorane needs to pseudorotate in order to allow for the isomerization. Figure 3 shows acid catalyzed degradation and isomerization of UpU.

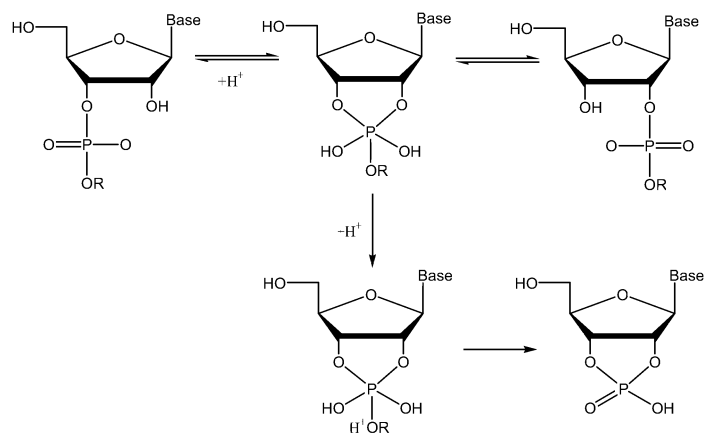


Figure 3: Acid catalyzed cleavage of UpU.

In addition, the degradation of RNA can be buffer catalyzed which is an even more mechanistically complex event¹⁵. The degradation of RNA by participation of the 2'-OH is also utilized by enzymes like RNase A¹⁶ and RNase T₁¹⁷ as well as Hammerhead ribozymes¹⁸. Enzymatic degradation of RNA by direct hydrolytic mechanisms is also possible, examples include: RNase H¹⁹, RNase V₁²⁰ and RNase III²¹. Interestingly, hydrolytic mechanisms are almost exclusively utilized for enzymatic degradation of double stranded RNA while transesterification based enzymes makes use of the 2'-OH for cleavage of single stranded RNA.

A slightly different mechanism is observed in large catalytic RNA's, including the group I introns²². Interestingly, the phosphate is now attacked by the 3'-OH of an external guanosine. The corresponding reaction does not occur in aqueous solution in case of non-ribozymic RNA but has been demonstrated when reactions on model

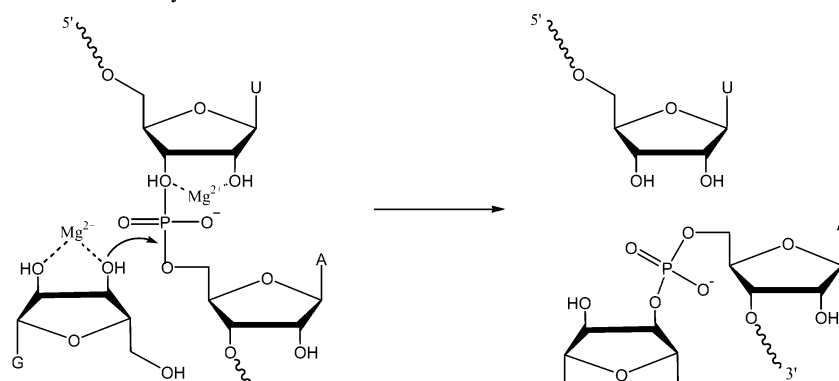


Figure 4: Schematic illustration of the first transesterification step of the self-splicing group I intron.

compounds is performed in organic media. This was suggested to be an effect of stronger hydrogen bonding in less polar media.²³ In the group I intron derived

ribozymes the 2'-OH instead of acting as a nucleophile has been suggested to catalyze the transesterification reaction by hydrogen bonding. Figure 4 shows the first transesterification step for the group I intron.

Metal ions are important both for stabilizing the structures of large RNAs and in catalysis of phosphate ester cleavage. Metal ions may act both as general acids²⁴ and bases^{25,26}, by e.g. protonating the leaving 5'-oxyanion or deprotonating the attacking 2'-OH by hydrated aqua ions. Furthermore, metal ions may electrostatically facilitate the intermolecular delivery of the 2'-OH by coordinating the phosphate and releasing electron density²⁵. Figure 5 shows different examples of metal ion catalyzed cleavage of phosphates.

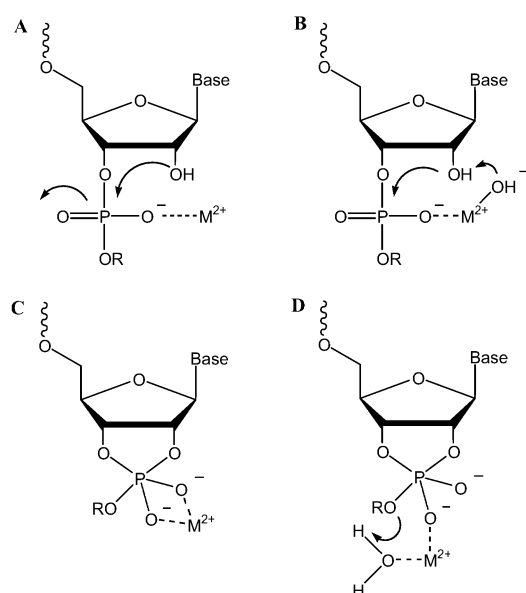


Figure 5: Mechanisms suggested for the metal ion promoted hydrolytic cleavage of phosphodiester. M^{2+} represents a divalent metal ion.

In **A** cleavage is catalyzed by a metal ion chelating to the negative phosphate and facilitating for the incoming 2'-OH. The 2'-OH may also be deprotonated by a metal hydroxide (case **B**) thus providing general base catalysis. In the situation depicted in **C** the metal ion is stabilizing the phosphorane intermediate while in **D** general acid catalysis is provided by a hydrated metal ion.

1.3 ANTISENSE REGULATION OF GENE EXPRESSION

Antisense is a natural mechanism by which cells using Watson-Crick base pairing can regulate gene expression²⁷. Natural messenger RNA is recognized by complementary RNA oligonucleotides, produced by transcription inside the cell. The mRNA of the resulting duplex is thereby shielded towards any further processing on the path to protein translation. Regulation of gene expression by antisense RNA is

observed in eukaryotes as well as in prokaryotes. Antisense regulation could also be introduced via transfection by antisense genes.

In 1978, Zamecnik and Stephenson demonstrated the use of synthetic oligonucleotides for the same purpose when they successfully inhibited the growth of Rous sarcoma virus in cell culture using a 13-mer DNA sequence complementary to the mRNA of the virus²⁸. Since this first pioneering achievement, antisense oligonucleotides have demonstrated pharmacological activity in a number of animal models related to human diseases including viral infections²⁹, autoimmune diseases³⁰ and cancers³¹. The methodology has also been commercialized with the ISIS Pharmaceuticals drug substance Vitrovane®³² which is used to treat cytomegalovirus-induced retinitis, a condition found in AIDS patients. Several antisense substances as well as ribozymes intended for pharmacological use are today in clinical trials^{33,34}. Further, antisense oligonucleotides have found widespread general use as tools in molecular biology.

The basic principle of action is that synthetic oligonucleotides, typically 20-22 nucleotides long, bind specifically to complementary RNA sequences through Watson-Crick base-pairing. The length of the antisense is important for two different reasons: Firstly, The stability of the resulting duplex between the targeted mRNA and the synthetic antisense oligonucleotide is proportional to the amount of hydrogen bonds holding it together. Hence, a greater amount of base pairs and a larger fraction of G:C base-pairs usually provides more stable hybrids. Secondly, in order to achieve site-specific cleavage, it has been calculated that a sequence of at least 16-17 nucleotides is statistically necessary to target a single sequence out from a gene pool having the size of the human genome. Thermal stability is readily determined by measuring of melting temperature (T_m) by means of UV hyperchromicity³⁵.

Translation may after duplex formation, be prevented by different mechanisms. The most common mechanism is RNase H dependent cleavage³⁶ where the cellular enzyme hydrolyzes the RNA part of a RNA : DNA duplex by a hydrolytic mechanism that uses Mg^{2+} as co-factor. Since the mRNA is digested and released from the antisense strand, which in turn can react with another substrate mRNA the reaction is also catalytic. RNase H is found in the nucleus and in the cytoplasm of eucaryotic cells. The translational arrest mechanism³⁷, which is similar to natural antisense inhibition, although shorter antisense fragments and modified RNA or DNA are used, relies on the generation of a stable duplex between antisense and RNA that provides a steric block at the site of the ribosomes thereby inhibiting translation of the mRNA. This method is however not catalytic and will at least require stoichiometric amounts of antisense DNA / RNA to accomplish inhibition.

Although the concept of utilizing RNaseH or the translational arrest mechanism for antisense approaches is attractive the methodologies suffer from some drawbacks. In order to overcome the inherent lability of oligonucleotides to naturally occurring nucleases present in cells and to improve hybridization properties several modifications of the nucleosides and / or the internucleosidic phosphate linkage have been studied. Some of the more outstanding achievements, illustrated in Figure 6, include LNA³⁸ (Locked Nucleic Acids) and PNA³⁹ (Peptide Nucleic Acids) that both have favorable hybridization properties as well as excellent stability against enzymatic degradation. The A-type helix that RNA duplexes or DNA-RNA hybrids usually forms may be more thermodynamically favorable if the antisense oligomers are pre-organized in a fashion that favors formation of an A-type helix.

Although it is still not clear how much this really influences duplex stability, the

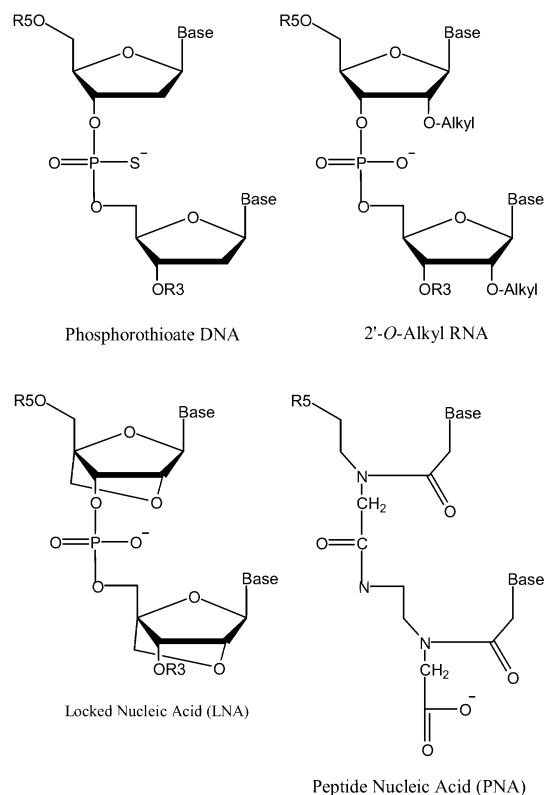


Figure 6: Structure of typical modifications introduced to RNA or DNA oligomers to achieve duplex stability and / or resistance towards cellular nucleolytic degradation.

pre-organization hypothesis has been a corner stone for many 2'-modifications designed to stabilize antisense oligonucleotide RNA duplexes. Typically, the sugars constituting the antisense strand have a preference for the *C3'-endo* (north) conformation. In general, electronegative substituents at C2' e.g. 2'-F, or 2'-*O*-alkyl groups shift the conformational equilibrium of nucleosides towards north while less electronegative e.g. 2'-H (deoxy), 2'-Alkyls or 2'-NH₂ have a higher preference for the *C2'-endo* (south) pucker⁴⁰. The design concept behind LNA was to "lock" the sugar moiety in the north conformation by introduction of a 2'-C,4'-C-oxymethylene linkage. Up to 8.5 °C increase in melting temperature per modification has been achieved in some cases⁴¹. The PNA modification was introduced 1991³⁹ as a novel achiral non-charged DNA modification with a pseudopeptidic N-(2-aminoethyl)glycine (Aeg) backbone. DNA-PNA and RNA-PNA duplexes were more stable than the native structures⁴². The non-charged nature of the PNA backbone-reduces electrostatic repulsion of duplexed strands and contributes to a large extent to the hybridization properties. Synthetic efforts, sometimes quite elaborate are however needed to produce

the monomeric building blocks for PNA and especially for LNA. Introduction of phosphorothioates at the internucleosidic phosphate linkage has been a popular and simple modification to non-modified DNA oligomers since phosphorothioates provide an improvement of the stability towards nucleolytic enzymes (100-300-fold increase in stability) and the resulting duplexes with RNA are recognized by RNase H⁴³. However, the T_m 's of the resulting duplexes are lower compared to their non-modified counterparts and the introduction of a new stereocenter at each phosphate creates a complex mixture of diastereomers that makes purifications difficult and the integrity of the product mixture complicated to reproduce if mixtures are used.

Introduction of 2'-*O*-alkyl modifications at the sugars results in increased stability towards cellular nucleases as well as to an increased stability of resulting duplexes.⁴⁴ Larger 2'-*O*-alkyls however destabilize duplexes due to steric interference in the minor groove. The break-even point is at the size of 2'-*O*-propyl, larger 2'-modifications tend to destabilize while smaller groups stabilize⁴⁵. Disruption of the hydration patterns has also been suggested as a source of destabilization⁴⁶.

The drawback with most modifications is that the resulting duplexes are not substrates for RNase H. This particular problem can be solved by introduction of a mixed backbone oligonucleotide that may e.g. have 2'-*O*-methyl or 2'-*O*-methoxyethyl modified nucleosides combined with an internal RNase H window containing either phosphorothioates or non-modified DNA⁴⁷.

