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Development of Oligonucleotide Based Artificial Ribonucleases 2'-*O*-MeOBAN's and PNAzymes

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to my parents

ABSTRACT

The present thesis is based on three parts. The first and second part describe the development of two different classes of metal-ion dependent artificial ribonucleases, 2'-*O*-methyl-ribonucleic acid based artificial ribonucleases (2'-*O*-MeOBANs) and peptide nucleic acid based artificial ribonucleases (PNAzymes). These studies may be regarded as a development of traditional antisense methodology. Antisense mediated inhibition of gene expression can be achieved by antisense oligonucleotide hybridization with the target mRNA causing a steric blocking or generating a substrate for the endogenous RNase H. In the case of artificial ribonucleases the catalytic transestrification unit is covalently attached to the oligonucleotide scaffold and this enables the catalytic cleavage of target mRNA without the assistance of cellular enzymes and allows for a larger variety of modifications to the oligonucleotide.

The developed 2'-*O*MeOBANs and PNAzymes are individually tailored artificial enzymes capable of sequence selective cleavage of target RNA. The general basis for the systems is that these artificial enzymes selectively hybridize with the target RNA, which brings the catalytic group to the vicinity of the scissile phosphodiester linkage thus inducing cleavage of the RNA. Several 2'-*O*-MeOBANs and PNAzymes have been developed and evaluated with respect to cleavage of a model of the leukemia related M-BCR/ABL mRNA. What is special with these artificial ribonucleases is that they are designed to induce formation of a non-base paired region (bulge) in the target RNA upon binding which is advantageous since RNA bulges are more predisposed to cleavage than fully duplexed RNA. In addition, when the catalytic unit is incorporated inside the duplex region the cleavage of the target RNA creates shorter complementary fragments and catalytic turnover is provided.

Particularly interesting is a new class of Cu(II) dependent PNAzymes. These are highly site selective RNA cleavers displaying multiple turnover of substrate (PNAzyme:RNA, 1:100). The fastest PNAzymes displayed half-lives down to ½ h for target cleavage, which makes these the fastest and most selective artificial ribonucleases reported so far and these can be regarded to be RNA cleaving restriction enzymes.

The third part of this thesis concerns peptides that can affect hybridization of double-stranded oligonucleotides. Several different cationic peptides have been synthesized and investigated for their ability to influence the thermal melting of 2'-*O*-MeRNA/RNA and DNA/DNA duplexes. These cationic peptides were shown to selectively increase the thermal stability of 2'-*O*-MeRNA/RNA duplexes, while leaving the DNA/DNA hybrids unaffected. The dramatic difference suggest that, although electrostatic interaction plays a role, there is another major and structurally related component influencing the properties of these oligonucleotide duplexes.

LIST OF PUBLICATIONS

This thesis is based on the following articles and manuscripts, which are referred to by their Roman numerals:

I **Murtola M**, Strömberg, R. 2'-*O*-methyloligoribonucleotide based artificial nucleases (2'-*O*-MeOBANs) cleaving a model of the leukemia related M-BCR/ABL mRNA. *ARKIVOC*, **2009**, (3), 84-94.

II Sandbrink J, **Murtola M**, Strömberg R. Solid support post-conjugation of amino acids and a phenanthroline derivative to a central position in peptide nucleic acids. *Nucleosides Nucleotides Nucleic Acids*. **2007**, 26, (10-12), 1485-9.

III **Murtola M**, Ossipov D, Sandbrink J, Strömberg R. RNA cleavage by 2,9-diamino-1,10-phenanthroline PNA conjugates. *Nucleosides Nucleotides Nucleic Acids*. **2007**, 26, (10-12), 1479-83.

IV **Murtola M**, Strömberg R. PNA based artificial nucleases displaying catalysis with turnover in the cleavage of a leukemia related RNA model. *Org Biomol Chem*. **2008**, 6, (20), 3837-42.

V **Murtola M**, Strömberg R. PNAzymes that are artificial RNA restriction enzymes. Manuscript.

VI **Murtola M**, Zaramella S, Yeheskiely E, Strömberg R. Cationic Peptides that Increase the Thermal Stability of 2'-OMeRNA/RNA Duplexes, but that do not affect DNA/DNA hybridization. Manuscript.

Additional publications

Ora M, **Murtola M**, Aho S, Oivanen M. Hydrolytic reactions of 3'-*N*-phosphoramidate and 3'-*N*-thiophosphoramidate analogs of thymidylyl-3',5'-thymidine. *Org Biomol Chem*. **2004,** 2, (4), 593-600.

Yousefi-Salakdeh E, **Murtola M**, Zetterberg A, Yeheskiely E, Strömberg R. Synthesis of 8-aminoadenosine 5'-(aminoalkyl phosphates), analogues of aminoacyl adenylates. *Bioorg Med Chem.* **2006**, 14, (8), 2653-9.

Murtola M, Strömberg R. Development of 2'-*O*-methyloligoribonucleotide and peptide nucleic acid based artificial ribonucleases. *Nucleic Acids Symp Ser*. **2007**, (51), 201-202.

Milton S, **Murtola M**, Sandbrink J, Yeheskiely E, Strömberg R. Making oligonucleotide conjugates and breaking oligonucleotides. *Nucleic Acids Symp Ser*. **2007**, (51), 61.

CONTENTS

LIST OF ABBREVIATIONS

A	Adenosine
Ac	Acetyl
Alloc	Allyloxycarbonyl
AMP	Adenosine monophosphate
AS-ON	Antisense oligonucleotide
Bz	Benzoyl
C	Cytidine
CE	Cyanoethyl
CPG	Controlled pore glass
Dap	Diaminopropionic acid
DCM	Dichloromethane
DMM	*N,N*-dimethylaminomethylidene
DMSO	Dimethylsulfoxide
DMT	Dimethoxytrityl [*bis*(4-methoxyphenyl)(phenyl)methyl]
DNA	Deoxyribonucleic acid
DVB	Divinylbenzene
Fmoc	9-fluorenylmethoxycarbonyl
G	Guanosine
HBTU	*O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HEPES	*N*-(2-hydroxyethyl)piperazine *N*'-(2-ethanesulphonic acid)
HPLC	High performance liquid chromatography
HPNPP	2-hydroxypropyl *p*-nitrophenyl phosphate
IE-HPLC	Ion exchange HPLC
*i*Pr	iso-propyl
LNA	Locked nucleic acid
M-BCR/ABL	Major brakepoint cluster region/Abelson tyrosine kinase
Me	Methyl
MES	2-(*N*-morpholino)ethanesulfonic acid
miRNA	micro RNA
MOE	Methoxy ethyl
mRNA	messenger RNA
MS	Mass spectroscopy
Mtt	4-methyltrityl
NMP	*N*-methylpyrrolidone
OBAN	Oligonucleotide based artificial nuclease
PEG	Poly(ethylene glycol)
PNA	Peptide nucleic acid
PNAzyme	Peptide nucleic acid based enzyme
PS	Polystyrene
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNase	Ribonuclease

RP-HPLC	Reverse phase HPLC
shRNA	short hairpin RNA
siRNA	small interfering RNA
T	Thymidine
TEA	Triethylamine
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TIS	Triisopropylsilane
T_m	Thermal melting temperature
U	Uridine
UNPP	Uridine 3'-*p*-nitrophenyl phosphate
UpU	Uridylyl-3', 5'-uridine

1. Introduction

1.1 Stability of RNA

Ribonucleic acids (RNA) are commonly thought of as intrinsically very unstable molecules for the reason that trace contaminations by natural ribonucleases cleave RNA phosphodiesters very effectively. In fact, phosphodiester linkages, those that join the nucleosides of DNA and RNA, are highly resistant to spontaneous hydrolysis.(*1*) It can be estimated that apparent water attack on the phosphorus atom of simple dialkyl phosphate anions (DNA phosphodiester mimics) have extrapolated rate constants typically around $k_{25} = 7 \times 10^{-16}$ s^{-1}, equivalent to a half-life of 31 million years at 25 °C.(*2*) It is also assessed that the spontaneous cleavage of DNA is dominated by pathways that occur through the formation of abasic sites, rather than by a P-O bond breaking mechanism.(*2*) Cleavage of RNA *versus* DNA is facilitated by the proximity of the RNAs 2'-hydroxyl to the phosphate diester. It has been approximated that RNA is cleaved 10^9 times faster than DNA under neutral conditions by hydroxide ion mediated cleavage.(*3*) In the absence of catalyst at neutral pH and physiological temperatures, RNA phosphodiester linkages have an estimated half-life of over 100 years.(*4, 5*) However, the rates of degradation of phoshodiesters are strongly influenced by the molecular environment. The secondary structure and base sequence influences the reactivity of phosphodiesters in RNA either by retarding the rate of cleavage or enhancing it.(*6-8*) Evidently, strong stacking between nucleic acid bases retards the transesterification reaction and not only the neighboring bases but also those further apart in the molecule contribute to this.(*6, 7*) Interestingly, the long half-life of RNA can be considerably reduced by catalyzing the phosphodiester cleavage with simple metal ion complexes or by RNA cleaving enzymes.(*9, 10*)

1.2 RNA transesterification

The mechanism of solvent or specific acid/base catalyzed (H_2O, H^+, OH^-) hydrolysis of RNA 3', 5'-phosphodiesters in aqueous solutions can involve two different intramolecular transesterification reactions (Figure 1): phosphate migration forming a 2', 5'-isomer of the starting material or cleavage to a 2', 3'- cyclic phosphate and release of the 5'-linked nucleoside, followed by the hydrolysis of the 2', 3'-cyclic phosphate intermediate to the 2'- or 3'-monophosphates.