Oligonucleotides are believed to cross the cell membrane through endocytosis⁴⁸ but the cellular uptake is poor if not uptake enhancers are not administered with the antisense. Various agents that have successfully been used include cationic lipids⁴⁹, liposomes⁵⁰ and peptides, either conjugated or not.

1.4 RNA SYNTHESIS

A number of different methods for synthesizing oligonucleotides are described in the literature. Older methods to synthesize RNA include the phosphodiester⁵¹ and the phosphotriester methods⁵² but the most commonly used method for synthesis of DNA is the phosphoramidite method. The most common version of this method relies on use of 5'-*O*-DMT protected nucleosides carrying a β -cyanoethyl *N,N*-diisopropylphosphoroamidite function at the 3'-position. Typically, 1*H*-tetrazole catalyses the condensation step and the resulting phosphitetriester is oxidized in presence of I₂ to the phosphor triester after each elongation cycle⁵³. The DNA adapted phosphoramidite synthesis has been adopted for RNA synthesis by introduction of 2'-*O*-TBDMS protection on the RNA monomers⁵⁴ but quality and reachable chain lengths has not been comparable to those obtained for DNA synthesis. Coupling yields are typically $\leq 98\%$ for RNA synthesis. More successful amidite methods have been developed where novel protective groups of 5'- and 2'-positions have been introduced as well as different condensation agents. The 2'-*O*-TOM method⁵⁵ uses the less steric 2'-*O*-triisopropylloxymethyl (TOM) group at 2' and condensations are carried out with benzylthiotetrazole. The improved performance has been attributed to less steric interference during the coupling event. In the 2'-*O*-ACE method⁵⁶ the mildly acid labile 2'-*O*-bis(2-acetoxyethoxy)methyl group was introduced together with fluoride labile 5'-*O*-bis(trimethylsiloxy)cyclododecyloxysilyl ether protection. Coupling yields higher than 99% could then be achieved. All RNA substrates used in this thesis were synthesized according to the 2'-*O*-ACE method. As an alternative to the amidite

method the H-phosphonate approach was developed⁵⁷ and this has lately been forwarded as particularly suitable for larger scale production of antisense oligonucleotides in solution. In solid phase synthesis of oligonucleotides the H-phosphonate function is readily coupled to the free 5' hydroxyl of the growing oligonucleotide chain in the presence of a condensation agent. This stepwise process is repeated until the desired amounts of monomers are incorporated (Figure 7). Oxidation of all H-phosphonate diesters is then carried out in a single step, prior to base deprotection and cleavage from support. All OBAN precursors used in this thesis were synthesized with the H-phosphonate method.

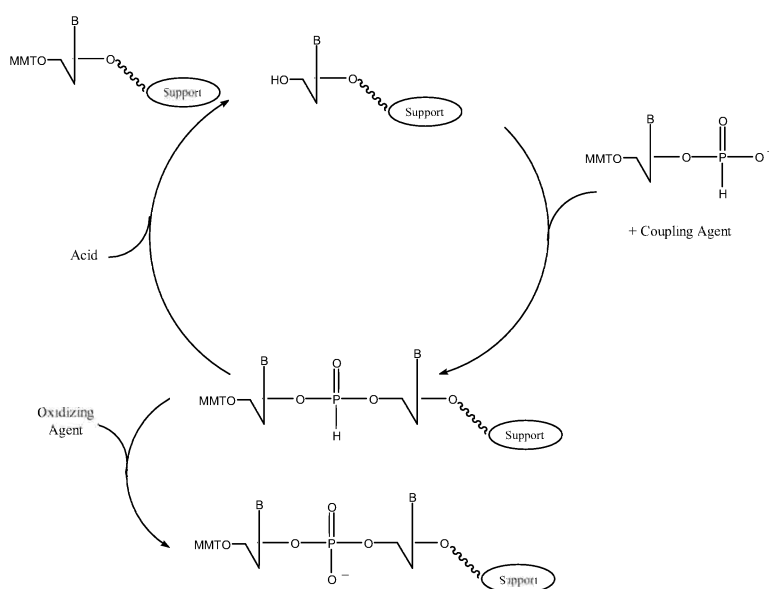


Figure 7: The coupling cycle for the H-phosphonate method. The first nucleoside is anchored to the solid support. The acid labile 5'-O-MMT is removed under acidic conditions and coupled to a nucleoside H-phosphonate monomer under the influence of a coupling agent. The cycle is repeated until the assembly is complete. Finally, the H-phosphonates are oxidized to phosphates by use of an oxidant.

2 OBAN'S

2.1 BACKGROUND

An attractive approach to regulation of gene expression is to attach to the antisense oligonucleotide a group that can cleave the target RNA after duplex formation. Using this approach, a catalytic group, typically a metal chelate⁵⁸ or an organic, metal independent, transesterification catalyst⁵⁹ is covalently attached to the antisense oligomer via a linker. Ideally, this can be developed into catalytic systems, i.e., artificial enzymes that will give turnover of the RNA substrate. Such oligonucleotide based artificial nuclease (OBAN⁶⁰) systems would, if efficient enough, have the advantage of being independent on cellular enzymes as well as allowing for incorporation of numerous modifications that can stabilize duplexes and protect against degradation. This could give more efficient suppression of gene expression at lower concentrations of antisense oligonucleotides.

The first reported oligonucleotide based cleavers capable of site-specific RNA degradation were however, enzymes covalently attached to DNA. In 1988-89, Zuckerman and Schultz published two groundbreaking articles describing DNA conjugates of Staphylococcal nuclease and Ribonuclease S. The former conjugate was constructed by covalent attachment of a staphylococcal nuclease mutant to the 3'-end of a DNA sequence complementary to a 59-mer single stranded RNA. In the presence of calcium ions fragmentation occurred in the region of RNA in close proximity of the recognition site⁶¹. Since the staphylococcal nuclease has activity towards both single-stranded RNA and DNA, side reactions from self-cleavage were observed. Hence, the RNA specific RNase S was attached to a 14-mer DNA and the competing side reaction from self-cleavage could be eliminated⁶². In later studies, Kanaya and coworkers attached RNase H to a 9-mer DNA and achieved degradation of a complementary RNA sequence⁶³. Variation of linker length was also evaluated and an optimum was achieved with a 27 Å linker appended from the 5'-position

While conjugation of natural or slightly mutated natural nucleases to oligonucleotides might appear to be an obvious solution to the problem of combining catalytic activity with sequence recognition, these constructs suffered from a few crucial limitations including disruptions of quaternary structures of the multi sub-unit enzymes at elevated temperatures. Further, difficulties in preparing them and their large molecular weight would make them difficult for practical applications.

Quite some efforts have, on the other hand been directed towards finding small molecules capable of catalyzing transesterifications. Metal aqua ions free in solution as well as complexed in metal chelates, have been reported to cleave phosphate esters both in model systems and in oligonucleotide based systems. A number of different mechanisms have been suggested. Figure 8 illustrates cleavage of a phosphodiester by a neocuproine-Cu (II) complex as described by Linkletter and Chin⁶⁴. The metal complex of the neocuproine is suggested to provide double Lewis acid activation of the phosphate. Hence, the electron density on phosphorus is reduced and this facilitates for the incoming intra-molecular 2'-hydroxyl. The mechanism is, however, not thoroughly studied and it is also conceivable that a coordinated metal hydroxide could act as a general base and deprotonate the attacking 2'-hydroxide or a hydrated water molecule could promote hydrolysis by protonating the leaving group. Evaluation of suitable

catalytic groups or non-catalytic cleavers has been extensively done in model systems. Hydrolysis of RNA dimers is a common method to probe reactivity of these groups towards RNA⁶⁵. However, the character and reactivity of oligomeric RNA differs from

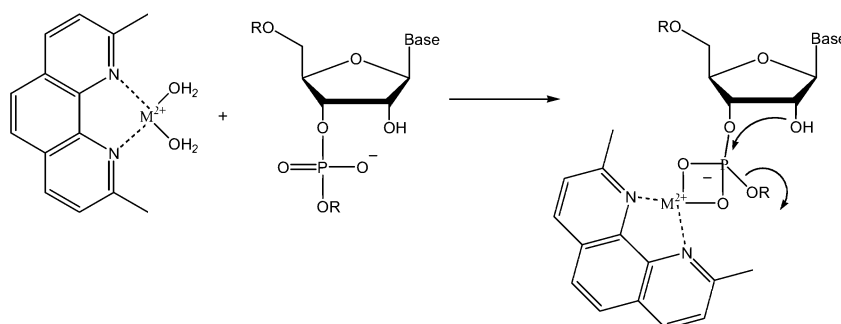


Figure 8: Illustration of catalytic degradation of a phosphodiester by a metal complex of neocuproine.

RNA dimers due to the polyanionic nature of RNA. Adjacent phosphates accelerate the rate of cleavage. Thus, ApAp is hydrolyzed two orders of magnitude faster than ApA¹¹ and the effect of rate enhancement by terminal phosphates is extended further down the chain from a given cleavage site. The most reliable results for probing reactivity are likely to be achieved using systems as similar as the ones that are intended to be used. The target RNA could be present as single stranded, duplexed or in various tertiary structures, e.g. hairpins, loops or bulges depending on the particular application. Some differences in reactivity between conjugated and non-conjugated catalysts are to be expected since the electronic properties may be somewhat different due to the attachment of the linker and the effective concentration of catalytic group at the cleavage site will be much dependent on the design and positioning of linker as well as the overall three dimensional structure of the associated OBAN-substrate complex. If a given OBAN-substrate hybrid is compared with the same system having the catalytic group free in solution, the effect of intra-molecularity can be evaluated. Caution in such comparisons is justified though, since conjugated and non-conjugated systems may have somewhat different three dimensional structures, which will be difficult to fully account for.

To succeed in getting phosphate ester cleavage by any catalytic group attached to a oligonucleotide via a linker, the catalytic group needs to be delivered in close proximity to the phosphate linkage / linkages one wishes to target. For metal chelates, the metal ion should preferably, form a stable enough complex with the ligand to obtain reasonable saturation at the metal ion concentrations available (in vivo, or what may be realistic in biotechnology applications). Metal-ions may be added as cofactors to incubated OBAN mixtures or, if the complex between the metal ion and the chelate is sufficiently strong, stoichiometric amounts of metal ion may be premixed with the OBAN, eliminating the need of cofactor addition. The latter method can greatly increase the number of metal ions available for utilization since rare metal ions or metal ions not abundant in the body may be considered. Finally, the catalytic group should not be too active if random cleavage before duplex formation is to be avoided.

The first examples of oligonucleotide-based metal ion dependant cleavers were independently reported in 1994 by four different research groups. Bashkin and coworkers⁶⁶ achieved degradation of a 159-mer RNA sequence derived from the *gag*-mRNA of HIV using a system with a Cu (II)–terpyridine ligand (A) conjugated to a 17-mer DNA scaffold. The group was linked at the C-5 of an internal uracil residue and 11% cleavage was achieved after incubation at 37°C over a period of 72 h. Matsumura and colleagues⁶⁷ synthesized a 15-mer, 5'-linked, lanthanoid-complexing iminodiacetate system (D) that after incubation for 8h, at pH 8 and 37°C, cleaved 17 % of a 39-mer RNA substrate. Magda et al⁶⁸ tethered a Eu (III)-pentadentate texaphyrin

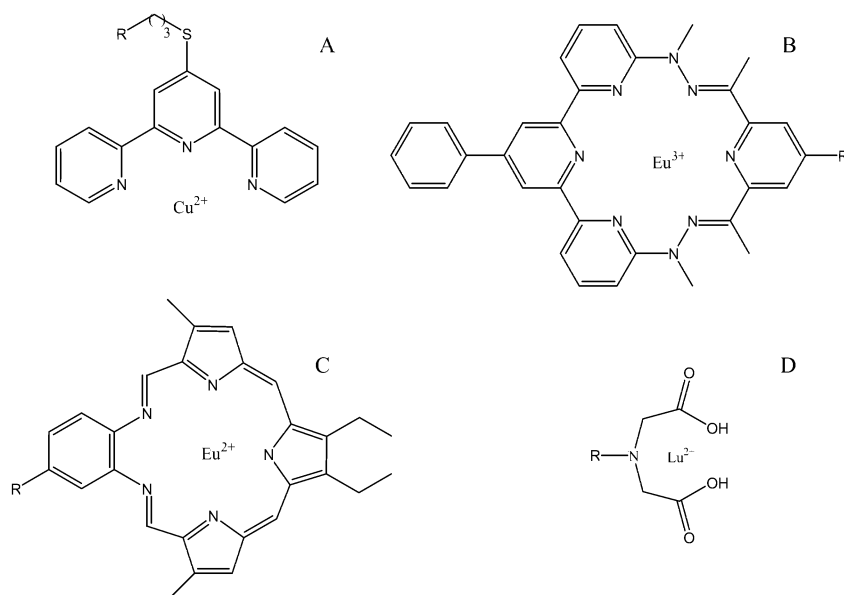


Figure 9: Illustrations of different RNA cleavers. A: Terpyridine Cu(II) complex, B: Terpyridine Eu(III) complex, C: Texaphyrin D: Iminodiacetate Lu(III). R denotes attachment point for the linker.

(C) both to the 5'-position and to the C-5 of an internal thymine residue of a 20-mer DNA probe. In the case of the 5'-linked OBAN 30% degradation of a 30-mer target was observed after 24 h at pH 7.5 and 37°C, while the OBAN with the internally conjugated complex failed to promote cleavage. The Eu (III)-pentadentate complex (B) has the advantage that the metal complex is strong enough to allow generation of the complex prior to incubation. Hence, the reaction is not dependent of metal ion cofactors which can be of importance in biological applications. The fourth example, presented by Hall et al.⁶⁹, was constructed from a terpyridine based macrocyclic Eu (III)-complex linked to the 5'-end of a 20-mer DNA, complementary to a 29-mer RNA target. 88% cleavage was observed after 16h at 37°C and pH 7.5. Figure 9 summarizes the structures of the above mentioned cleavers.