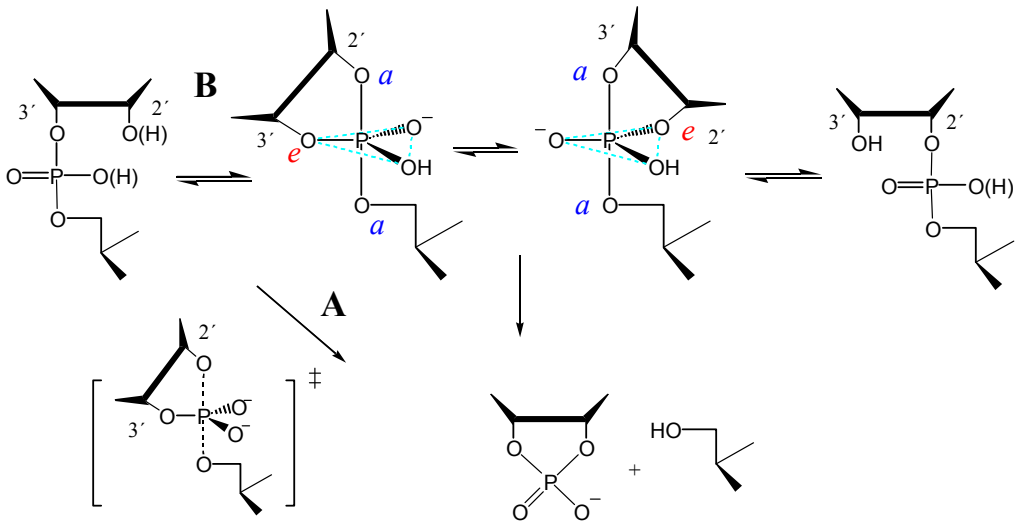


Figure 1. Cleavage and isomerization of RNA phosphodiester bond by intramolecular transesterification.

The reaction is initiated by a nucleophilic attack of the 2'-hydroxy group on the phosphate, which in principle could be a concerted process (A) or result in the formation of a pentacoordinated phosphorane intermediate (B) (Figure 1). The phosphorane intermediate may then decompose to give either cleavage or the isomerization products. The formation and breakdown of the phosphorane intermediate obey Westheimer´s rules.(*11*) Phosphorane intermediates have a trigonal bipyramidal geometry, with ligands occupying either apical (*a*) or equatorial (*e*) positions, apical P–O bonds being longer and weaker than equatorial P–O bonds. According to the rules proposed by Westheimer(*11*), the 2'-oxy nucleophile can enter and the 5'-oxy leaving group depart only through apical positions i.e., being co-linear with the phosphorus atom (in-line reaction). In the case of RNA, two of the ligand atoms are members of a same five-membered sugar ring, geometrically forcing one of them to be apical and one equatorial. Under acidic conditions RNA 3', 5'-phosphodiester linkages undergo competitive isomerization and hydrolysis, whereas in basic conditions phosphodiester hydrolysis is the only reaction detected. Displacement at phosphorus with good leaving groups can involve concerted mechanisms,(*12*) which could explain the absence of isomerization (path A, Figure 1). However, there is evidence that this is not the case for RNA and that hydrolysis even at basic conditions involves a phosphorane intermediate.(*13*)

Base catalysis

Under strongly basic conditions a dianionic phosphorane species would be generated. Due to its dianionic nature this phosphorane would have a high barrier towards pseudorotation, since in order to bring the 5'-oxy leaving group to an apical position an electron rich anion would also have to occupy an apical position, which is strongly unfavored according to the apicophilicity scale(*14*). In addition the phosphorane dianion would be a high energy intermediate that decomposes rapidly to cleavage products before any isomerization is possible. Cleavage of the phosphate diester backbone is a transesterification reaction (rather than hydrolysis that would have water or hydroxide as a nucleophile), which is followed by hydrolysis of the 2', 3'-cyclic phosphate to 2'- and 3'-mono phosphates.(*15*)

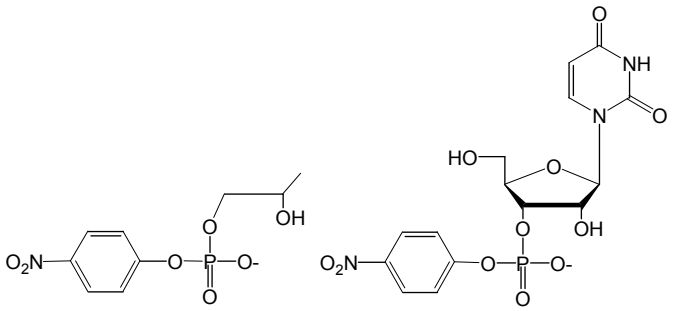
Acid catalysis

The transesterification reaction that predominates under neutral and acidic conditions has two possible alternative reaction paths. First a pentacoordinate phosphorane intermediate is formed upon the nucleophilic attack of the 2'-hydroxy function. The neutral or monoanionic phosphorane intermediates can undergo two possible reactions, cleavage or isomerisation *via* pseudorotation, accordingly forming a 2', 3'-cyclic phosphate and 5'-alcohol or the 2', 5'-isomer of the starting material. Under acidic conditions these two reactions are approximately as fast showing that the two energy barriers are approximately as high, where the phosphorane breakdown can be considered as the reverse of the nucleophilic attack.(*15*) Under neutral conditions the departure of the leaving group is clearly the rate limiting step of uncatalyzed cleavage.(*13, 16, 17*)

Metal catalyzed hydrolysis of RNA

It is known that metal ions have dual importance in stabilizing large structured RNAs and in catalyzing the hydrolysis of RNA phosphodiester bonds in catalytic centers of many RNA cleaving enzymes.(*18, 19*) To obtain phosphodiester hydrolysis in RNA within minutes, enormous rate acceleration is necessary (10^8-fold). Understanding of detailed mechanisms of metal catalyzed hydrolysis of phosphodiesters of RNA would be of help in the rational design of RNA cleaving artificial enzymes. RNA transesterification and hydrolysis is catalyzed by a wide range of metal ions like Mg(II), Ca(II), Fe(III), Co(III), Ni(II), Cu(II), Zn(II), Pb(II) and trivalent lanthanide

ions. The catalytic activity of the metal ion complexes generally depend on the identity of the metal ion and on the activity of the aquo ligands of the catalyst. Metal complexes with acidic aquo ligands are usually efficient catalysts.(*15*) In general it is difficult to study the hydrolysis of biologically important phosphate diesters with poor leaving groups (*i.e.* RNA oligomers) because of their inherent stability. For this reason, activated phosphate diesters with good leaving groups are often used for activity studies. Numerous studies on the mechanisms of metal ion promoted cleavages of phosphodiesters in simple artificial model systems or with RNA dimers have been carried out.(*4, 15, 20-25*) Metal ion promoted cleavage of aryl phosphates is clearly faster than for the corresponding alkyl esters and are commonly used to study possible metal ion interactions with the 2'-hydroxyl function (Figure 2). Despite the simplicity and convenience offered by these activated phosphodiesters, there are some drawbacks associated with their use as RNA phosphodiester models, e.g. metal ion binding sites might differ and the stability of the phosphorane is changed by the presence of an electron withdrawing group (good leaving group). In addition there is a change in rate-limiting step when going from a poor to a good leaving group in cleavage of RNA.(*13*)



HPNPP **UNPP**

Figure 2. Common model substrates used for studying RNA transesterification reactions.

There are three direct (or indirect) modes of activation by which metal ions can catalyze the hydrolysis of phosphodiester bonds; nucleophile activation, leaving group activation and rate accelerations due to Lewis acid activation. Cooperation between these activation modes is possible and would in principle give the highest rates of accelerations. One suggested mechanism for metal ion promoted cleavage of RNA phosphodisters is similar to the base-catalyzed reaction.(*15*) This and other plausible alternatives are shown schematically in Figure 3. The fact that the phosphate migration

is usually not enhanced by metal ion catalysis suggests that the reaction is either concerted or the phosphorane intermediate doesn´t pseudorotate. The cleavage rate in the presence of metal ions is often enhanced upon increase of the pH. Mechanisms where a metal ion assist the deprotonation of the 2'-hydroxyl as a general base catalyst or lowers the pKa of the 2'-hydroxyl *via* direct coordination are possible. As departure of the leaving group is rate limiting in the absence of metal ions, assistance of this departure by intracomplex general acid catalysis or *via* direct coordination of the metal ion to the leaving group oxyanion would be an efficient way to catalyze the overall rate of the cleavage. If breakdown of an intermediate metal ion coordinated phosphorane (Figure 3, D) would be rate limiting one could expect the reaction to be pH independent as long as the pH is well below the pKa of water bound to the metal.

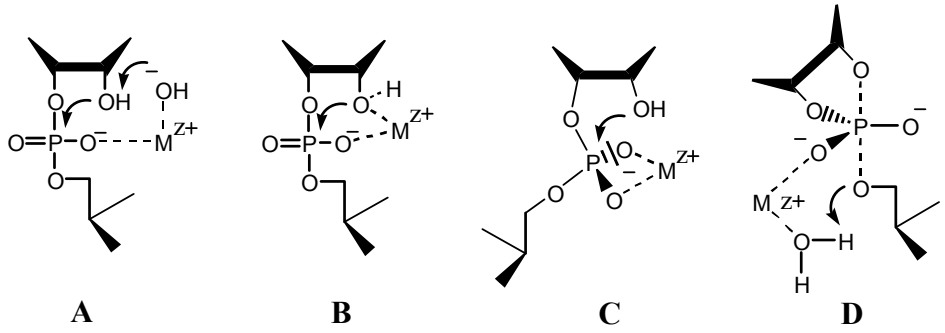


Figure 3. Possible mechanisms for metal ion catalyzed nucleophile activation (A, B), Lewis acid activation (C) and assistance of leaving group departure (D).

Metal ions and metal ion complexes could give Lewis acid activation by interaction with one or both phosphodiester non-bridging oxygens. By interaction with the phosphate oxygen(s), the phosphorus center becomes more electrophilic and thus susceptible to nucleophilic attack. The interaction should be more prominent in the dianionic phosphorane state which should stabilize this intermediate or transitions state. This is an important catalytic strategy and, in principle, dinuclear metal complexes could provide double Lewis acid activation for hydrolyzing phosphodiester bonds by bridging the two metal ions between the two phosphoryl oxygens.(*26*) The crystal structure of diphenyl phosphate where both non-bridging oxygens of the phosphodiester are bound to metal ions of a dinuclear complex has been reported.(*27*) The crystal structure data suggest μ-1,3-bridged coordination of the phosphate unit in this active complex, which then may provide double Lewis acid activation for the substrate (Figure 4). An interesting question is whether the reaction passes through a

transition state or a formal intermediate and whether such intermediates can pseudorotate.

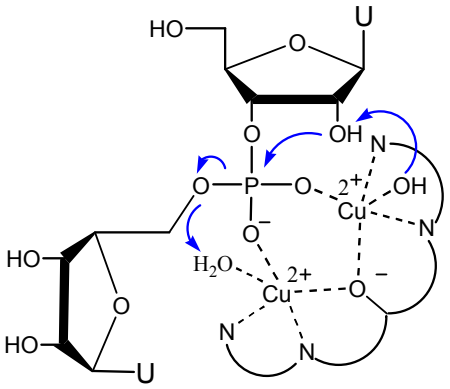


Figure 4. A proposed mechanism for a UpU transesterification reaction including double Lewis acid activation.