Further developments have been made since the first pioneering achievements were presented. In the majority of cases presented to date the catalytic group has been tethered from the 5'-end of the oligonucleotide thus targeting phosphates that are located outside the duplex region. In cases where phosphates inside the duplex region have been targeted, including those mentioned above, the degradation has been poor or difficult to achieve.

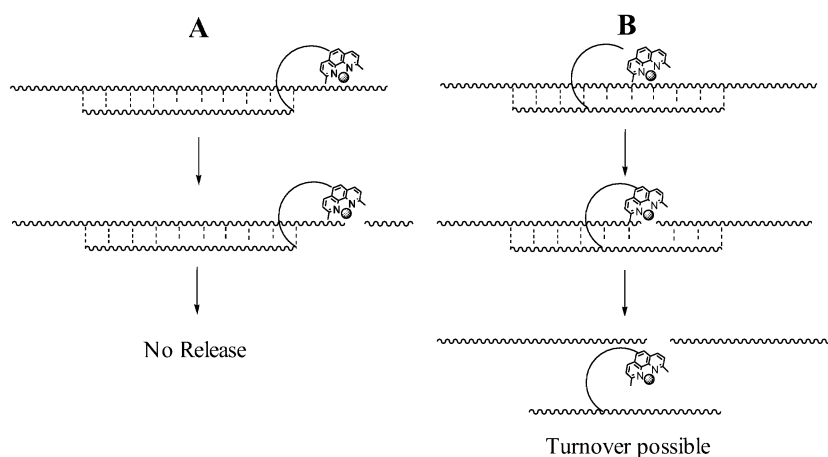


Figure 10: Illustration of **A** cleavage in a single stranded region and **B** cleavage of a base-pair within the duplex region. In case of **B** catalytic turnover is promoted.

The benefit of cleaving an internal phosphate as opposed to an external is illustrated in Figure 10. In the latter case cleavage takes place outside the duplex region and after the cleavage event all base-pairs between antisense oligonucleotide and substrate are intact. Hence, the associated antisense-substrate complex will not readily dissociate and a catalytic turnover is likely inhibited due to poor product release. In case B cleavage inside the duplex results in two, or more, shorter fragments that are likely to dissociate at 37°C. Thus promoting catalytic turnover. Without doubt, cleavage inside the duplex region would be the best strategy for design of an efficient OBAN. In practice it has been difficult to achieve efficient cleavage within a duplexed region. The conformational rigidity of the duplex prevents the RNA to adopt a conformation with the 2'-hydroxyl in-line with the adjacent phosphate, which is needed for the transesterification reaction⁷⁰. A parallel can be drawn to the mechanism of natural enzymes where the 2'-OH is never utilized by enzymes having ds-RNA as substrate (see chapter 2). A possibility is to target RNA bulge structures or non-base pairing regions inside a duplex. Since the bulged-out region is single-stranded it can be flexible enough to go through the conformational change necessary for cleavage. Husken et al. showed with an inter-molecular external cleaver (free in solution) that phosphates in bulged out regions were particularly susceptible towards cleavage⁷¹ and later Muth et. al showed the same in cleaving a 23S rRNA with a phenanthroline derivative⁷². It has also been reported that metal-ions promote cleavage of the single stranded regions of bulges⁷³ and hairpins at a rate comparable to that of single stranded RNA⁷⁴. Husken et al. later extended their study to direct a linked catalyst towards a

duplex having a 4-nucleotide bulge. Several groups have introduced non-nucleosidic windows inside the OBAN sequence to generate a more flexible substrate side⁷⁵. Bashkin and co-workers later introduced the concept of "minimal nucleoside displacement" where the internal attachment point for the linker catalyst system is instead of a nucleoside a serinol derivative⁷⁶. The serinol linker has as opposed to a normal nucleoside no hydrogen bonds to the substrate and is therefore believed to render the opposite phosphate more flexible and hence more susceptible to cleavage.

2.2 DESIGN OF SYSTEMS

RNA bulge loops are commonly occurring secondary structural motifs of large RNA's. Bulges are characterized by a non base-pairing stretch of one or more nucleotides interrupting an RNA double helix. Bulges are usually conserved and have been shown to have different functions including interactions with RNA binding proteins⁷⁷ and facilitating tertiary folding of structural domains⁷⁸.

In the context of designing artificial nucleases, an appealing characteristic is that the bulge part of the structure is single-stranded, hence the phosphodiester backbone is more conformationally flexible, rendering it prone to intramolecular cleavage by the 2'-hydroxyl. Thus, we have chosen to design our OBAN systems so that the target RNA would form a bulged out structure.

The parent bulge structure chosen for these studies is a structure derived from the *Tetrahymena thermophila* group I intron whose structure has been determined by NMR⁷⁹ in the form of a 25-nucleotide hairpin sequence. Figure 11 shows the structure of the parent bulge loop (A) and the derived antisense-target duplex (B).



Figure 11: 25-mer hairpin bulge loop system from the *Tetrahymena thermophila* group I intron. The parent loop structure (A) was used for the design of the two piece bulge system (B).

According to the NMR study, the sugar conformation of some of the nucleosides in the bulge, including A6 and A8 are to high degree (50%) C2'-endo; this conformation brings the attacking 2'-hydroxyl in closer proximity to the phosphate. The energy needed to adopt the conformation needed for reaction may then be reduced.

To be able to construct thermally- and hydrolytically stable OBAN systems a few modifications were introduced. Three nucleotides were added to the 5'-side of the bulge containing

RNA strand to improve the hybridization properties. Furthermore, one G-C base-pair was changed to C-G to avoid self-complementarity. Finally, the hairpin loop was deleted in order to create a two-piece antisense-target system. The RNA sequence complementary to the bulge sequence was used as the scaffold for the OBAN's. The nucleoside building blocks used to synthesize the OBAN's were modified by 2'-*O*-methyl groups to be more resistant to enzymatic degradation and to provide higher affinity for the substrates.

Different attachment points for linker/catalyst were defined through molecular modeling. The central uridine of the OBAN strand was used to attach linkers either from the 2'-position of the sugar moiety (OBAN 2) or from the C5-position of the nucleobase (OBAN 1, 3 and 5). In one case (OBAN 4) the central uridine was exchanged for a cytidine, and a linker was attached to the N4. The structures of the OBAN's are summarized in Figure 12.

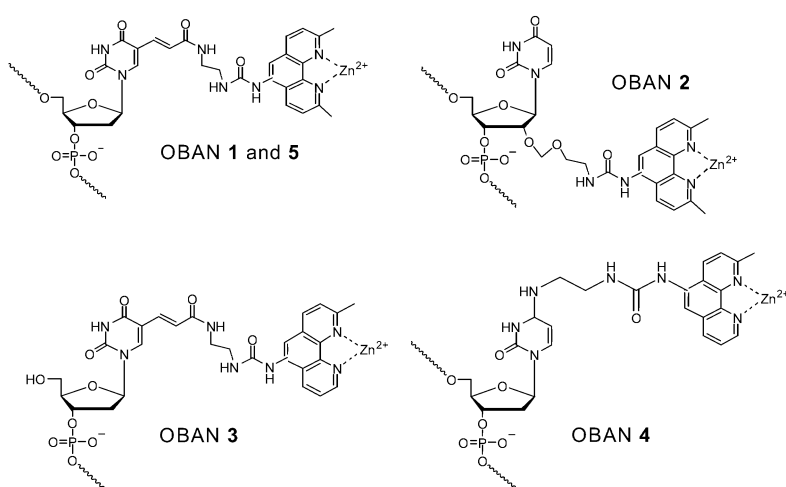


Figure 12: Structures of the different OBAN's. OBAN 3 and 5 contained an extra building block attached to the 3'-end of the OBAN's. All OBAN's contained 2'-*O*-methylated nucleosides.

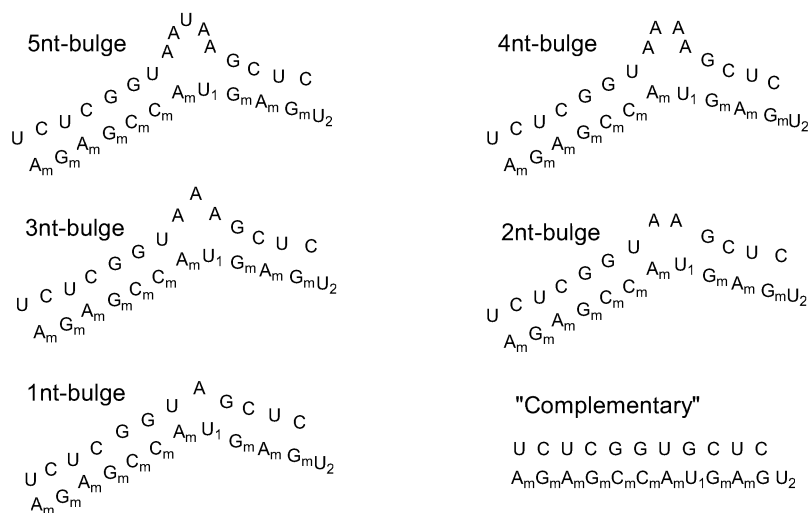
OBAN 3 had the same linker/cleaver as OBAN 1 with the difference that it was appended from the 5'-end of the OBAN strand, making it one nucleoside unit longer. OBAN 5 can be visualized as a combination of OBAN 1 and 3 having two neocuproine units conjugated one to the central uridine and the second from a uridine at the 5'-end.

From molecular modeling it seemed plausible to reach the bulge with all OBAN's even from the 5'-end of OBAN and specifically over the minor groove (in the duplex) In the case of OBAN 5 a cooperative action of the two conjugated neocuproines was conceivable.

Obviously, any successful design must bring the catalytic group in proximity to the phosphate one wishes to cleave and this may be difficult due to many factors including the natural flexibility of the bulge-loop.

To be able to investigate proximity factors for the different OBAN's, the size of the bulge was varied by incubating the five OBAN's with substrate RNA's containing a

non base-pairing window varying from one to five nucleosides. Thus, upon hybridization OBAN-substrate complexes having 1-5 nucleotides in the bulge are



N_m denotes 2'-O-Me nucleotide residues

Figure 13: Illustration of the OBAN-target RNA complexes. Nucleotides carrying linker/neocuproine units were introduced either at position U_1 or U_2 . OBAN **1**, **2** and **4** all had modifications at U_1 . OBAN **3** had linker/catalyst at U_2 while the dual conjugated OBAN **5** had modified building blocks both at U_1 and U_2 .

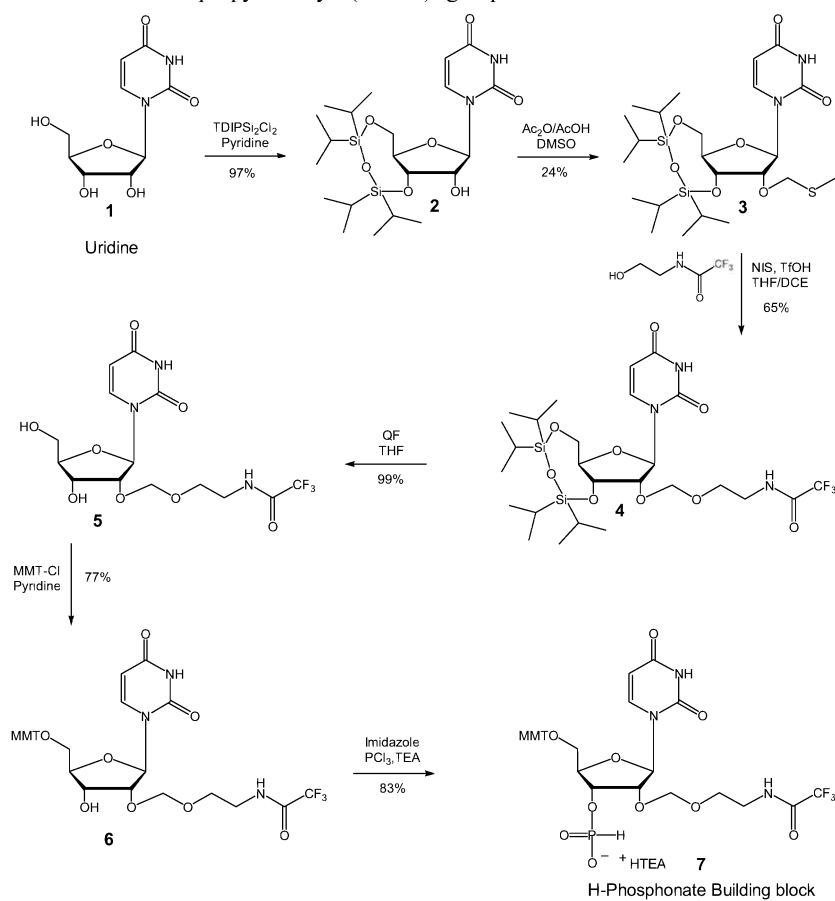
formed. Figure 13 illustrates these structures. The metal chelate used in the study was Zinc (II) 5-amino-2,9-dimethyl phenanthroline (neocuproine). This chelate was previously proven to be an active cleaver of phosphodiester in the presence of Cu (II) ions in model systems e.g. nucleoside dimers such as ApA⁸⁰ prior to our interest. It has subsequently been shown to be active as an RNA cleaver when conjugated to DNA⁸¹ and PNA⁸² and in our case as an OBAN (i.e. displaying catalytic turnover) when conjugated to 2'-O-methyl RNA⁶⁰.

Cleavage reactions were performed with the different OBAN's in the presence of metal ions. All reactions were performed at pH 7.4 in the presence of 0.1-M NaCl in HEPES buffer. Cleavage reactions were in case of OBAN **1** carried out in the presence of varying amounts of Zn (II) to determine evaluate the effect of concentration on activity and rate of background metal ion induced cleavage. Once that had been established all following OBAN systems were for ease of comparison incubated with $[Zn^{2+}] = 100 \mu M$.

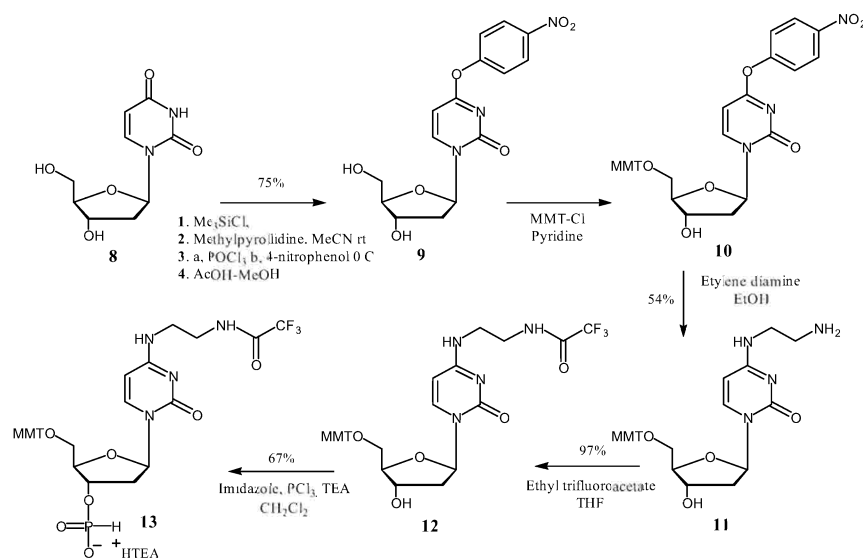
2.3 SYNTHESIS OF OBANS

2.3.1 Synthesis of Nucleoside Monomers for the OBAN's

The oligonucleotide precursors to OBAN **1**, **3** and **5** were purchased as assembled 2'-*O*-methyl oligonucleotides having linkers carrying free primary amines that subsequently could be conjugated to the neocuproine ligand. OBAN **2** and **4** were synthesized according to Scheme 1 and Scheme 2. The OBAN **2** containing building block was synthesized from uridine. The 5'- and 3'-hydroxyls of uridine were protected with the tetraisopropyl disilyl (TIPDS) group. Reaction of **2** with DMSO/acetic



Scheme 1: Synthesis of monomeric H-phosphonate building block used to synthesize OBAN **2**



Scheme 2: Synthesis of building block used in the construction of OBAN 4.

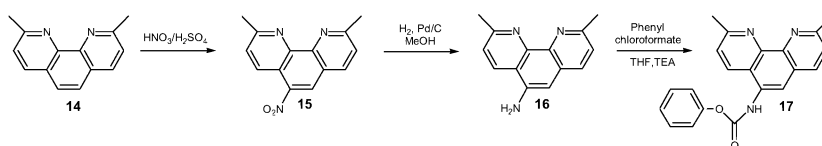
anhydride/acetic⁸³ acid gave after silica gel chromatography and crystallization from hexane-ether, the desired product **3**. Coupling of donor **3** with protected amino ethanol acceptor using the NIS/TfOH activation^{84,85,86,87} gave the desired product **4**. Product **3** had to be crystallized, to remove traces of impurities for the coupling reaction to work. The silyl protection was then removed using fluoride ion and the deprotected compound **5** was converted into building block **7** ready for automatic machine synthesis of RNA using H-phosphonate chemistry. Compound **5** was first protected in the 5'-position using the monomethoxy trityl group (MMT). After purification, **6** was converted into the H-phosphonate **7** using standard procedures.⁸⁸

The C-4 modified building block **13** used for the construction of OBAN **4** was synthesized by a reaction sequence starting with 2'-deoxyuridine (Scheme 2), first the 4-(4-nitrophenoxy) derivative **9** was produced by treating a suspension of **8** with 1-methylpyrrolidine followed by chlorotrimethylsilane in dry acetonitrile following the convertible nucleoside approach described by Reese and co-workers⁸⁹ i.e. phosphorus trichloride and 4-nitrophenol was added. Product **9** could be precipitated from an acetic acid-methanol solution by addition of diethyl ether. In a first attempt compound **9** was reacted with ethylene diamine in ethanol producing the substituted product, however this compound was difficult to purify due to its high polarity. Instead, the 5-hydroxyl was first monomethoxy tritylated in pyridine producing **10**. After concentration and without any further purification ethylene diamine in ethanol was added. After stirring for 2 h the reaction was complete and the crude reaction mixture was chromatographed. The free amine **11** was then trifluoroacetylated by using the methyl ester of trifluoroacetic acid in methanol⁹⁰ producing **12**. This reaction proceeded readily and with high yield, however NMR analysis in CDCl₃ gave a complicated pattern that indicated internal hydrogen bonding between the N4 proton and the carbonyl function of the trifluoroacetate. This spectra were, however, simplified by changing to the more

polar solvent, DMSO. In the next step the H-phosphonate function could be introduced by means of standard conditions⁸⁸ producing building block **13**.

2.3.2 Synthesis of the Neocuproine Reagent

The metal chelate used in this study is a phenanthroline derivative, 5-amino-2,9-dimethyl-1,10-phenanthroline. Scheme 3 describes the synthetic route for the phenanthroline and for the derivative used for attachment to the oligonucleotides.



Scheme 3: Synthesis of neocuproine reagent.

Neocuproine (**14**) was reacted with SO₃/H₂SO₄, HNO₃ at 168°C⁹¹ to produce nitro derivative **15** (Scheme 2). The purification of **15** was complicated due to many by-products being formed during the reaction. The isolated yields from these reactions were relatively low but satisfactory enough for our purposes. **15** was then reduced to 5-amino-2,9-dimethyl 1,10 phenanthroline (**16**). Reduction to the amino neocuproine was readily carried out at ambient temperature with H₂, Pd/C. Finally, the amino compound was coupled to phenyl chloroformate, this was carried out in THF using 1.5 eq of triethyl amine and 1.5 eq of phenyl chloroformate. The reaction mixture was left at -20°C to precipitate out carbamate **17**.

2.3.3 Synthesis of Oligonucleotides

The RNA precursors of OBAN **2** and OBAN **4** were synthesized using H-Phosphonate chemistry⁹² on solid polystyrene support. Nucleosidic building blocks carrying acid labile mono-methoxy-trityl groups at the 5'-position, base labile amides on exocyclic amines of the nucleobases and 3'-H-phosphonate functions were synthesized, Figure 14. The H-phosphonate function is readily coupled to the free 5'-hydroxyl of the growing oligonucleotide chain in the presence of a condensation agent. This stepwise process is repeated until the desired amounts of monomers are incorporated. Oxidation of all H-phosphonate diesters is then carried out. Pivaloylic chloride was used as condensation agent and I₂ in aqueous pyridine was used for the oxidation step. After oxidation is completed, base protection and the succinyl ester linkage to the solid support are cleaved off in the presence of ammonia in ethanol. The crude oligonucleotides are purified on ion-exchange and reversed phase HPLC and finally concentrated by lyophilization.

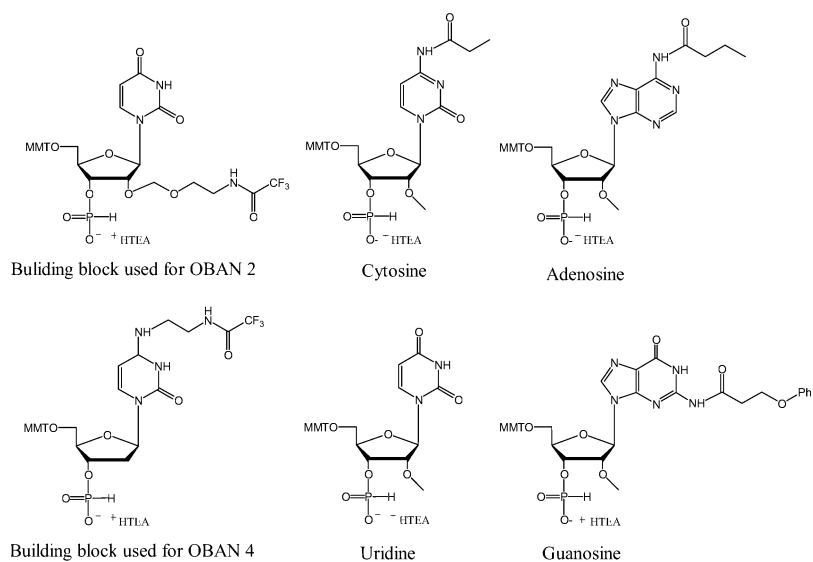


Figure 14: H-phosphonate building blocks used to produce OBAN 2 and OBAN 4

2.3.4 Conjugation of Neocuproine to Oligonucleotides

The conjugation reactions were performed in aqueous sodium tetraborate buffer (pH 8.5) and it is our observation that it is essential that the amino group about to be conjugated is sufficiently deprotonated (pKa of aminogroup not too high) or else the conjugation is less efficient (probably hydrolysis of the reagent then becomes too prominent). The neocuproine reagent **17** was first dissolved in DMSO. Borate buffer and the oligonucleotide bearing a free primary amine were added (Figure 15). The mixture was put on gentle vortexing for two hours after which the reaction in all cases, were complete.

After conjugation the OBAN's were diluted with water, filtered and purified by reversed phase HPLC. In all cases studied when the linker had either a β -oxygen or nitrogen the conjugation reaction was highly efficient, proceeding fast and with

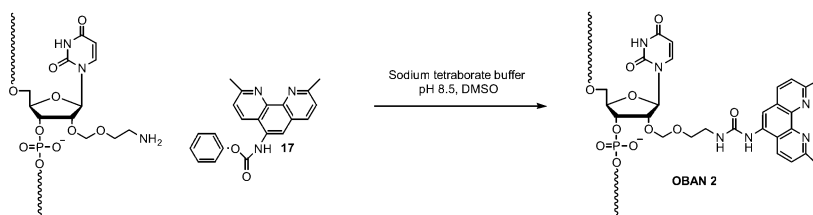


Figure 15: Conjugation reaction demonstrated for OBAN 2. Fully deprotected oligoribonucleotides bearing free primary amines were reacted with neocuproine phenyl carbamate in aqueous solution producing the conjugated species.

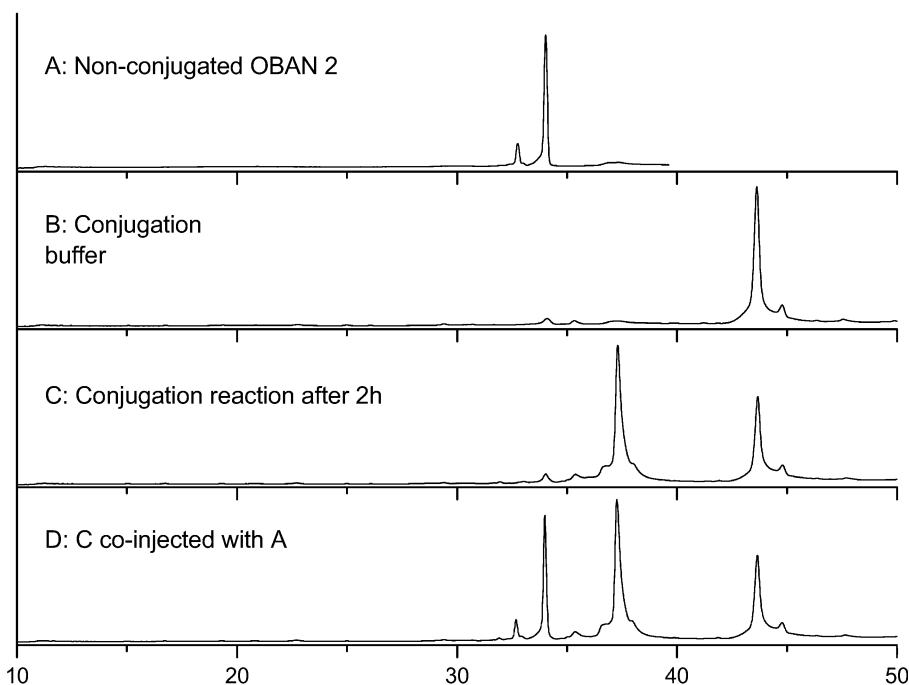


Figure 16: Conjugation reaction as performed for OBAN 2.

virtually quantitative yields. Figure 15 illustrates the formation of OBAN 2 and Figure 16 shows the actual conjugation reaction for OBAN 2.