An example of metal ion catalyzed phosphodiester isomerization close to neutral pH was shown with UpU cleavage mediated by dinuclear metal ion complexes.(*28*) This observation implies that dinuclear metal ion complexes may stabilize the formation of a phosphorane intermediate sufficiently to allow it to pseudorotate. However, generally it´s difficult to quantify this mechanism because the hydrolysis reaction may proceed *via* a different mechanism, e.g. involving singe Lewis acid activation and metal hydroxide activation or single Lewis acid activation and leaving group activation.

1.3 Natural RNA cleaving enzymes

Studies on different natural RNA cleaving enzymes have revealed a substantial diversity in kinetic behavior as well as in catalytic mechanism. In many cases these enzymes use one or two divalent metal ions (e.g. Zn^{2+}, Mg^{2+}) close to each other in the active sites.(*18*) Alternatively, mechanisms relying on catalytic amino acid residues are common. This diversity of different catalytic strategies has complicated mechanistic studies of individual enzymes. Bovine pancreatic ribonuclease A (RNase A) is one of the classic examples of RNA cleaving enzymes and it has been the object of extensive studies. It is a relatively small protein (124 residues, ~13.7 kDa) and it was the first enzyme for which a correct amino acid sequence was determined.(*29-31*) RNase A catalyze the cleavage of P-O5' bonds in target RNAs. The geometry of the active center plays a significant role in substrate binding specificity and cleavage efficiency (Figure 5).

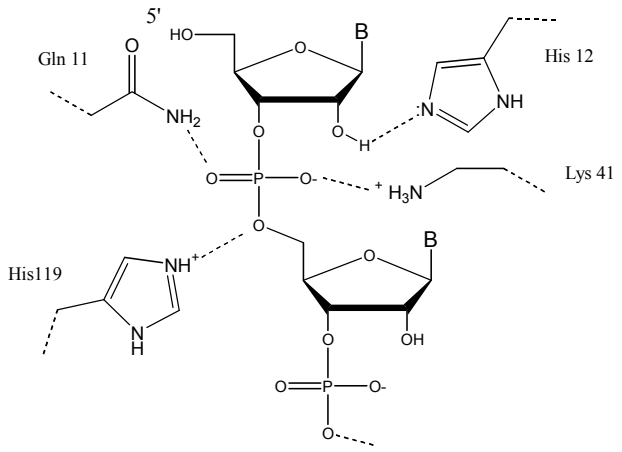


Figure 5. Schematic presentation of the catalytic center of RNase A showing the amino acid residues that play the key role in the catalysis.

Ribozymes

RNA molecules have an enormous potential for structurally complex folding and therefore have the capacity needed for forming different catalytic and binding sites. Catalytic RNA molecules, ribozymes, are an interesting class of RNA cleaving enzymes and recent discoveries of ribozymes involved in regulating gene expression in both bacteria and eukaryotes highlight their importance in biology (*32, 33*). Natural ribozymes catalyze often either the hydrolysis of their own phosphodiester bonds, or the hydrolysis/ligation of phosphodiesters of other RNA molecules.(*34-36*) These RNA molecules are also active components in ribosomes, capable of catalyzing the aminotransferase reactions.(*37*) Mechanistic studies of these enzymes are highly challenging for the reason that even slight changes in the structural conformation can have dramatic effect on the folding and activity of ribozymes. Seven different types of ribozymes are found in nature and those can be divided into two categories on the basis of their size and catalytic mechanism used in RNA cleavage: small and large ribozymes. (*38-40*) The first group consists of small catalytically active nucleic acids which range from ~40-160 nucleotides in length, generating products with a 2',3'-cyclic phosphate and a 5'-hydroxyl groups. This category includes the hammerhead, hairpin, hepatitis delta virus (HDV) and varkud satellite (VS) ribozymes. The second category, large ribozymes consist of several hundreds of nucleotides, including the self-splicing group I and group II introns as well as the RNA component of endoribonuclease P (RNase P). This category of ribozymes generates reaction products with a free 3'-hydroxyl and 5'-phosphate groups. The first category, small ribozymes, catalyzed RNA

transesterification reactions proceeds *via* attack on the phosphate of the vicinal 2'-OH group and the second category, large ribozymes, utilizes transesterification reaction initiated by attack of an external nucleophile on a 3',5'-phosphodiester bond. The external nucleophile can be a water molecule (hydrolytic reaction) or another 2'/3' OH-group.(*41*) Ribozymes typically require magnesium ions for catalytic activity, and in most cases the metal ions have been suggested to participate in the actual catalytic process and not only to play a structural role(*19*). The transesterification process is promoted by one or more divalent metal ions, magnesium being the most common choice, and maximum k_{obs} values are often 0.1-1 min^{-1} and can be up to ~10 min^{-1}.(*10, 42*) For example, RNase P, utilizes several Mg^{2+} ions at and in the vicinity of the cleavage site.(*43*) It is speculated that at least two Mg^{2+} ions are directly involving in the RNA cleavage, where a metal bound water molecule acts as nucleophile producing the 5'-phosphate and 3'-OH cleavage products.(*44*) The metal dependent RNase P plays an important general role in the processing of precursor RNAs in the cells, an important function being tRNA processing.(*44, 45*) On the other hand, ribozymes are not limited to the use of metal ions as functional groups in catalysis, but can also utilize nucleotide bases, sugar hydroxyls and phosphate backbone as groups that contribute to catalysis. The catalytic power of the known natural ribozymes is impressive, giving half-lives measured on a timescale of seconds, but is still relatively poor compared to efficient protein based enzymes,(*46*) e.g. RNase A that promotes the same phosphodiester cleavage reaction with a maximum k_{obs} of 80 000 min^{-1}, corresponding to a rate that is nearly 10^4 faster than the fastest ribozymes.(*47*) The stability of ribozymes is, however, limited due to the susceptibility to RNase mediated hydrolysis. These molecules have great potential to be used as effective biotechnological tools/therapeutic agents, but substantial improvements in the properties of the ribozymes are generally needed to make them more suitable for use *in vivo*. The mechanistic principles used by ribozymes can be utilized in artificial enzymes and an understanding of detailed mechanisms of ribozyme action could contribute to the development of artificial ribonucleases. Natural enzymes have advanced three dimensional structures but the efficiency in catalysis can be obtained with only a few active groups, since these can act in a concerted and/or complementary fashion and with high intramolecularity by exact positioning. In addition, structural events can influence the overall catalysis. It is quite a challenge to try to mimic these complex systems, but by using one or few of the factors contributing to catalysis, it is possible to build up relatively simple systems that are capable of cleaving RNA enzymatically.

1.4 Oligonucleotide based therapeutics and biotechnological tools

Oligonucleotide based applications have the potential to make revolutionary contributions to basic science and medicine. Particularly interesting are approaches to suppress expression of genes, which are linked to diseases, by oligonucleotide therapeutics,(*48-50*) Figure 6 summarizes different oligonucleotide based approaches. Oligonucleotides are suitable tools for the reason that they can be synthesized with sequence complementarity to a unique target and their properties can be tailored by chemical modifications. Problems related to these different applications are remarkably similar, concerning efficient delivery, enhanced stability, minimization of off-target effects and identification of sensitive sites in the target mRNA.

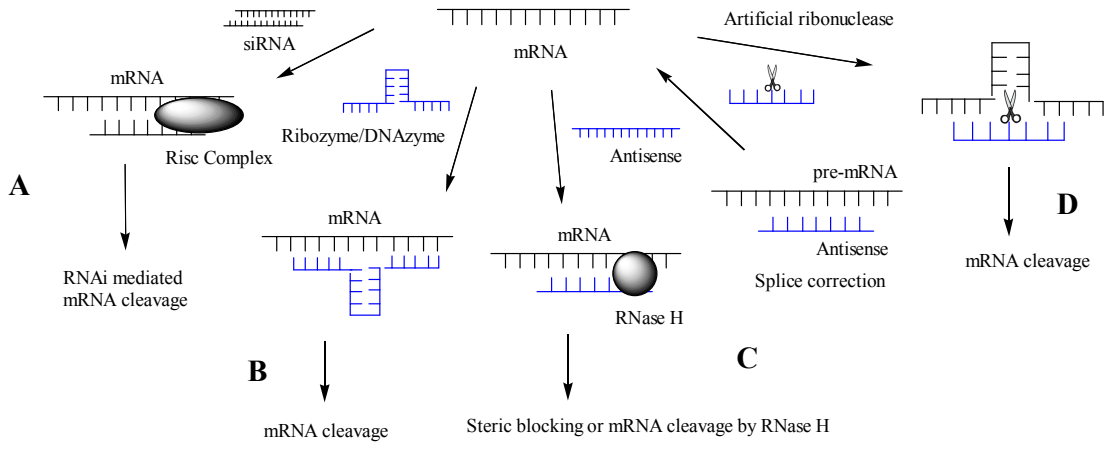


Figure 6. Approaches for the sequence specific knockdown of mRNA.

siRNA

The RNA interference approach (path A, Figure 6) has received an enormous attention in recent years and is one of the fastest developing approaches in molecular biology. Small interfering RNAs (siRNA) are short double-stranded RNA molecules that participate in a natural cellular post-transcriptional silencing mechanism called RNA interference (RNAi). This phenomenon was first seen in plants and later in invertebrates, and is believed to be an innate reaction to the foreign double-stranded RNA structures that result from viral infection. A multimeric complex of argonaute

family of nucleases called the RNA-induced silencing complex (RISC)(*51, 52*) [uses also short hairpin RNA (shRNA)(*53*) and micro RNA (miRNA)(*54*)] incorporates the antisense strand of the siRNA and degrades mRNA sequences to which it is complementary. Several clinical trials using RNAi to treat human diseases are currently ongoing worldwide.(*55*) However, the various protocols involved have not yet been optimized. Issues that need to be addressed so that therapeutic strategies can be optimized include; how to improve the systems that deliver synthetic siRNAs or expression vectors to the targeted cells, how to modify siRNAs for longer durability and how to minimize off-target effects.(*56*)

Synthetic ribozymes

An interesting class of potential oligonucleotide therapeutics is catalytically active ribozymes that degrade complementary mRNA molecules. Synthetic ribozymes (path B, Figure 6) became appealing for therapeutical and biotechnological use when it was proven that it is possible to produce trans-acting ribozymes directed against specific RNA sequences of interest. The idea of synthesizing ribozymes *in vitro* and introducing modifications in the natural ribozymes has been explored to broaden the use of ribozymes, extend the target pool, increase the nucleolytic stability, improve the accessibility to the target RNA and to elucidate the phosphodiester cleavage mechanisms.(*57, 58*) Development of *in vitro* selection has provided a powerful approach to explore the catalytic abilities of nucleic acid enzymes. *In vitro* selection combinatorial libraries of e.g. 10^{14} random oligonucleotide sequences are generated by solid phase synthesis using appropriate phosphoroamidate mixtures. In essence, designed selection methods separates the catalytically active molecules from the inactive sequences, and with each round of the selective amplification the ratio of active *vs* inactive molecules in the oligonucleotide pool is increased and finally narrowed down to a few active sequences. Also several allosterically controlled ribozymes, wherein the binding of an effector molecule controls catalytic function, such as biosensors(*59*) and nuclease-resistant synthetic ribozymes against therapeutically important targets(*58, 60, 61*) have been developed. For example Angiozyme, a chemically modified 35 nucleotide antiangiogenic ribozyme, has shown significant antitumor effect in animal models. Size is an important factor for ribozymes designed for therapeutic use. Synthetic hammerhead ribozymes are one of the most commonly used class of ribozymes for gene inactivation assays due to its small size and catalytic efficiency. The “hammerhead” motif, approximately 30 nucleotide long, is the

smallest natural endonucleolytic cis-acting ribozyme structure.(*62*) Incorporation of LNA nucleotides and other modified nucleotides in the binding arms at the distal ends of the ribozymes has allowed the shortening of the arms while keeping the hybridization sufficiently strong and holds promise for future development.(*57*) A close relative to modified ribozymes is autocatalytically operating RNA cleaving DNAzymes,(*63*) which also can be obtained by *in vitro* selection (also referred to as SELEX when used to isolate molecular recognition elements called aptamers).(*64-66*) In the future, rationally designed DNAzymes has the potential to manipulate the functions of biological systems, but currently available DNAzymes are more suitable for applications *in vitro*, e.g. utilizing DNAzymes as biosensors(*67, 68*) or molecular-scale computational elements(*69*). Aptamers are RNA/DNA based oligonucleotide therapeutics which can selectively recognize specific target molecules. The therapeutic potential of aptamers lies in the fact that, whereas other conventional oligonucleotide therapies targets the mRNA or chromosomal DNA *via* sequence selective hybridization, aptamers binds to various specific targets (e.g. small molecules or proteins) on the basis of 3D-structure complementarity.(*48*) SELEX with modified nucleotides may allow the possibility of generating active molecules with outstanding properties compared to conventional DNA and RNA structures.(*70*)

Antisense oligonucleotides

Oligonucleotides and oligonucleotide mimics which inhibit the expression of selected genes through Watson-Crick base pairing are commonly referred as antisense oligonucleotides (AS-ON) (path C, Figure 6) or gene knockdown agents.(*71, 72*) These are usually modified oligonucleotides which are considerably smaller in size compared to ribozymes. AS-ON strategies represent one of the most successful approaches in oligonucleotide based therapeutics.(*73*) The effect of synthetic AS-ON sequence for potential therapeutic purposes was shown by Zamecnik and Stephenson in 1978.(*74*) Antisense oligonucleotides block translation of target mRNAs in a sequence specific manner and in a broad meaning the modes of action can be divided into two main categories: antisense agents taking advantage of endogenous enzyme RNase H that degrade the cellular RNA, thus giving a turnover and re-use of the AS-ON, and AS-ONs functioning solely by a steric blockade mechanism *via* hybridization to the target. Possible mechanisms for steric blockade are translational arrest, inhibition or modulation of splicing of pre-mRNA and formation of a triple helix through hybridization to the genomic DNA, resulting in inhibition of transcription. The most

widely accepted/used mechanism is the generation of AS-ON/mRNA hetoroduplex structures that leads to the destruction of mRNA by Ribonuclease H. RNase H is an endonucleolytic enzyme present in the nucleus and cytoplasm of all cells, which hydrolyzes RNA in RNA-DNA duplexes. In antisense technology the AS-ON is taking the role of DNA strand mediating the RNA cleavage. Standard nucleic acids (RNA or DNA) are rapidly degraded by the action of exo- and endonucleases, so oligonucleotides used in these applications are usually modified in order to optimize their pharmacokinetic and pharmacodynamic properties. Considering the RNase H mediated mechanism, chemically modified antisense drugs that are designed to have optimal chemical qualities often do not retain the ability to activate RNase H as effectively. Generally, the chemically modified antisense oligonucleotides can be divided into three different generations (Figure 7).

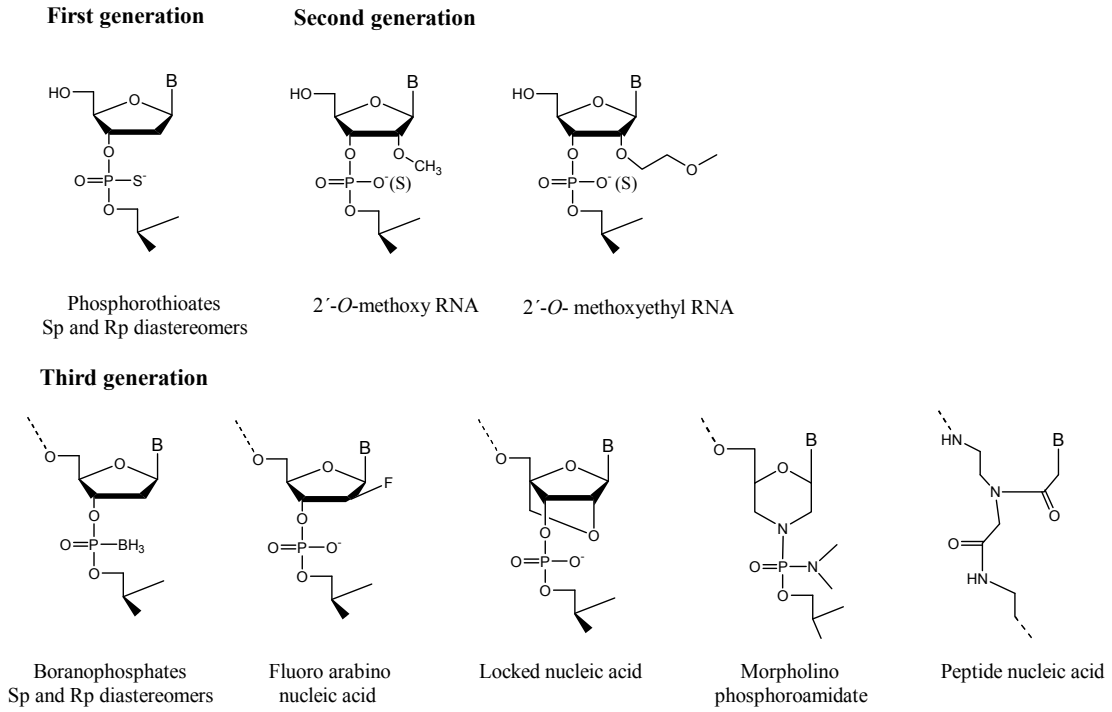


Figure 7. Examples of AS-ON modifications.

The first-generation AS-ONs (phosphorothioates PS-ON) confer enhanced nuclease resistance through a relatively simple change in the oligonucleotide backbone chemistry, (PS linkage instead of PO), by slowing down the nuclease action while still retaining the RNase H activity. Nevertheless, phosphorothioate-ONs are still relatively short lived *in vivo* showing some undesirable effects, like strong protein binding capacity.(*75*) Second-generation AS-ONs were designed with more modifications, such

as oligonucleotides containing 2'-*O*-methyl (2'-*O*-Me) and 2'-methoxy ethyl (2'-MOE) modifications, providing increased stability and tighter binding to the target RNA. These agents are only active through a steric blocking mechanism, unless "gapmer technology" is used. Gapmers contain a central block of deoxynucleotides (for. ex. PS-ON) which is sufficient to induce RNase H cleavage and flanking ends with 2'-modifications to protect the internal block from nuclease degradation and increase the duplex stability.(*76-78*) Third-generation AS-ONs usually contain modified phosphodiester linkages or totally different chemical moieties which replace the native ribose of the nucleotides. Peptide nucleic acids (PNA), morpholino phosphoramidates and locked nucleic acids (LNA) are among the most studied members of the third-generation. These analogues have been developed to improve the overall properties of antisense molecules e.g. target affinity, nuclease resistance and pharmacokinetics.(*79-81*) One of the interesting properties of PNA oligonucleotides is that polypyrimidine PNAs form stable triplexes with single-stranded oligonucleotides and can overcome the obstacles of strand invasion of double stranded-DNA. Strand invasion within duplex DNA by polypyrimidine PNAs can occur through formation of a four-stranded complex in which one PNA strand binds by Watson-Crick pairing and a second binds by Hoogsteen base pairing to the opposite direction. This strand invasion can be enhanced by use of bis-PNAs in which the two hybridizing strands are connected together by a flexible linker. The rate of formation of this stable $(PNA)_2$/DNA complex is greatly enhanced by reducing the entropic cost through linking PNA strands to form a bisPNA.(*82, 83*) The absence of multiple negative charges in the backbone of PNA and morpholino oligonucleotides also prevent binding to proteins that have a affinity for polyanions, thus avoiding one source of nonspecific interactions *in vivo*. In principle, properties of gapmers/mixed backbone AS-ONs can be modulated by combining appropriate segments and modifications at defined sites to optimize the effectiveness of these therapeutics. This kind of flexibility opens up possibilities in the antisense field. Developing short-length AS-ONs based therapeutics hold great promise and is likely to have wide applications in the future. However, many challenges remain. Improvements are needed for techniques related to oligonucleotide delivery, target selectivity, target finding and toxicity. Especially *in vivo* delivery is a remaining challenge. Accessing the cytosol or nucleus of cells through the surrounding membranes is one of the main obstacles for therapeutic oligonucleotides. It is speculated that PS-ON *in vivo* delivery may be rarely achieved and reported gene knockdown results may actually be due to various off-target effects.(*84*) There is an

ongoing research in the field of cellular delivery, heretofore the transport of negatively charged oligonucleotides across membranes is typically achieved with methods that can have toxic effects or poor efficiency, *e.g.* lipofectamine and microinjection. Alternative approaches are oligonucleotide conjugation with cell penetrating peptides (CPP) or other signal molecules, like 5' trimethyl cap-structures for nuclear localization,(*85*) that aim at development of systems where cellular uptake and intracellular distribution can be designed and optimized.(*86-88*) In addition, peptide nucleic acids and morpholino oligomers, which are highly stable and uncharged molecules, have been delivered by conjugation to various CPPs.(*89-91*) When toxicity is found for antisense agents it is often dose dependent. Information about the harmful effects of these compounds is hard to generalize, because many antisense oligonucleotides are chimeras of different modifications and toxicity is also dependent on the method of delivery.(*92*)

If only one specific RNA sequence could be recognized from the pool of different RNA sequences *in vivo* and selectively cleaved at a desired site with a low concentration of a stable antisense agent and without the help of cellular enzymes, this would open up new possibilities in therapy and biotechnology. Natural and chemically modified ribozymes are closest applications to reaching this so far, but the limitations are vulnerability to ribonucleases, need of special target sequences, target finding (steric hindrance by tertiary structure of target RNA), limitations in sequence-selectivity and structure complexity (especially for chemically modified ribozymes). Development of artificial ribonucleases, possessing sequence- and site-selective RNA cleaving activity, is an attractive alternative approach to ribozymes and step towards new therapeutic possibilities.