2.4 OBAN ACTIVITY

By changing the RNA substrate of the OBAN-RNA hybrids from complementary sequences to substrates having 1 to 5 nucleosides in a bulged out region we thought that we more efficiently could find an optimum with respect to rate and selectivity for a given linker/catalyst system. Furthermore, we wanted to evaluate if the bulge structures would facilitate release (to give catalytic turnover) after the cleavage event. An initial screening carried out with OBAN 1 and the 6 substrate RNA's in the presence of 100 μM Zn (II) at pH 7.4 and 37°C showed that a maximum in rate was achieved with the 3- and 4-nt bulge systems. Due to more selective cleavage patterns for the 3-nt bulge compared to that of the 4-nt bulge the former was singled out for further evaluation. The first factor to study was the dependence of the Zn (II) cofactor. Hence, reactions were carried out with Zn (II) concentrations varying from 10-500 μM while keeping the concentrations of OBAN 1 and the 14-mer RNA substrate constant at 4 μM . As clearly illustrated by Figure 17, the reaction has a strong dependence for Zn (II). This is

to be expected since the stability constant is only about 10^4 . The reaction is most accelerated in the region 0-100 μM and then the dependence reaches a point of saturation at Zn (II) levels exceeding 200 μM . To be able to compare our neocuproine

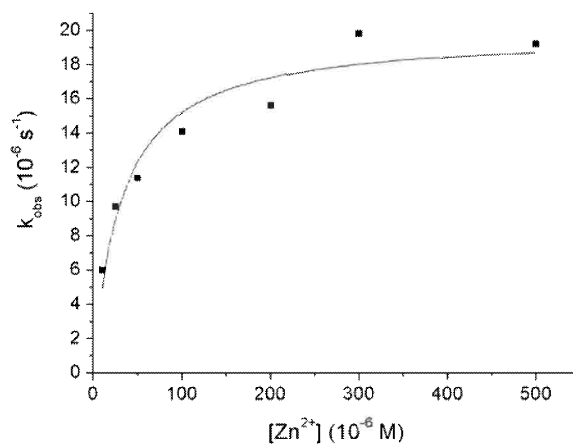


Figure 17: Metal ion dependence of the rate of degradation of the 3-nucleotide bulge with OBAN 1

tagged OBANs we wanted to set a fixed level of Zn (II) for the forthcoming experiments. Although the level of Zn (II) in human serum that has been determined to be in the range 10-20 μM ⁹³ is quite sufficient to promote cleavage by OBAN 1 we decided for our following experiments to use a concentration of 100 μM to provide at least half saturation of our complexes and hence faster kinetics. The next issue to address was the dependence on OBAN concentration. The 3-nt system was again chosen as the test system. 4 μM substrate was incubated with OBAN 1 concentrations varying from 0.5-12 μM in the presence of 100 μM Zn (II). The results are shown in Figure. 18. As can be seen the rate of the reaction reaches a plateau at OBAN 1 concentrations exceeding the concentration of substrate which in this case was 4 μM .

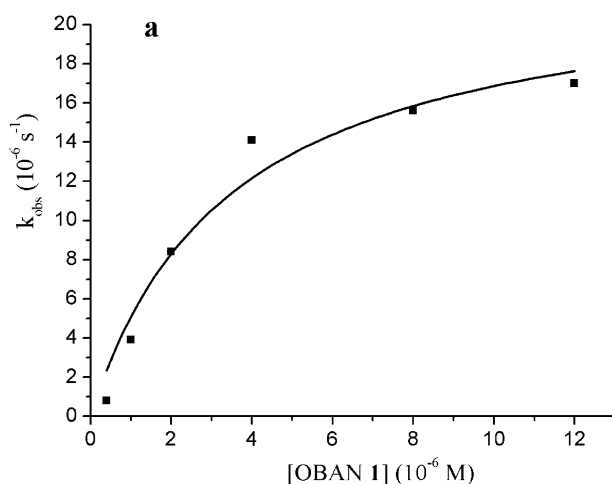


Figure 18: Enzyme dependence on the rate of degradation of the 3-nt bulge. Experiments carried out in 10 mM HEPES buffer containing 100- μ M Zn (II), 0.1 M NaCl, 4- μ M substrate and 0.5-12 - μ M OBAN 1

This result is logical with the associated OBAN-substrate complex as the reactive species. The result also emphasize the fact that cleavage is due to the intra-molecular activity of the neocuproine Zn (II) complex after duplex formation as opposed to random inter-molecular cleavage by free non-hybridized OBAN in solution. In the latter case the rate dependence of OBAN concentration would continuously increase instead of reaching the saturation plateau depicted in Figure 18. The rate of cleavage displays Michaelis-Menten behavior on the concentration of OBAN 1. Curve fitting of Figure 18 gives a $V_{max} = 20-22 \times 10^{-6} \text{ s}^{-1}$ and K_M of approximately $3-3.5 \times 10^{-6}$ M.

The prospect of using the OBAN's in a catalytic fashion is perhaps the most appealing feature of the RNA bulge design. Indeed, when incubated with excess substrate at ratios of 1:2, 1:4 and 1:10 OBAN 1 displays true enzyme behavior with turnover of substrate. The turnover of substrate is in fact not limited to the reaction of OBAN 1 but to the effect of degradation of non-duplexed substrate RNA by Zn (II). The metal aquo ion catalyzed degradation is faster for the single-stranded RNA than in duplexed regions or in bulge regions (at least partially due to the statistical effect) so that, at higher ratios substrate to OBAN this background reaction predominates. Nevertheless, OBAN 1 displays true catalysis with turnover of substrate which was what we wanted to achieve with the introduction of the bulge. Higher turnover numbers will be plausible to achieve if a catalyst with similar activity but higher complex constant would be appended to the RNA scaffold. However, much more important for potential applications, is to achieve a higher overall cleavage rate.

Having set the boundaries for the reaction our next aim was to evaluate if rate and selectivity could be tailored by changing the length/directionality or append point

of the linker/catalyst system. The structures of the different bulges-containing systems are illustrated in Figure 13. The RNA substrates were first incubated with OBAN **1** in equimolar concentrations (4 μM). All cleavage reactions were monitored by reversed phase HPLC. Figure 19 shows the HPLC chromatograms of the reaction mixtures with the different bulge systems after 12h and the bulge size dependence is striking. In the complementary system (no bulge) as well as for the two smallest bulges the cleavage rate is very low, after 12h little cleavage is detected. For the 3-nt bulge considerable cleavage has taken place and for the 4-nt bulge a maximum in rate is achieved. In the case of the largest 5-nt bulge, the rate of cleavage is clearly lower than for the 3- and 4-nt bulges. Observed first order rate constants are summarized in table 1. The trend clearly indicates that proximity factors are here the main influence for the efficiency of cleavage, in case of the smallest systems there can also be an inhibiting factor arising from the systems being conformationally rigid. It has been shown in studies on dinucleotides that cleavage of ApA in the presence of Cu (II) bi- or terpyridyl chelates can be considerable faster than cleavage of dinucleotides with other base combinations⁹⁴. This apparent base selectivity has been attributed to a π - π interaction between the adenines and the bi or terpyridyl system. If a π - π interaction would influence also in the present case we could obtain a substantial influence on the cleavage rate of the 5-nt bulge if the uridine residue in the middle of the bulge is changed to an adenosine. Hence, an oligoribonucleotide forming a 5-nt all adenosine bulge was incubated together with OBAN **1** under identical conditions as those for the AAUAA bulge forming substrate. The first order rate constant for the 5-A system was lower but quite similar to that for the AAUAA system. ($7.10 \pm 0.2 \times 10^{-6} \text{ s}^{-1}$ and $9.1 \pm 0.2 \times 10^{-6} \text{ s}^{-1}$ respectively). This indicates that there is no strong influence from a π - π interaction between the neocuproine and the adenosine containing bulge. The directionality and length of the linker is clearly more important for positioning of the neocuproine complex. Control experiments with free non-conjugated neocuproine Zn (II) complex present with bulge systems lacking the linker have been carried out under otherwise identical conditions. These experiments demonstrated a slow increasing rate of substrate degradation proportional to the amount of phosphates in the bulge⁹⁵. Since both inter- and intra-molecular experiments had been carried out a calculation of the effective concentration for the OBAN **1** system could be made. The rate of cleavage of the 3-nt bulge system was $14.1 \times 10^{-6} \text{ s}^{-1}$. A system of the same substrate RNA hybridized to an all 2'-*O*-methyl RNA having the same sequence as OBAN **1** was cleaved at $1 \times 10^{-6} \text{ s}^{-1}$ in the presence of 100 μM Zn (II) and 10 μM neocuproine. Thus, since the concentration of external cleaver was 2.5 higher in the latter experiment the intra-molecularity could be approximated to 50. It should be stressed that this is a lower limit since the degradation of RNA in the 2'-*O*-Me-RNA system by 100 μM Zn (II) concentration is almost as high. The next step was to vary the attachment point and linker length. OBAN **2** had the linker appended from the 2'-position of the same internal uridine unit and OBAN **3** had the linker attached from an extra nucleoside added to the 5'-end of the OBAN sequence, thus creating a 12-mer OBAN. In the reported three dimensional structure of the 5-nt bulge the 2'-position is less ideally positioned with respect to the bulge. However, we thought that smaller bulges could be better structures for OBAN **2**. Kinetic experiments carried out in the same fashion as for OBAN **1** revealed that the 2-nt, 3-nt and 5-nt were cleaved fastest whereas the rate was decreased for the 4-nt bulge.

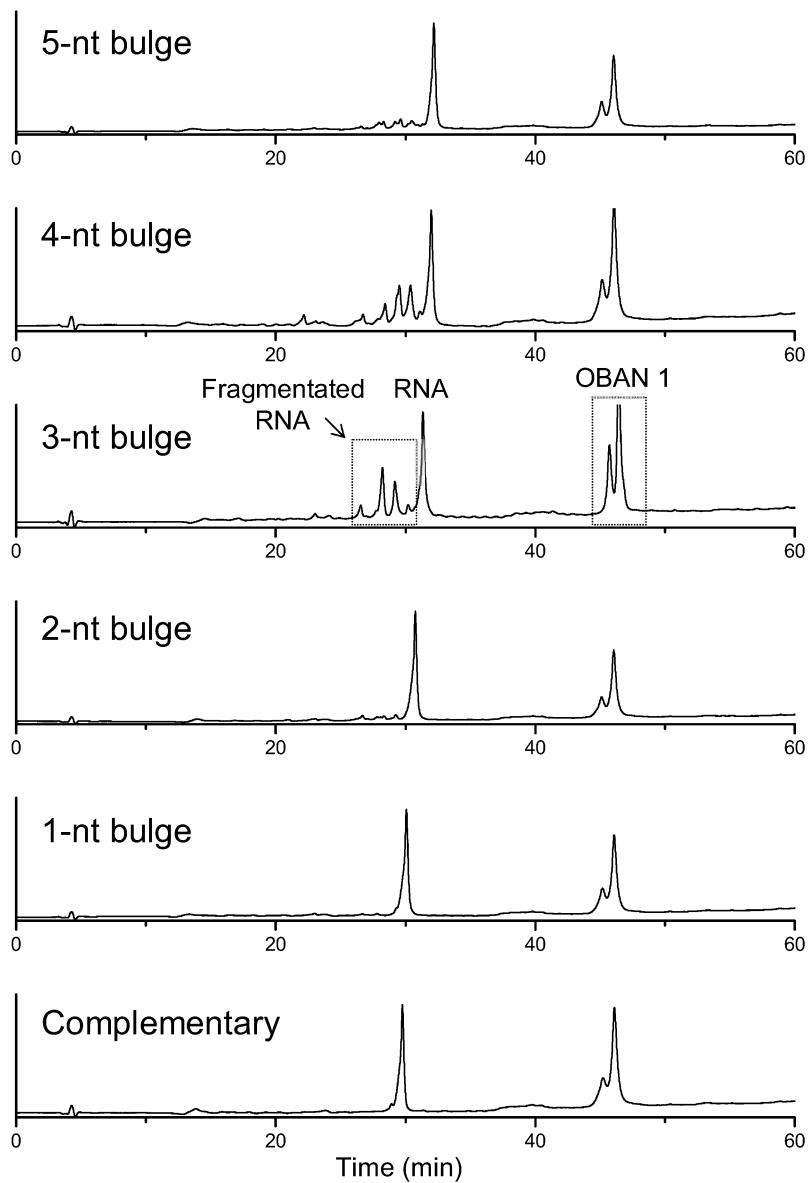


Figure 19: HPLC analysis from the analysis of reaction mixtures of OBAN 1 and RNA substrates after 12h, from the 5-nt bulge forming complex (top) to the complementary duplex (bottom). All reactions were carried out with a 4 μM conc. of both OBAN 1 and substrate RNA, in 10 mM HEPES buffer (pH 7.4) containing 0.1 M NaCl and 100 μM $\text{Zn}(\text{NO}_3)_2$ (The additional shoulder on the peak arising from OBAN 1 is because the HPLC analysis (see Experimental section) is carried out at 6.5, which is close to the $\text{p}K_a$ of the chelate. At higher pH this is not present but the target RNA and fragments from cleavage are then less well separated).

The rates were overall lower than for OBAN 1, except for the 2-nt bulge that was more readily cleaved by OBAN 2. The bulge size preference may again be attributed to proximity factors. The complementary system where the neocuproine is likely to reach the substrate through the minor groove again failed to promote cleavage, illustrating the difficulties in obtaining cleavage within duplexed regions.

In the 3-D structure of the 5-nt bulge the 5'-end of the part that corresponds to our OBAN points toward the bulged out region and provides another attractive option for a linker attachment. OBAN 3 was believed to have a preference towards larger bulges and indeed, a slow increasing preference with bulge size from the 2-nt to the 5-nt bulge is seen. The 5'-linker, with the extra nucleoside, is probably too long to work efficiently which also is reflected by the rate constants for OBAN 3 (Table 1). Molecular modeling suggests that a shorter linker, perhaps the neocuproine attached directly to the 5'-end. Nevertheless, the bulge is still reachable from the 5'-position. With OBAN 4 we wanted to further explore the positioning of the neocuproine ligand by introducing a linker that was one carbon shorter, tethered to the same nucleoside position but pointing in a slightly different direction, compared to OBAN 1, to evaluate if any changes in cleavage patterns, bulge preference or overall efficiency could be observed. As expected, cleavage was exclusively observed in the region of the bulge and interestingly, the bulge size preference for OBAN 4 was almost the same as for OBAN 1 as were the rates (Table 2). A maximum in rate was again observed for the 4-nt bulge, the 5- and 3-nt bulges were cleaved less rapidly and the 2-nt was degraded least efficiently. Figure 20 illustrates the cleavage assays for OBAN 4. Neither the 1-nt bulge nor the complementary sequence was affected to any significant extent. The difference in cleavage patterns is likely due to the fact that the catalytic group is positioned slightly differently compared to OBAN 1 placing different phosphates of the substrate side closer to the neocuproine-Zn (II) complex. A plausible explanation for the similarities in rate could be that the bulge region is flexible to move and will be equally close in space to linkers of not too dissimilar lengths and directionalities. From a mechanistic point of view, the neocuproine co-ordinated Zn (II) ion is likely to coordinate to the target phosphate and electrostatically facilitate an intramolecular attack from the adjacent 2'-hydroxyl. However, if the bulge structure flexes between different conformations the phosphate-metal interaction will probably not be strong enough to fix the bulge in a single conformation. Hence, more than a single cleavage site is observed and systems of equal lengths and directionalities are likely to behave quite similarly. A more efficient system may involve more interactions through e.g. hydrogen bonds between the OBAN and the bulge part of the substrate.

OBAN 5 can be visualized as a combination of OBAN 1 and 3. The basic idea was to evaluate if the combination of the two linker systems would yield an OBAN that displayed an additive or even synergistic rate enhancement compared to OBAN 1 and 3. However, this kind of effect could not be observed with the dual neocuproine containing OBAN 5. Instead, OBAN 5 was less prone to induce cleavage than either OBAN 1 or 3. Thermal melting studies confirmed that all OBAN's used in this study formed stable bulge structures with the substrate RNA's. Differences in melting points were observed but all were higher than 44°C, ensuring a minimum of 80-90% duplex at 37°C. An explanation for the low activity could be that the two neocuproine units provides a steric block for each other and do not allow either catalyst to efficiently reach their target phosphates. Another explanation could be that the two neocuproines

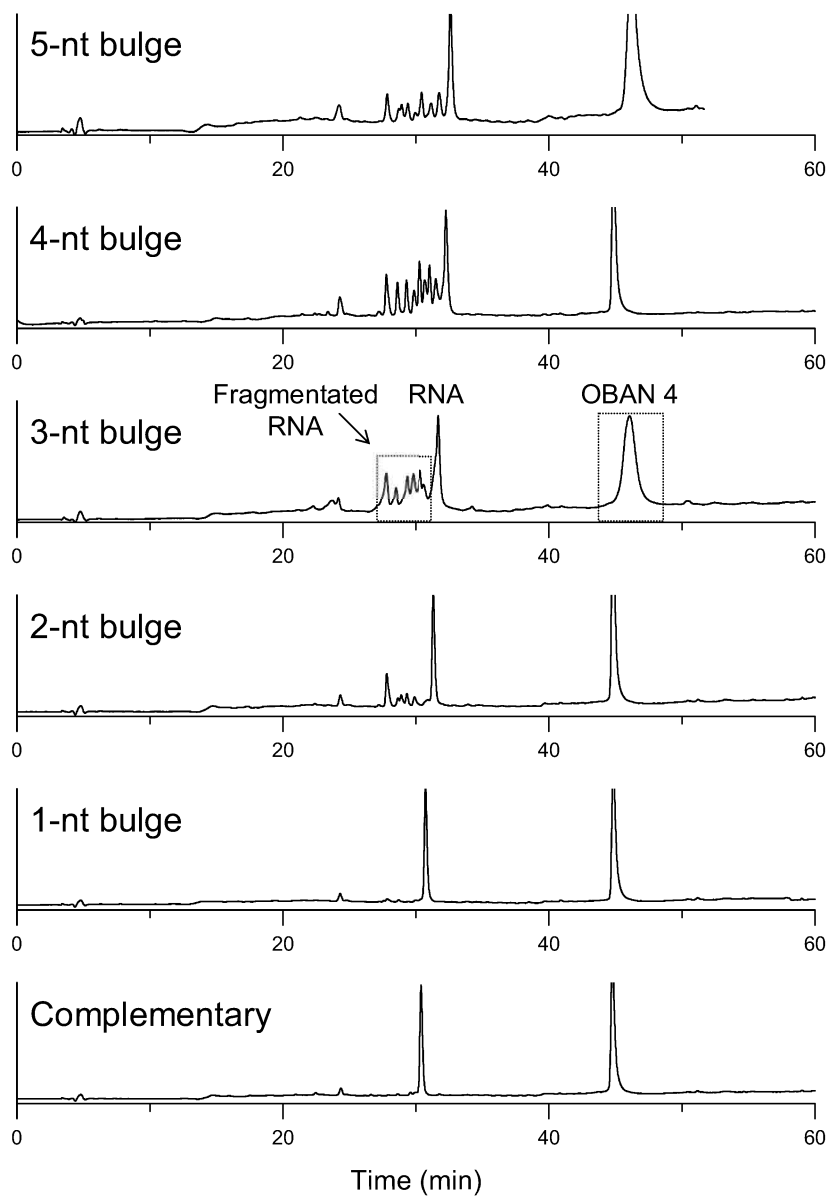


Figure 20: HPLC analysis from the analysis of reaction mixtures of OBAN 4 and RNA substrates after 24h, from the 5-nt bulge forming complex (top) to the complementary duplex (bottom). All reactions were carried out with a 4 μM conc. of both OBAN 4 and substrate RNA, in 10 mM HEPES buffer (pH 7.4) containing 0.1 M NaCl and 100 μM $\text{Zn}(\text{NO}_3)_2$

coordinate the same metal ion and thus form a sandwich complex that is likely to be inactive. Such interactions have been observed for 1,10-phenanthroline induced cleavage of RNA dimers e.g. ApA but was not believed to occur for the more steric 2,9-dimethyl 1,10-phenanthroline (neocuproine). 1:st order rate constants for the OBAN **1-5** are summarized in Table 1.

Table 1: Observed 1:st order rate constants (k_{obs}) summarized for the OBAN induced degradation of differently sized bulges.

Bulge size	$k_{\text{obs}} (10^6 \text{s}^{-1})$				
	OBAN 1	OBAN 2	OBAN 3	OBAN 4	OBAN 5
5	9.1 ± 0.2	7.5 ± 0.7	5.8 ± 0.1	9.5 ± 0.5	4.4 ± 0.1
4	17.1 ± 0.4	4.8 ± 0.3	5.1 ± 0.1	13.4 ± 0.5	3.3 ± 0.2
3	14.1 ± 0.2	6.2 ± 0.4	4.0 ± 0.1	9.3 ± 0.9	4.6 ± 0.5
2	3.3 ± 0.2	7.5 ± 0.4	2.4 ± 0.1	6.7 ± 0.3	n.d.
1	n.d.	n.d.	n.d.	n.d.	n.d.
0	n.d.	n.d.	n.d.	n.d.	n.d.

Experiments carried out in 10 mM HEPES (pH 7.4), 0.1 M NaCl and 100 μM $\text{Zn}(\text{NO}_3)_2$ (total ionic strength = 0.11 M). Substrate RNA and OBAN 1-5 were present at 4 μM , all reactions were incubated at 37°C. Due to some variation in melting points of the associated complexes the extent of bound RNA varies from 82 to 95%. Thus, differences in k_{obs} smaller than 10-15% are insignificant.

2.5 CLEAVAGE SELECTIVITY

The preferred cleavage sites for the two most efficient 4- and 3-nt bulges with OBAN 1 and the 4-nt bulge systems of OBAN 4 were studied in more detail by use of LC-MS. With OBAN 1 and the 3-nt bulge, a pronounced site selectivity could be seen. The phosphate linkage between A8 and A9 (counting from the 5' side of the substrate) was degraded to almost 70% (Figure 21).

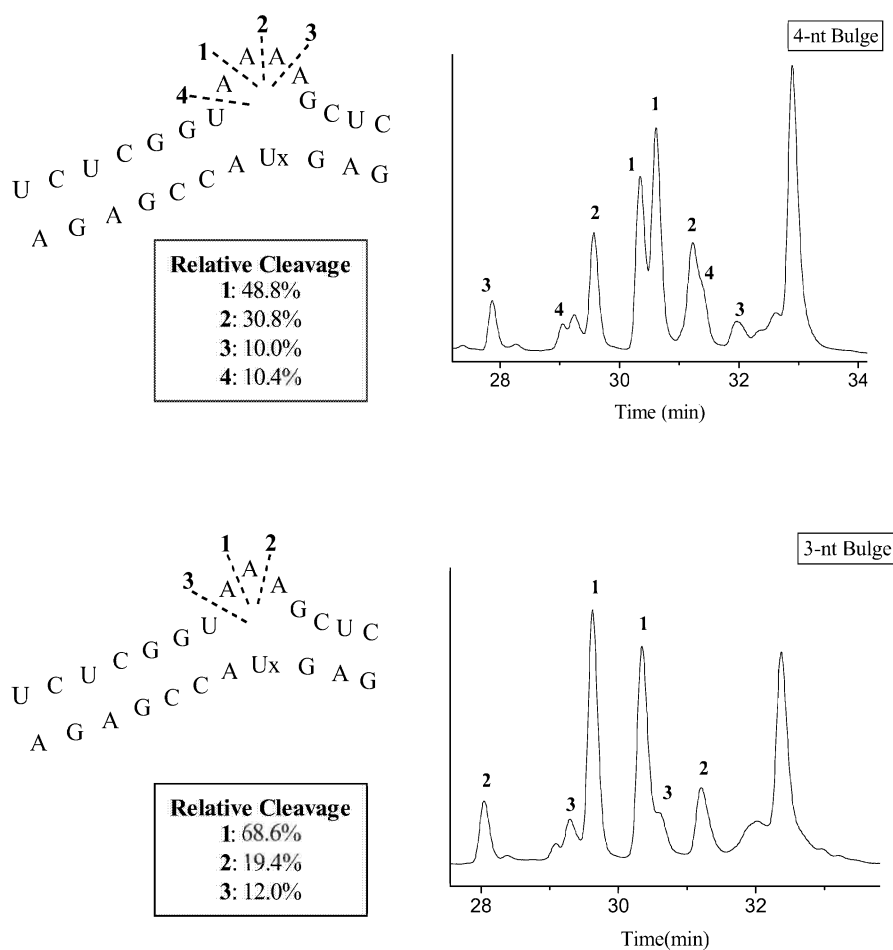


Figure 21: Preferred cleavage sites in 4-nt (top) and 3-nt (bottom) systems by OBAN 1. The numbers refer to specific cleavage sites on the bulge

Further cleavage takes place between A9 and A10 to an extent of 19% and between U7 and A8 to 12%. In the case of the larger 4-nt bulge the same general trend is seen, 49%

cleavage between A8 and A9, 31% cleavage between A9 and A10, whereas the phosphates between A10-A11 and U7-A8 are both responsible to 10% of the cleavage products. Interestingly, the phosphate expected to be the most vulnerable, considering the conformational preference in the published three dimensional structure, would be the one between A8-A9. The three dimensional structure is however determined for the 5-nt bulge system so the conformations of individual sugars may be quite different in the 3- and 4-nt bulges. In case of OBAN 4, there was little site selectivity which rather indicates that the positioning of the neocuproine is more responsible for the selectivity

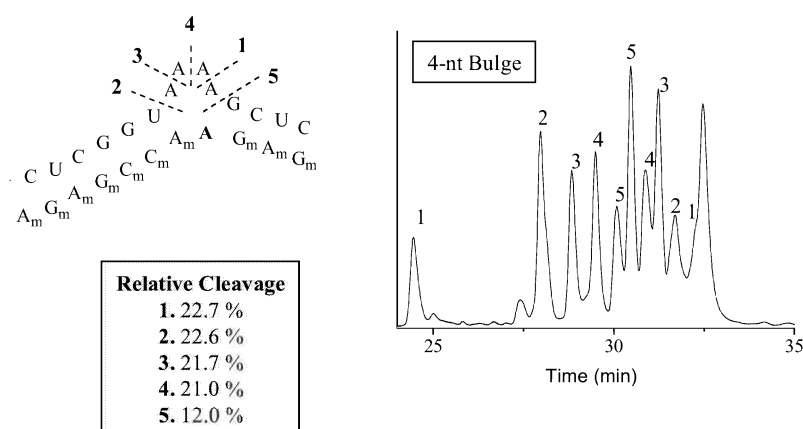


Figure 22: Preferred cleavage sites in 4-nt (top) and 3-nt (bottom) systems by OBAN 4. The numbers refer to specific cleavage sites on the bulge.

than vulnerability/conformational preference at a specific position. Figure 22 illustrates the cleavage sites on the 4-nt bulge induced by OBAN 4. Cleavage appears to be quite uniform between the first 4 phosphates in the bulge (from 5'-end). Relative cleavage around 21% is seen for all phosphates except for the last A-G pair that is degraded to an extent of 12%. As for the OBAN 1 systems and likely also for the other OBAN's, cleavage exclusively takes place in the bulged out region.