2. Artificial ribonucleases

2.1 Introduction to artificial ribonucleases

Artificial ribonucleases (path D, Figure 6) are synthetic mimics of natural ribonucleases. In an ideal case these agents would be able to cleave RNA oligonucleotides with high efficiency, site- and sequence-selectivity and catalytic turnover. These artificial ribonucleases could be used as conformational probes in the determination of large RNA structures or as customized RNA restriction enzymes in

molecular biology. In principle, due to the controllable sequence specificity they may even be used as therapeutic agents able to block the gene expression at the mRNA level. There has been quite some efforts in the development of these synthetic molecules capable of nonrandom RNA cleavage.(*93-96*) Artificial ribonucleases typically consist of two parts, a sequence recognizing moiety responsible for site- and sequence-selectivity and a catalytic group responsible for cleavage of the phosphodiester bonds. For oligonucleotide based artificial nucleases hybridization with the target RNA is the initial recognition event which increases the effective concentration of the catalytic moiety in the vicinity of phosphodiester bonds, converting the otherwise random cleavage of the target RNA to a sequence selective event (Figure 8). Duplex formation between this type of artificial nuclease and the target RNA can be exploited to shape the secondary structures of the target to the optimal for cleavage (e.g. to create bulges).

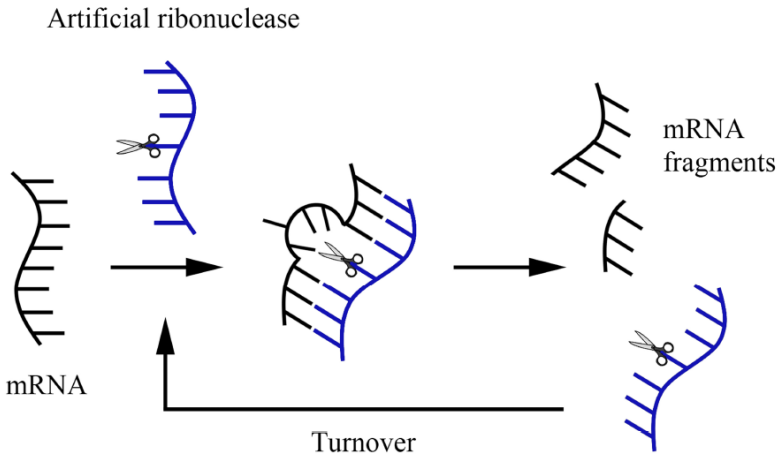


Figure 8. Schematic representation of artificial ribonuclease action.

The sequence recognizing moiety can in principle be any mimic or analog of DNA oligomers, similar to what is used for antisense technologies (Figure 7). Important properties are affinity, specificity, chemical and enzymatic stability i.e. as for antisense applications. The oligonucleotide based artificial nucleases (OBANs) developed so far can be categorized according to type of catalytic group. Most extensively used strategy makes use of metal ion chelates that cleave RNA phosphodiesters *via* transesterification reaction followed by hydrolytic cleavage of the produced cyclic-monophosphate (*94, 95, 97*). Another interesting approach is metal ion independent cleavers (*93, 94, 98-101*), using purely organic structures for

hybridization and hydrolysis of target RNA. A combination strategy using noncovalent interactions for activation of the target phosphodiester linkage and addition of an external catalyst also seems promising for *in vitro* use.(*102*) Catalytic turnover is a key issue in designing artificial ribonucleases. In many reported studies the catalytic unit is positioned at the termini of the sequence recognizing moiety and phosphodiester scission is performed outside of the complementary duplex region. This keeps the hybridization intact thus preventing the RNA fragment release and arrests or severely retards turnover. In a more developed systems the catalytic unit would cleave within the duplex region and upon cleavage of the target RNA shorter complementary fragments are created and catalytic turnover is more favored.

Lanthanide ion complexes have been demonstrated to be effective mediators of RNA phosphodiester backbone cleavage under physiologically relevant conditions.(*103, 104*) This is consistent with the Lewis acidity of the lanthanide ions and macrocyclic complexes are favored for their inertness to lanthanide dissociation. One of the most efficient class of artificial cleavers reported so far is lanthanide ion based conjugates, 5'-tethered Dy^{3+}-texaphyrin oligonucleotide conjugates, exhibiting a half-life of around 2 hours when used in excess compared to RNA target.(*105*) An internally derivatized Dy^{3+}-texaphyrin conjugate also exhibits catalytic turnover.(*106*) One of the most efficient artificial ribonucleases utilizing intracellularly occurring metal ion, Zn(II), as a monomeric metal complex is intrachain 2,9-dimethyl-5-amino-phenanthroline oligonucleotide conjugates showing half-lives around 10 hours.(*107*) Artificial oligonucleotide based cleavers using same chelate as Cu(II) complex has been used for sequence-selective RNA cleavage displaying similar half-lives, when used in excess compared to target RNA.(*108*) The high efficiency of natural metallonucleases often utilizes the cooperative action of two or more metal ions or other functional groups. This has inspired the development of RNA cleavers with multiple catalytic entities.(*9, 97*) Synthetic bimetallic artificial ribonuclease complexes have the possibility to take simultaneously advantage of different activation modes that metal ions can provide for the RNA transesterification reactions (Figure 3). For example, tethering of two azacrown-Zn^{2+} ligands to the 3'-end of a 2'-*O*-Me-oligonucleotide backbone has been reported to increase the cleaving activity 1000-fold compared to the free monomeric azacrown-Zn^{2+} complex. Half-lives of around 10 h are achieved at equimolar concentration of target and cleaving agent under physiological conditions, turnover is also shown when target is used in 4-fold excess compared to cleaving agent in spite of

the fact that the cleavage site is outside the complementary region.(*109*) 2'-*O*-Me-oligonucleotide conjugates with two contiguous terpyridine-Cu^{2+} complexes gave substantial rate enhancement suggested to be due to cooperative action of the complexes.(*110*) However, for any given combination of a specific artificial cleavers and its target RNA, the nature of the catalytic process and the importance of each individual step can vary dramatically and therefore complicate the rational design of artificial ribonucleases.

Design of metal ion-independent artificial ribonucleases(*93*) is exceptionally challenging, but an important objective. The systems described above have the advantage that metal-ion complexes are notably attracted to negatively charged phosphates, thus increasing the effective catalyst concentration in the vicinity of the scissile bond, while nonmetal systems can be more dependent on precise positioning. Metal independent artificial ribonucleases have several advantages for *in vivo* applications that motivates the development of this category of nucleases. Such ribonucleases are not dependent on that metal ions are available at sufficient concentrations in the intracellular environment or alternatively that stability constants are sufficiently high. Metal ion chelates may also retard cell penetration of oligonucleotide conjugates and increase the toxicity of these compounds. Attempts to develop metal ion-independent ribonucleases often involves peptide or peptide-like oligomers conjugated to DNA mimics; containing e.g. carboxy, imidazole, amino, amide or guanidinium groups arranged at specific distances from each other. At physiological conditions, some of the amino residues of these peptide mimetics exist as neutral amines, with potential to function as base catalyst, and some existing as protonated ammonium ions that could act as acid catalysts. The right positioning of active groups is crucial for the efficiency of these molecules. Development of purely non-metallic ribonucleases is a complicated task, with some reports giving motivation to further achievements in this field.(*98-101*) For instance, DNA oligomer based tris(2-aminobenzimidazoles) conjugates have shown to cleave RNA with half-lives of down to 12-15 hours, with substrate specificity and partial site selectivity.(*98*) Also PNA conjugates of neamine have been shown to cleave a 96-mer TAR RNA in the vicinity of its binding site at physiological conditions. Attachment of the neamine core imparts greater solubility and increase cellular uptake of the PNA.(*99*)

Artificial RNA cleaving enzymes could be used as restriction enzymes in molecular biology research, functioning as footprinting reagents to map the structure of nucleic acids or as accurate genotyping tools for single nucleotide polymorphisms and insertion/deletion polymorphisms. Combination strategies using "pinpoint activators" and an external catalyst has been successfully used for these purposes.(*111, 112*) These systems consist of two major components; a DNA based sequence recognizing moiety with an acridine as interchelating group for phosphodiester activation and an external catalyst that cleave RNA phosphodiester bonds randomly but the "pinpoint activator" converts this reaction to a sequence-specific by shaping the secondary structure of the target RNA which results in selective cleavage. Watson-Crick hybridization protects the stems against the external catalyst and acridine perturbs the conformation of the target RNA backbone by interchalation between adjacent nucleobases, which causes flipping out of the opposite base in an extrahelical position. A similar approach has been used by tethering phenylurea at the N6 position of deoxyadenosine by an amide linker. This modified nucleotide is incorporated into a suitable DNA strand which promotes site-selective cleavage of the target RNA in the presence of Mg^{2+}-ions.(*113*)

2.2 2'-*O*-Me-oligoribonucleotide based artificial nucleases *(Paper I)*

2.2.1 Design of 2'-*O*-MeOBANs

Optimizing the specificity of AS-ON binding to the given target oligonucleotide sequence is of special importance in the field of antisense and artificial nuclease applications. Specific target finding and in the case of artificial nucleases also the cleavage can be further enhanced by making the target sequence more sensitive. It is known that in a single-stranded form, which includes also bulges and loops, the RNA phosphodiester backbone is conformationally more flexible than in duplexes making it more prone to intramolecular cleavage by the 2'-hydroxyl function.(*114-119*) It was expected (and later confirmed) (*107, 110, 120*) that introduction of a certain degree of flexibility into an oligonucleotide based artificial nuclease (OBAN)-RNA complex may result in increase in cleavage efficiency. Flexible unpaired RNA bulges can more easily rearrange to adopt a conformation necessary for an in-line transesterification reaction to occur. The presence of several bulged out nucleotides in an otherwise complementary duplex leads to substantial destabilization of the complex.(*121*) It is known that 2'-*O*-modified nucleic acids in which the 2'-*O*-modifications drive the ribose to a preferential

C3'-endo (North-type) conformation show increased affinities to the RNA target (Figure 9).(*77*) 2'-*O*-methyl modification give considerable duplex stabilization and is notably more stable towards nuclease mediated degradation in cells especially when used in combination with phosphorothioate modifications. To be able to create thermally- and hydrolytically stable artificial ribonuclease-RNA target complexes 2'-*O*-methyl modifications were introduced to all nucleotides in the antisense strand, except for the cleaving group carrying nucleotide (dT).

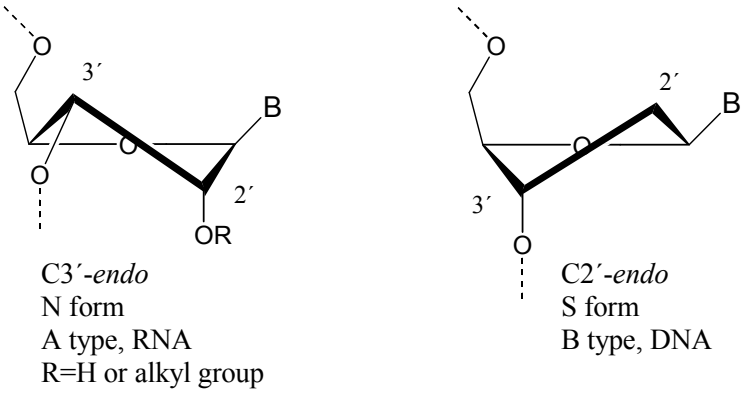


Figure 9. Preferred 2'/3'-endo sugar puckering in RNA, 2'-modified RNA and DNA, displaying the effect of electronegative 2'-substituents on the sugar conformation.

In our initial approach towards creating OBAN sensitive RNA targets(*107, 122*) assistance was taken from a model RNA where the 3D structure(*123, 124*) has been reported. The RNA structure with a five nucleotide (AAUAA) bulge loop originates from the Group I ribozyme domain of *Tetrahymena thermophilia*, and this structure was used to provide guidance in the design of the first artificial ribozyme mimics. (Figure 10. showing 5nt bulge complex). Goal was to find an optimum structure with respect to cleavage rate, selectivity and release of the RNA fragments after the cleavage event to enable catalytic turnover. It was of particular interest to use intrinsically less reactive but biologically relevant metal ions such as Zn^{2+}-ions.

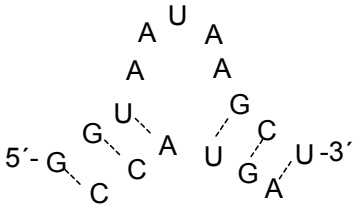


Figure 10. The original five nucleotide (AAUAA) bulge loop from the Group I ribozyme domain which was used for the design of 2'-*O*-MeOBAN-RNA complexes.

This structure contains wobble G·T (G·U if both strands are RNA) base pair(*125*) in the vicinity of the bulge (3'-side), as present in the parent structure. Interestingly, a wobble base pair at the site of cleavage in Group I introns is nearly universally conserved,(*126*) pointing out that a wobble pair near a cleavage site can substantially influence the rate of RNA transesterification. One distinctive property of G·U base pairs is the different geometry compared to ordinary base pair and the increased conformational flexibility. G·U pairs are found in somewhat different conformations depending on the environments and can be described as conformationally soft structures when compared to common Watson-Crick G·C base pairs.(*125*) For this reason the RNA structure can be more easily altered at sites containing wobble pairs, which then could contribute to phosphodiester cleavage. Interestingly, also RNAs that have been selected from a pool of random sequences (RNA libraries) using *in vitro* selection to perform different biological functions often contains G·U pairs.(*127-130*) We postulated that keeping the native G·T pair will introduce more conformational flexibility in the bulge. As expected, thermal melting point experiments (complexes between 2'-*O*-Me-oligonucleotides and target RNAs) show that the thermal melting stability decreases with growing bulge size.(*131*) Replacing the wobble base pair with the natural Watson-Crick G·C base pair increases the thermal stability somewhat but the difference in Tm is only about 1 degree for 4nt bulges and almost non-existent for a 5nt bulge.(*131*) Based on Tm-studies and additional molecular dynamics simulations(*132*) of these complexes it seems like the lower degree of hydrogen bonding in the wobble pair can be compensated by the more flexible ends that facilitate stacking within the bulge.

The reason for choosing the above model system was, apart from having an available 3D structure, the sequence similarity to a real potential therapeutic target, *i.e.* to the junction point of the M-BCR/ABL mRNA.(*133, 134*) In fact a 4A-bulge, very similar to the one that was cleaved most efficiently in the initial model system(*107*), can also be constructed with the BCR/ABL mRNA by choosing an appropriate antisense (OBAN) strand. The bulge consist of the actual junction point with two of the adenosines originating from the BCR part and two from the ABL part (Figure 11). Importantly, also the wobble G·T base pair at 3'-side of the bulge is present. Although there is some sequence differences these are mainly in the more rigid duplex stems and it was expected that much of the knowledge obtained in the model systems can be transferred with only minor differences in absolute efficiency of cleavage.

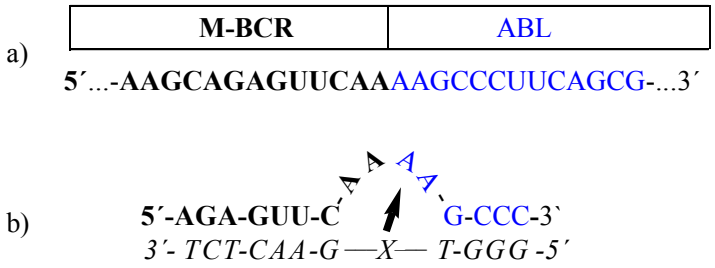
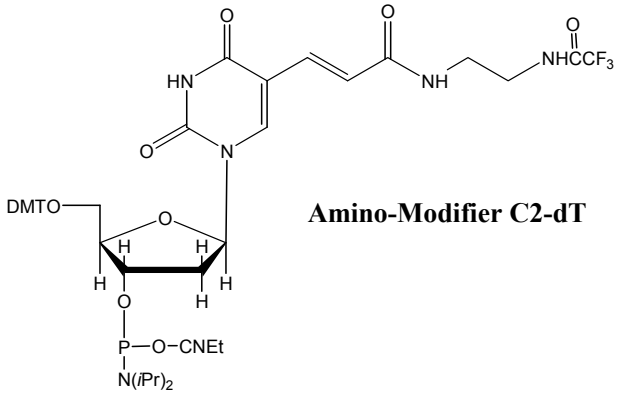


Figure 11. a) Schematic structure and sequence of the M-BCR/ABL mRNA fusion site. The sequence in bold originates from the M-BCR (Major Brakepoint Cluster Region) gene and the other part from the ABL (Abelson tyrosine kinase) gene (b_3a_2 transcript variant). b) OBAN-BCR/ABL model complex forming a 4 nt bulge loop.

2.2.2 Synthesis of 2'-*O*-MeOBANs

Synthesis 2'-*O*-methyl oligonucleotides (OBAN precursors) were performed on an Applied Biosystems A392 DNA/RNA synthesizer and oligonucleotides were assembled on pre-loaded CPG cartridges (2'-*O*-Me-U-RNA-CPG) using 2-cyanoethyl phosphoramidite monomers [5'-DMT-N-3'-P(OCE)N*i*Pr$_2$ 2'-*O*-Me N = U, A^{Bz}, C^{Ac}, G^{DMM}, Amino-Modifier C2-dT] at 1.0 µmol scale using the manufacturers protocols with 10 min coupling time.



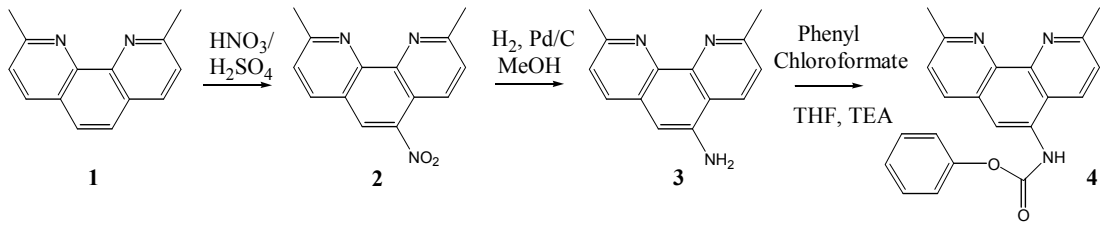
Amino-Modifier C2-dT

Figure 12. Amino-Modifier C2 dT building block

The amino-modifier C2 dT building block (Figure 12) was included to provide a free primary amine that subsequently could be conjugated to 5-amino-2,9-dimethyl-1,10-

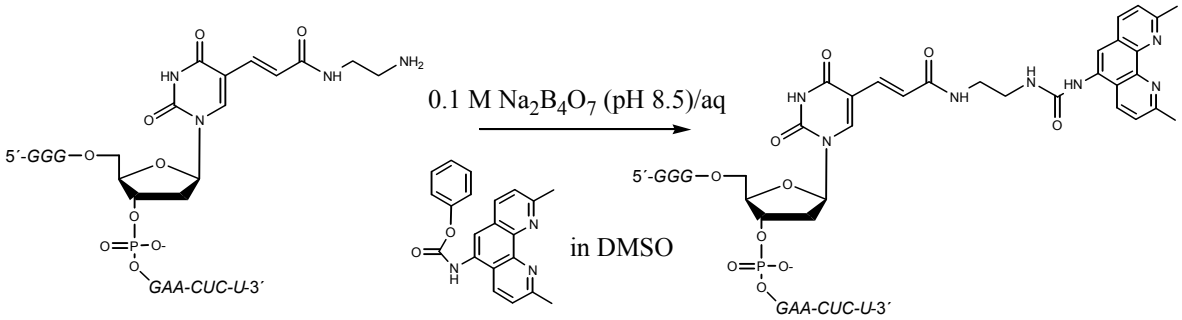
phenanthroline. The TFA protecting group can be removed during the standard ammonium hydroxide deprotection, but the presence of the amine functionality can cause a minor side reaction during ammonia deprotection and lead to irreversible capping (2-5%) of the amine. To prevent this side-reaction, 2'-*O*-methyl oligonucleotide precursors were synthesized using acetyl-protected cytidines and cleavage from the support was performed using 32% aqueous ammonia/40% aqueous methylamine 1:1 (AMA) for 10 minutes at room temperature, followed by CPG resin filtration and filtrate incubation at 65 °C for 15 min. Lastly the AMA reagent was evaporated and the crude product lyophilized.