2.6 CONCLUSIONS

Five different OBANs have so far been evaluated. The higher efficiency of cleavage is shifted towards larger bulges. With none of the five OBAN's could cleavage of fully complementary or 1-nt bulge substrates be observed to any measurable extent. The fastest systems were the OBAN 1 and OBAN 4 designs which both degraded the 4-nt bulge most readily. For a 2-nt bulge target, however, the OBAN 2 design becomes interesting being the faster and more selective for this bulge-size. A major factor for differences in rate and selectivity is likely to be how well the catalytic group is positioned which is also displayed in the differences for substrate (bulge-size) preferences. What is quite clear though is that the difference in absolute numbers is remarkably small. The difference between the highest and lowest rate constants for all systems in cleavage of substrates forming 2-5 nt bulges is just short of an order of magnitude. One plausible explanation for the similarities in rate could be that the bulge

region is flexible and can be reached quite readily. This flexibility also means that the intramolecularity will be relatively modest. The phosphate-metal interaction is not likely to be strong enough to fix the bulge in a single conformation. This is also supported by the observation of more than a single cleavage site as well as that there is some correlation between site selectivity and overall rate. There are numerous possibilities to vary linkers and linker positions and this may well lead to improvements. However, it does not seem unlikely that these improvements will still be somewhat modest. This reasoning is further justified by comparison with other reported Zn (II) dependent, oligonucleotide based, cleavers. Komiyama achieved 5% cleavage in 3h using a binuclear (TPBA) system in 25 eq. excess to substrate RNA and 50 eq. Zn (II).⁹⁶ Putnam and Bashkin⁹⁷ degraded 25 % of a 28-mer and 18 % of a 159-mer RNA substrate in 15 and 10 h respectively using a 50 eq. excess of a neocuproine tagged oligonucleotide and the same concentration of Zn (II). Whitney et. al.⁹⁸ conjugated neocuproine to PNA backbones and cleaved 12-30% (internal and external phosphates) of an RNA substrate based on human telomerase in 12-24 h with 50 eq. PNA-chelate and 100 eq. of Zn (II). More recently, Niitymäki et.al. demonstrated catalytic turnover using an azacrown Zn (II) complex.⁹⁹ Substrate was present in 2 fold excess and Zn (II) in 20% excess to OBAN giving a half-life for cleavage of 20 h. With equimolar amounts of our fastest systems (see Table 1), i.e., with approximately 80-90% substrate saturation and about 70-80% Zn (II) saturation of the neocuproine complex, we obtain half-lives of 11-14h. Since the neocuproine complex does not give strong complexing of zinc (II) ions, the rates at lower zinc concentration are also somewhat lower. In any case the rates for most published systems display a very modest distribution, even though different catalytic groups have been used. A clear possibility to overcome this could be to introduce oligonucleotide modifications that specifically rigidify the bulged out region of the RNA substrate. If more interactions were introduced e.g. hydrogen bonds or stacking interactions between the OBAN and substrate site it is plausible that the bulge would be fixed in a single conformation. This conformation must however be suitable for intra molecular attack by placing the attacking 2'-hydroxyl in a suitable position for cleavage. A more active metal chelate should also be tested. A high binding constant for the metal is also preferred since that widens the perspective on what metals may be used in a biological application. Needless to say, future work in this field will be challenging and will also require help from precise molecular modeling. This would in turn benefit from structural determination of the present bulges by NMR and/or crystallography.

3 pKa DETERMINATION OF ATP'S AND SUBSTITUTED ADENOSINES

3.1 BACKGROUND

The importance of sugar modifications for the stability of antisense and OBAN based oligonucleotide systems have been discussed earlier in this thesis. The motivation for such work has been to achieve oligonucleotides that are stable in cell systems and have favorable association properties for complementary RNA sequences. In addition, incorporation of modified nucleosides in monomeric, dimeric as well as in oligonucleotides have found widespread use in investigations of enzyme mechanisms^{100,101,102}, nucleosides carrying modifications at the 2' and 3'-positions (e.g. amino, fluoro, thio, methoxy) have been of particular interest. Naturally, to understand the molecular basis of how a certain modification affect the physical properties at the nucleoside level is of importance. Of particular importance are the ionization properties of the nucleoside. The secondary hydroxyls take part in transesterification reactions at phosphate esters both as nucleophiles and leaving groups. In case of the self-splicing group I introns, the 3'-OH of an external guanosine residue attacks a phosphate in the first transesterification step for removal of the intron. A two metal ion mechanism has been suggested for the reaction^{103,104,105}. It has been suggested that a hydrogen bond between the 2'-OH and the 3'-oxygen would be of importance in catalyzing the transesterification^{106,107} reaction. Although a hydrogen bond between the 2'-OH and the 3'-oxygen has not been reported in water, it has been reported to be detected in aprotic solvents¹⁰⁸. The energy gain for such stabilization, especially when the 3'-oxygen carries a partial negative charge as it would in a transition state with the 3'-oxygen as a leaving group, should be reflected by differences in pKa between secondary hydroxyls in nucleoside derivatives. If the hydrogen bond is significant this effect should be enhanced when there is a fully developed charge on the neighboring oxygen and there would then also be a substantial difference in acidity between compounds having this

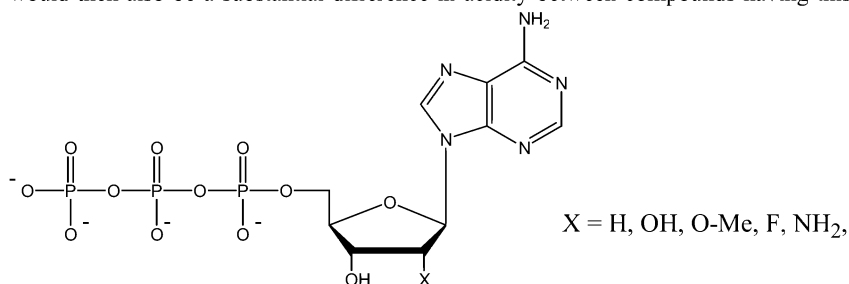


Figure 23: Structures of the 5 ATP derivatives for which the pKa of the 3'-OH was determined.

hydrogen bonding possibility and those without.

We have determined the exact pKa of the ATP derivatives in Figure 23 to supply more information for mechanistic work, especially to the discussion around the group I intron. In addition, we have determined the pKa values of adenosine, 2' and 3'-O-methyl adenosine (Figure 24) in MeOH and DMSO buffers. The catalytic site of an enzyme may render the local micro-environment more or less free of water, i.e., lipophilic and hence differences in pKa measured in water may not always be as relevant as those measured in organic solvents. The pKa measurements in aprotic media were intended

to compensate for such changes in lipophilicity and would be expected to enhance any intra-molecular hydrogen bonding since there is less competition from inter-molecular hydrogen bonding by the solvent.

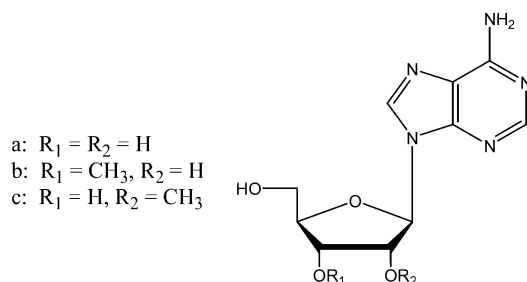


Figure 24: Structure of the adenosine derivatives for which the pKa was determined in MeOH and DMSO buffers.

3.2 DETERMINATION OF PKA

3.2.1 General considerations

All nucleoside derivatives were commercially available. Buffers were prepared having different pH and constant ionic strength. Natural abundant ^{13}C NMR shift titration was used to determine the pKa values. To enhance carbon sensitivity, DEPT 90 or DEPT 135 pulse sequences were used for the NMR measurements. For measurements carried out in aqueous and methanolic buffers we used an inner tube containing D_2O /pyridine for lock and reference respectively. DMSO buffers were prepared using deuterated DMSO. The pKa values were determined using the chemical shift variant of the linear form of the Henderson Hasselbach equation: $pH = pK_a - \log(\delta_H - \delta_{obs}) / (\delta_{obs} - \delta_L)$. The pKa values were then obtained from the intercepts of plots of $\log(\delta_H - \delta_{obs}) / (\delta_{obs} - \delta_L)$ vs pH, where δ_L corresponds to the chemical shift of the protonated (ROH) form at the lower plateau of the curve and δ_H to the deprotonated (RO⁻) form at the upper plateau¹⁰⁹. When the upper plateau of the titration curve was incompletely defined, the δ_H values were obtained from the relationship: $\delta_{obs} = \delta_H + 1/K_a (\delta_L - \delta_{obs}) aH^+$, thus the intercept from plots of δ_{obs} vs $(\delta_L - \delta_{obs}) aH^+$ gives δ_H . In cases where the upper plateau was reached, sigmoidal curve-fits could be used to verify the results obtained with the linear curve-fits.

3.2.1.1 ATP's in water buffers

The presence of the triphosphate function made the differently substituted ATP's sufficiently soluble in aqueous buffers. NMR studies confirmed that the triester function was intact throughout the time of measurement and over the whole pH range studied (pH 9-13.65). NMR measurements were performed and the ^{13}C shift of the C3' was plotted against pH.

As shown in the extrapolated titration curves in Figure 25, the upper plateau is only reached in the case of ATP and 2'-F-dATP, sigmoidal curve fits then agrees well with those obtained using the linear Henderson Hasselbach method (0.07-0.03 pKa units). In the other cases the upper end value needs to be iterated to perform a sigmoidal curve-fit and consequently the errors become larger (0.2-0.25 pKa units). It is generally more accurate to use linear evaluations of the pKa values. Figure 26 illustrates linear plots for 2'-NH₂ and 2'-O-methyl ATP.

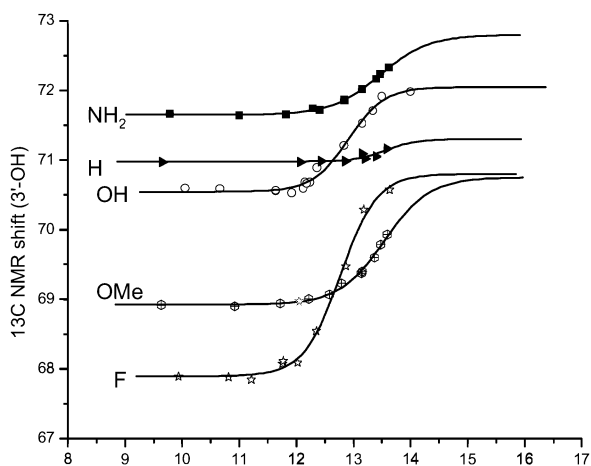


Figure 25: Plots of observed chemical shift as a function of pH for the differently substituted adenosines illustrated in Figure 23.

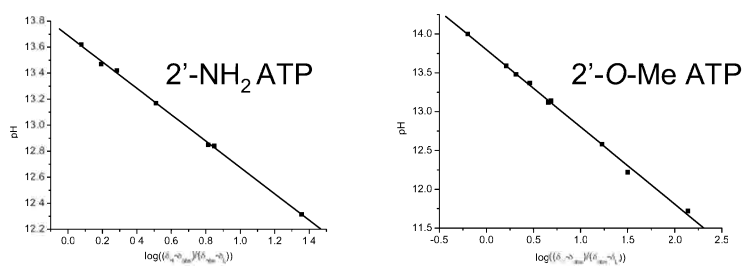


Figure 26: Linear regression analysis of plots of pH vs $\log(\delta_H - \delta_{obs}) / (\delta_{obs} - \delta_L)$ based on the ¹³C chemical shift (δ) changes with pH for 2'-NH₂ and 2'-O-Me in H₂O buffers.

The determined pKa values are summarized in Table 2.

Table 2: pKa values determined for substituted ATP derivatives in aqueous buffers.

Compound	pKa
ATP	12.98 ± 0.04
dATP	14.30 ± 0.13
2'-O-Me ATP	13.80 ± 0.02
2'-H ₂ N-dATP	13.69 ± 0.02
2'-F-dATP	12.74 ± 0.02

The pKa values were obtained by linear regression analysis of plots of $\log(\delta_H - \delta_{\text{obs}})/(\delta_{\text{obs}} - \delta_L)$ vs pH, where δ_L corresponds to the chemical shift of the protonated (ROH) form at the lower plateau of the curve and δ_H to the deprotonated (RO⁻) form

3.2.1.2 Adenosines in water, methanol and DMSO

The pKa values for the adenosines depicted in Figure 24 were further determined. The pKa values in aqueous buffers were determined as for the ATPs. Further, pKa values were also determined in methanol buffers (pH_{MeOH} 10-16.6) and in DMSO buffers (pH_{DMSO} 25.2-27.8). In all cases the pKa values were as above determined by the chemical shift variant of the linear form of the Henderson-Hasselbach equation. Figure 27 shows shift vs. pH plot and the linear regression analysis for 2'-O-methyl adenosine in DMSO.

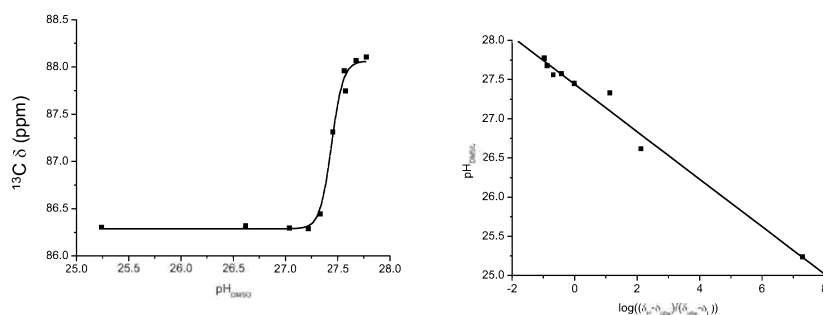


Figure 27: Plots of chemical shift vs pH_{DMSO} (left) and linear regression of plots of pH vs $\log(\delta_H - \delta_{\text{obs}})/(\delta_{\text{obs}} - \delta_L)$ based on the ¹³C chemical shift (δ) changes analysis for 2'-O-Me adenosine

Table 3 summarizes the pKa values determined for the adenosine derivatives.