Synthesis of the activated metal chelator, the phenyl carbamate of 5-amino-2,9-dimethyl-1,10-phenanthroline (**4**), started from neocuproine (**1**) and was synthesized as published.(*135, 136*) Activation of 5-amino-2,9-dimethyl-1,10-phenanthroline (**3**) was readily carried out with phenyl chloroformate in THF and the resulting carbamate was purified by silica column chromatography. (Scheme 1)



Scheme 1. Synthesis of activated metal chelate, phenylcarbamate of 5-amino-2,9-dimethyl-1,10-phenanthroline (**4**).

The conjugation reactions between **4** and aminolinker containing 2'-*O*-methyl oligonucleotide precursors were performed in solution at pH 8.5 to ensure sufficient deprotonation of the linker amino group ($-NH_3^+$) (Scheme 2). Excess of phenyl carbamate reagent was dissolved in DMSO and added to the aqueous solution of oligonucleotide precursor. Reaction proceeded quantitatively in 2 hours at room temperature and conjugated 2'-*O*-MeOBANs were purified with RP-HPLC. Five different aminolinker containing 2'-*O*-methyloligoribonucleotide precursors were synthesized and conjugated to form 2'-*O*-MeOBANs **1-5** (Figure 13).



N = 2'-*O*-methyl nucleotide

2'-*O*-Me-oligonucleotide precursor **1**

2'-*O*-MeOBAN **1**

Scheme 2. Conjugation of 2'-*O*-methyl oligonucleotide precursor **1** with *N*-phenylcarbamoyl-5-amino-2,9-dimethyl-1,10-phenanthroline (**4**).

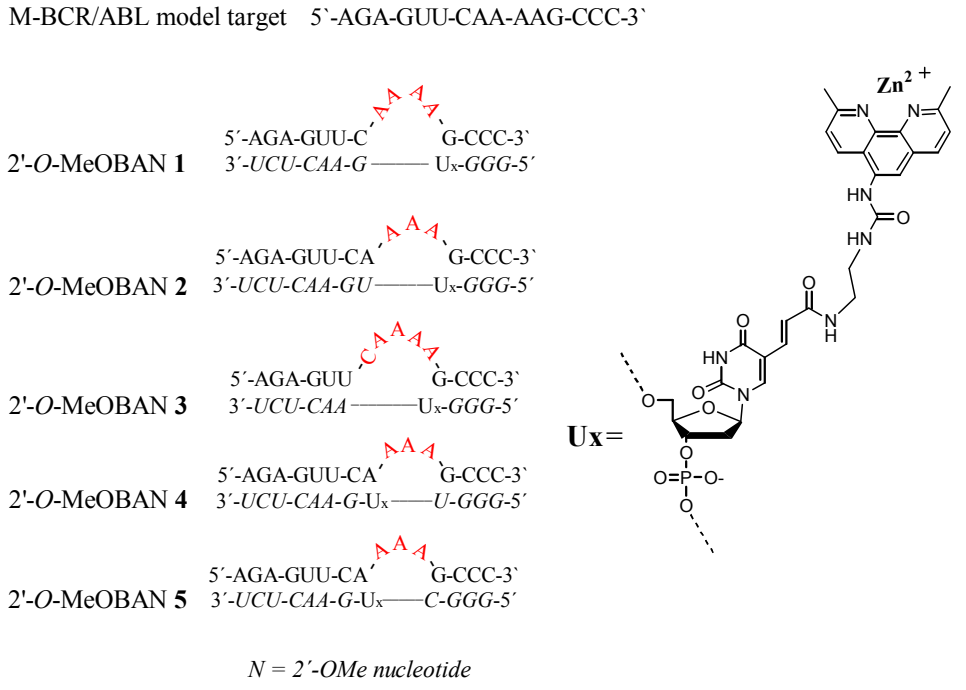


Figure 13. Different complexes of 2'-*O*-MeOBANs (**1-5**) with the M-BCR/ABL RNA model. $\mathbf{U_x}$= 5-amino-2,9-dimethyl-1,10-phenanthroline conjugated Amino-Modifier C2 dT.

2.2.3 Catalytic cleavage of M-BCR/ABL mRNA model

Zn(II) ions are biologically relevant metal ions that are known to enhance cleavage of phosphodiesters and are commonly used to study cleavage of RNA phosphodiester linkages. In the initial OBAN systems 100 μM zinc concentration was used to provide

largely saturated metal complexes. To be able to compare our experiments with these systems,(*107, 122*) we chose to use identical experimental conditions including the same concentrations of Zn(II)-ions. The kinetics measurements were performed at 37 °C, pH 7.4. The efficiency of 2'-*O*-MeOBANs **1-5** promoted cleavage of the M-BCR/ABL RNA model was evaluated by determination of the extent of RNA degradation using RP-HPLC analysis. The products were identified by MS and the observed first order rate constants for the RNA cleavage were determined (Table 1).

Table 1. Rate constants and half-lives for cleavage of the M-BCR/ABL mRNA model by 2'-*O*-MeOBANs **1-5** at 1:1 ratio (4 μM of each).[a]

	k_{obs} (10^{-6} s^{-1})	$t_{1/2}$ (h)
2'-*O*-MeOBAN **1**	13.8 ± 0.1	14
2'-*O*-MeOBAN **2**	22.6 ± 0.2	8.5
2'-*O*-MeOBAN **3**	4.2 ± 0.1	46
2'-*O*-MeOBAN **4**	9.6 ± 0.6	20
2'-*O*-MeOBAN **5**	9.0 ± 0.6	21

[a] The experiments were performed in 100 μM Zn^{2+}, 10 mM HEPES buffer, 0.1 M NaCl at pH 7.4, t = 37 °C. In the same buffer single stranded BCR/ABL RNA was cleaved at a rate of $2x10^{-6}$ s^{-1} and rates of cleavage of BCR/ABL RNA in presence of 2'-*O*-Me-oligonucleotide precursors 1-5 (1:1) were 1-2 $x10^{-6}$ s^{-1}.

The 3-A bulge system (2'-*O*-MeOBAN **2**) of the M-BCR/ABL RNA model was cleaved somewhat faster than any of the model systems. However, the rates are of the same order. This suggests that mainly the bulged out part of the RNA influences the cleavage rate and that the sequence in the duplex stems can be selected according to the sequence of the target RNA. The system which is most efficient in cleaving the M-BCR/ABL mRNA model also gives the most selective cleavage, showing 70% site-selectivity at the bulged out region of the RNA (Figure 14). MS analysis of the main cleavage site with 2'-*O*-MeOBAN **2** revealed that this is actually at the exact junction point of M-BCR/ABL.

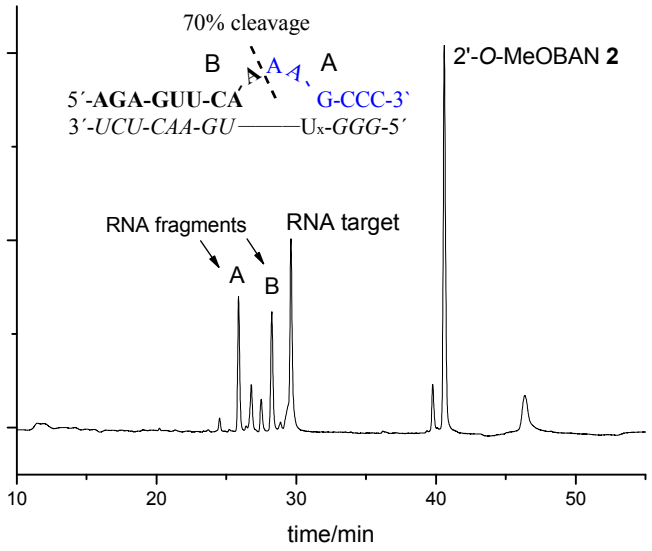


Figure 14. RP-HPLC analysis of the 2'-*O*-MeOBAN **2** promoted cleavage of the M-BCR/ABL RNA model. The insert shows the most prominent cleavage site as determined by MS of fragments A and B (ESI-TOF MS: A 1857; B 2923).

In previous studies 2'-*O*-MeOBAN systems were proven to behave as real enzymes capable of substrate turnover.(*107*) A limitation in these studies was that, as the amount of OBAN decreased, the excess of single stranded RNA (ssRNA) reacted sufficiently fast with free zinc aquo ions in a background reaction. One reason is that ssRNA is more prone to intramolecular cleavage by the 2'-hydroxyl function and ssRNA is present in excess during the turnover experiments. In order to reduce the background reaction with free zinc aquo ions we lowered the Zn(II)-ion concentration in the turnover experiments from 100 μM to 50 μM. Turnover experiments were done using 2, 4 and 10 times excess of RNA target *vs.* 2'-*O*-MeOBAN. In all cases our leukemia related RNA target was cleaved by 2'-*O*-MeOBAN **2** and the substrate was almost completely consumed demonstrating that turnover takes place.

Considering both this work and previous studies with 2'-*O*-MeOBAN, it seems that the 2'-*O*-MeOBAN sequence can be altered according to the specific target with cleavage rates essentially retained. These 2'-*O*-MeOBANs can be described as artificial enzymes as they display catalysis of the cleavage of RNA. These artificial enzymes have also proven to be versatile in accepting different RNA targets, including substrates with different bulge sizes, showing different preference for different bulges.

2.3 Peptide nucleic acid based artificial ribonucleases *(Papers II& III)*

2.3.1 Design of PNAzymes

In the 2'-*O*-MeOBAN studies we confirmed that the stem sequences of the double-stranded OBAN-RNA complexes can be altered according to the specific target with cleavage rates essentially retained. An interesting question was if we could also change the backbone of the OBANs to one with more desirable qualities while retaining the activity of OBANs. The most interesting candidate of several DNA mimics(*137*) was the peptide nucleic acid (PNA) structure which was first described by P. E. Nielsen *et al.* in 1991.(*138*) PNA´s backbone is composed of repeating N-(2-aminoethyl)-glycine units linked by peptide bonds. PNA is a structural analog of DNA in which the deoxyribose phosphate backbone has been replaced by an achiral polyamide chain (Figure 15).