Table 3: pKa values determined for Adenosines in water, methanol and DMSO

Buffer	Adenosine	2'-O-Me Adenosine	3'-O-Me Adenosine
H ₂ O	12.79 ± 0.05	13.53 ± 0.07	13.69 ± 0.09
MeOH	16.10 ± 0.09	16.80 ± 0.04	16.40 ± 0.09
DMSO	27.18 ± 0.06	27.44 ± 0.01	27.57 ± 0.02

The pKa values were obtained by linear regression analysis of plots of $\log(\delta_{\text{H}} - \delta_{\text{obs}})/(\delta_{\text{obs}} - \delta_{\text{L}})$ vs pH, where δ_{L} corresponds to the chemical shift of the protonated (ROH) form at the lower plateau of the curve and δ_{H} to the deprotonated (RO⁻) form

3.3 DISCUSSION

The acidities of the secondary hydroxyls of the ATP's (Table 2) follow a trend that is related to the electron withdrawing properties of the 2'-substituent. The presence of a vicinal hydroxyl does not seem to have a large effect on the pKa. The Δ pKa value between ATP and 2'-O-Me-ATP is less than one pH unit (0.8). The hydroxyl being more electron withdrawing than the methoxy and the 2'-OH more acidic than the 3'-OH, suggests that the stabilization of the oxyanion by the 2'-OH is either absent or very small. When evaluating the other modifications by plotting pKa's vs group electronegativity¹¹⁰ it can be seen that there is a good linear correlation. This suggests that the main influence on the pKa values is through inductive effects from the vicinal position. The possibility that the hydrogen bonding interaction could be quenched by surrounding water was evaluated by pKa determinations of adenosine, 2'-O-Me and 3'-O-Me adenosine in methanol and DMSO. The differences in relative acidities between the compounds in Figure 24 are similar in all solvents. Noteworthy is also that DMSO which could be expected to be the solvent where the biggest effect on pKa would be achieved actually displayed the smallest relative difference in pKa values.

The stabilization of a transition state involving a partially charged 3'-oxygen by hydrogen bonding to the 2'-hydroxyl is likely to be very small or insignificant. It is however possible that the 2'-OH can contribute to catalysis in such reactions by hydrogen bonding to a phosphoryl oxygen or through a coordination of a metal aquo ion.

4 STUDIES ON PHOSPHOTRIESTER TRANSESTERIFICATIONS RELATED TO THE GROUP I INTRON DERIVED RIBOZYME CATALYSIS

4.1 BACKGROUND

Model compounds have greatly increased the knowledge that we have about the reactivity and mechanisms by which phosphate esters are processed in biological systems. In the present case three phosphotriesters have been synthesized and studied. The structures of these compounds are displayed in Figure 28.

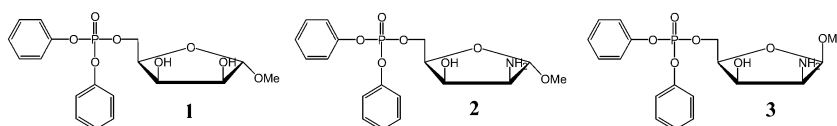


Figure 28: Structures of the triester model compounds that were synthesized and studied as model compounds for the metal ion catalyzed transesterification of the group I intron

These compounds can serve as model compounds for a part of the event when large catalytic RNA's (e.g. from group I introns) carry out metal ion catalyzed transesterification of RNA. As confirmed by ^{31}P -NMR analysis the 3-OH will perform an intra molecular attack on the triester function in analogy with the 3'-OH of the external guanosine that performs an inter molecular attack in the first transesterification reaction of the group I intron derived ribozymes. Figure 29 shows the cyclization step for the model compounds.

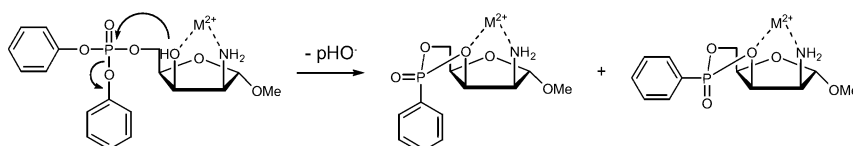


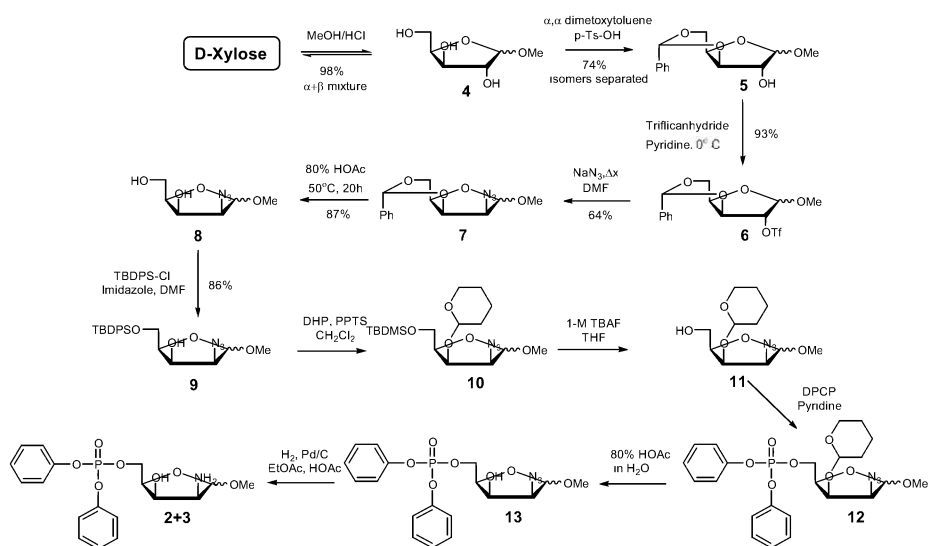
Figure 29: Intra-molecular metal ion catalyzed cyclization step studied for the model compounds. M^{2+} symbolizes a hypothetical divalent metal ion catalyzing the reaction.

In the ribozymes the nucleophilicity of the incoming guanosine hydroxyl is believed to be complex resemble that of a triester¹¹¹. The model compounds have either an amino enhanced by an increased degree of ionization through coordination of a metal ion. In addition we are suggesting that the mechanism may involve a two-metal ion complex that gives an intermediate / TS resembling a triester. Studies leading to this hypothesis involved use of a 2'-aminoguanosine as co-substrate in order to map the metal ion preferences of the reaction¹⁰⁵. Consequently, we include here model compounds that have an amino group vicinal to the attacking hydroxyl as well as a reference compound with two hydroxyl groups. Soft metal ions like Zn (II), Ni (II) or Co (II) are expected to coordinate the amino-group containing compounds and

accelerate rate of cleavage for these compounds compared to those of the corresponding lyxose derivative. The hard Mg (II) ion was not expected to provide much catalysis as it will bind poorly to the substrate. Furthermore, the amino compounds were prepared both as α and β methyl glycosides to detect any influence of the 1'-O-Me group. The presence of phenyl groups on the triester function increases the susceptibility towards cyclization and provides a convenient way of monitoring the reaction by the release of phenoxide ion ($\epsilon 270$) and makes potential acid catalysis of the leaving less important. Susequent inter-molecular cleavage of the second phenoxide group that can disturb the analysis could be suppressed by performing the reaction in a 50 / 50 water / dioxane buffer at pH 5.

4.2 SYNTHESIS OF MODEL COMPOUNDS

The model compounds were synthesized according to Scheme 4 by a reaction sequence starting from D-Xylose. The methyl glycoside was produced by treatment of the sugar with methanol in the presence of HCl. The product was obtained as an α, β mixture (4) which, after chromatography, was reacted further with α, α dimethoxytoluene to form the benzylidene derivatives 5. The two anomeric forms could then be readily separated by chromatography.



Scheme 4: Synthetic pathway for the construction of the amino lyxose triesters.

From this point on, the two anomeric forms were reacted further separately. In the next two steps the azide precursor of the amine was introduced in two steps. First by formation of triflate 6. This compound was after purification by chromatography reacted with sodium azide to form azido derivative 7. At the same time the 2, 3-cis configuration is established. The benzylidene group was then removed by treatment with 80% acetic acid in water. TBDPSCl was further used to protect the 5-hydroxyl and a THP function was introduced at the 3-hydroxyl. The silyl ether was then cleaved

off and the resulting derivative **11** could after purification on silica be reacted with diphenyl chlorophosphate (DPCP) to create the protected triester compound **12**. The THP group was then removed by acidic treatment and finally the azide could be reduced to the corresponding amine (**2** and **3**) by catalytic hydrogenation in presence of Pd/C in EtOAc/HOAc. The products could then, after extraction, be used directly for the kinetic experiments. The lyxose derivative was synthesized in a similar fashion. After introduction of the glycosidic bond, was the, 5-hydroxyl protected as before. The hydroxyls in position 2 and 3 were subsequently protected as THP ethers, after which the silyl ether was cleaved off. The resulting compound was then reacted with DPCP to form the triester. After purification the THP groups were detached by treatment with 80% HOAc.

4.3 RESULTS AND DISCUSSION

The rate of reactions were followed by measurements of increase in UV absorbance with time. Due to insufficient solubility of several metal ions at neutral pH we chose to carry out this study at pH 6. The experiments were done in HEPES buffer (pH 6, set at 22°C). The ionic strength was adjusted to 0.1 with sodium perchlorate. 0.2 mM of each triester and 10 mM of either Mg (II), Mn (II), Zn (II), Co (II) or Ni (II). The reaction temperature was set to 50°C. Due to insufficient complex constants between the compounds and the metal ions, concentrations needed for saturation could not be reached and a single concentration has been used in these comparisons.

The intra molecular attack of the 3-hydroxyl on the diphenyl phosphate triester is catalyzed by metal ions and the relative rate constants for respective triester and metal ion are summarized in Table 4.

Table 4: Rate constants for metal ion catalyzed degradation of compounds 1-3

Metal ion	1 <i>α-lyxose</i>	2 <i>α-aminolyxose</i>	3 <i>β-aminolyxose</i>
None	1	1	1
Mg ²⁺	1	1	2
Mn ²⁺	3.3	11	26
Zn ²⁺	7	21	56
Co ²⁺	1.3	150	160
Ni ²⁺	1.2	93	120

Relative rate constants: Rate constants obtained without catalytic metal ions present are set to 1 and all other rate constants are then compared to the respective non-catalyzed reaction within the same group. Metal ions were present in 10 mM, ionic strength was set to 0.1 M, all reactions were carried out at pH 5.8 at 50°C.

The difference in rate between the two anomeric forms of the amino lyxoses (**2** and **3**) is minor and can largely be explained by relative differences in pKa between the α and β lyxofuranosides¹¹². The orientation of the 1-O-Methyl (α or β) does not seem to have an effect on metal ion coordination and the rate of cleavage. When comparing the relative observed rate constants obtained for the amino sugars with different metal ions, it is evident that the nitrogen preferring soft metal ions: Mn (II), Zn (II), Co (II) and Ni (II) have the most profound effect on rate enhancement while the hard Mg (II) ion hardly

induces any accelerating effect. The same general trend is observed for both anomeric forms of the amino sugars although the rates for the β -lyxo-derivative are slightly higher for all metal ions. This difference in rate is to be expected since the different anomeric lyxofuranoses display difference in pKa values. When the corresponding metal ion experiments were carried out on the non-modified lyxose, the same metal ion dependence could not be seen. Poorer binding constants for the cis-diol system compared to the amino ethanol system explain the observed results. What factors could then be of importance for efficient catalysis? The pKa of the coordinated metal-aqua ion is obviously important since a more acidic metal ion would help in ionizing the attacking 3-hydroxyl. Further, a higher metal ion-sugar binding constant would give higher concentration of the reactive species which certainly would accelerate the rate of reaction. However, when the rate constants are plotted against the pKa values of the metal aqua ions a linear correlation is only obtained for the less acidic metal ions. No binding constants are available for the model compounds used in this study but when comparing binding constants for various simple alcohols, phosphates and amino alcohols the effect of the amino group seems to totally dominating. Hence, a simple amine or ammonia can be used to get approximate relative binding constants for the different metal ions. Consequently, ammonia was chosen since binding constants were available for all metal ions and under the same conditions. When the rate constants are corrected for these binding constants there is no strong correlation between these and the pKa values for the metal aquo ions. Two alternative explanations for this can be considered. One is that other factors are counteracting the effect of the enhanced nucleophilicity. This appears to be less likely since the magnitude of the relative rate enhancement with the most active metal ions is quite substantial. A more plausible explanation is that there is no coordination to the 3-hydroxyl and thus little influence on the acidity. This suggests that the reaction is catalyzed through metal ion coordination to the anionic phosphoryl oxygen and concomitant stabilization of the anionic intermediate/transition state. It is not unlikely that this interaction could be dependent on atomic radii and coordination geometries of the specific metal ion and thus give rate differences that do not correlate with the pKa values of the aquo ions of the different transition metal ions, which is also found.

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