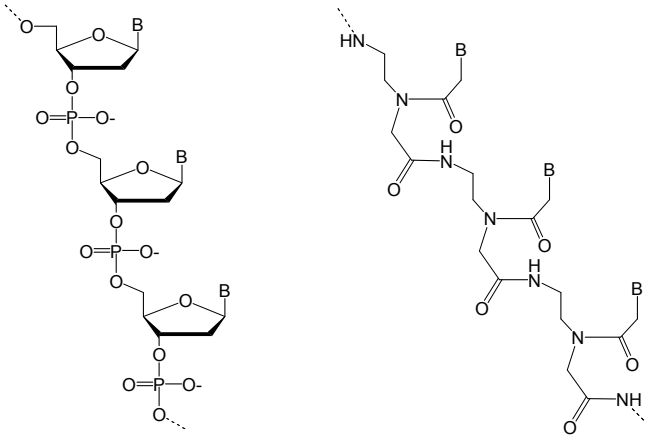


Figure 15. DNA and PNA oligomer backbone structures.

PNA was considered as a backbone because of its many interesting properties.(*139, 140*) PNA has been shown to hybridize in a sequence-specific manner with DNA and RNA targets and since the *N*-(2-aminoethyl)-glycine backbone doesn't carry negative charges, electrostatic repulsion of the target strand is avoided. PNA oligomers also form very stable duplexes with complementary RNA and DNA chains. This enables shorter PNA oligomers to reach the same affinity to the target RNA as longer 2'-*O*-MeOBANs have. However, the non charged PNA backbone gives both advantages and drawbacks. The neutral PNA oligomers have limited solubility in water and can form

hydrophobic aggregates. Conjugation to cationic amino acids is commonly used to enhance water solubility. Furthermore, PNA does not easily pass through cell membranes, limiting their applications as therapeutic agents. PNA technology is currently being refined with various delivery methods in order to overcome these limitations(*141-143*) and is tested, e.g. as antiviral agents against HIV.(*144*) We reasoned that since 2'-*O*-MeOBAN-RNA complexes, where the presence of several bulged out nucleotides leads to substantial destabilization of the complex, additional stabilization of the duplex could be beneficial and this would be possible with PNA modifications. In addition, a particularly attractive feature of PNA is its chemical stability and high resistance towards natural nuclease- or protease-mediated degradation. Moreover these oligomers can be synthesized by following solid phase peptide synthesis protocols(*140*) and can be readily modified with amino acids or other functional groups. This offers additional freedom in designing and optimization of PNA based artificial ribonucleases (PNAzymes). The simplest examples of PNA based RNA cleavers reported are urea linked diethylenetriamine conjugates.(*145*) A half-life of 8 h was reported at pH 7.0 (40 ºC, with excess of cleavers over RNA), displaying surprisingly high efficiency despite the relatively simple and flexible structure of cleaving group. In that report it was speculated that higher cleavage rates with the PNA based system compared to the corresponding DNA conjugates,(*100*) was attained by the decrease in the freedom of complex motion. The PNA backbone has also been used in non-catalytic cleavage employing 5-amino-2,9-dimethyl-1,10-phenanthroline as metal chelating agent.(*146*) This change to a PNA backbone did not give any marked change in activity, but the levels of RNA cleavage are as previously been observed for this Zn^{2+}-cleaving unit.(*147*) It was also shown that PNA-neamine conjugates exhibit RNA cleaving activity when used in 4-fold excess. In comparison to the above PNA systems, the observed cleavage of target RNA appears somewhat more efficient.(*99, 148, 149*) The polycationic neamine moiety, which is a part of the aminoglycoside antibiotic neomycine B, allows increase in cellular uptake, confers the RNA cleaving property and gives higher solubility of the PNA conjugate. These interesting reports encouraged us to investigate how PNA would behave as a sequence recognizing backbone structure for catalytic OBANs.

PNA complexes with RNA, where the former forms different bulges upon hybridization, are more forgiving that the corresponding 2'-O-MeRNA/RNA complexes, i.e., giving a less severe loss of thermal stability as bulges are introduced. In

addition, the introduction of a glycine unit in the PNA, (opposite a 3-4 nt RNA bulge) while keeping the rest of the PNA backbone intact, is accepted with very little further loss of complex stability (J. Sandbrink, R. Strömberg, unpublished data). This, then provides the possibility to introduce a handle for attachment of catalytic group by introducing an L-diaminopropionic acid (L-Dap) as linker position in place of the central glycine unit.

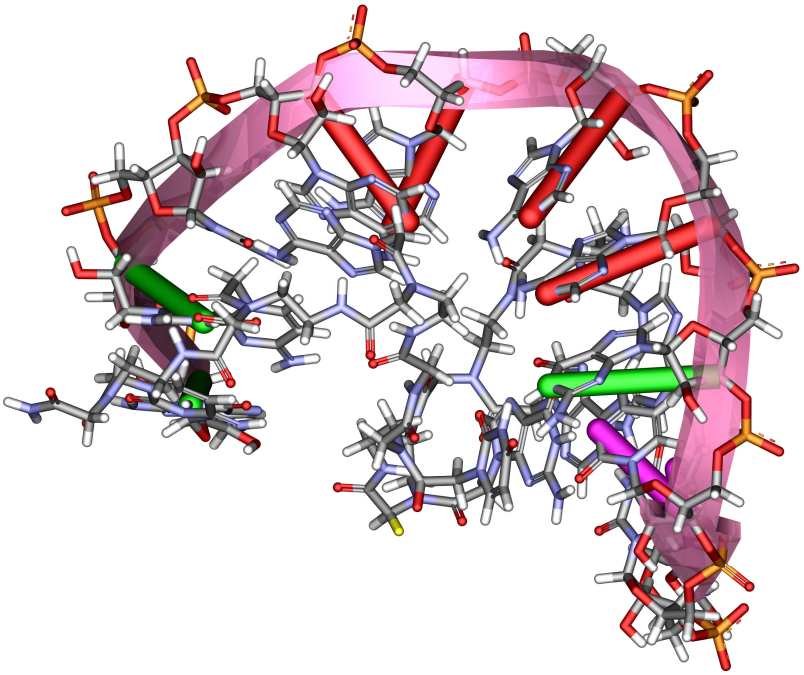


Figure 16. Model of a 4A-bulge RNA target hybridized with PNA-glycine-PNA oligomer (built in collaboration with Professor Lennart Nilsson and Dr. Boel Nyström-Macchion, unpublished data).

Molecular dynamic simulations of RNA-PNA complexes with internal glycine unit indicated that the structures are relatively flexible while still retaining similarities in base stacking compared to 2'-*O*-methyl-OBANs (unpublished data, Figure 16).

2.3.2 Synthesis and initial studies on PNAzymes

Solid support synthesis of PNAs is commonly done on functionalized polystyrene resin to which an amino-protected amino acid is attached *via* its carboxyl group. It is advisable to link PNA to the solid support *via* an amino acid in order to avoid the potential ketopiperazine type side-reaction during the first deprotection step, and the

addition of hydrophilic amino acids or short peptides greatly enhances the otherwise lower solubility of PNA compared to both DNA and RNA.(*138, 150-152*) We chose to use the basic amino acid lysine as PNA solubility enhancer, providing one positive charge at the *C*-terminus of PNA. TentaGel S RAM resin was used as solid support. This resin has a hydrophobic core, polystyrene crosslinked with divinylbenzene (PS-DVB), to which the rink amide is linked with a hydrophilic poly(ethylene glycol) (PEG) chain. This acid-labile support has good swelling properties(*153*) and cleavage from the resin is done by single step treatment with 95% TFA, providing peptide/PNA amides in high purities. In the present study we loaded the resin with Fmoc-lysine under conditions allowing the functionalization to be under 0.2 $mmolg^{-1}$. PNA sequences were assembled automatically on a solid support using Fmoc-chemistry (HBTU as coupling reagent) and following the manufacturer's protocol for the Applied Biosystems 433A peptide synthesizer. We synthesized modified PNA sequences (PNA-Dap-PNA) with an internally placed diaminopropionic acid (Dap) unit and with a glycine placed at the N-terminal end, both serving as amino linkers for post-conjugations for different phenanthroline derivatives.

Prior to the post-conjugation, the amino protecting group of the Dap-linker has to be removed selectively. Two different protecting groups, allyloxycarbonyl (Alloc) and 4-methyltrityl (Mtt), were evaluated for this purpose. The Alloc protecting group is reported to be fully orthogonal to Fmoc chemistry,(*154*) but in our hands, application of Alloc was accompanied by deprotection problems(*155*), as well as difficulties in getting the subsequent post-conjugation reaction to work. The post-conjugation resulted in very low yields or no product at all, possibly due to remains from the Alloc cleaving cocktail on the support. It is reported that reiterative deprotection of the Alloc groups with tin hydride and palladium reagents leads to the precipitation of palladium, which can catalyze the decomposition of Bu_3SnH, thus leading to different side reactions.(*154*) The highly acid-labile methyltrityl (Mtt) group on the other hand, was readily removed by mild acidic treatment without noticeable cleavage of the PNA from the solid support and the post-conjugation of 5-amino-2,9-dimethyl-1,10-phenanthroline was nearly quantitative (Figure 17).

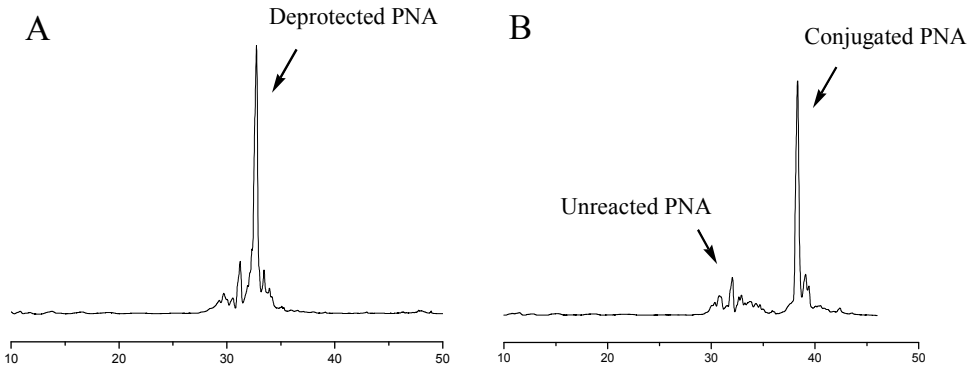
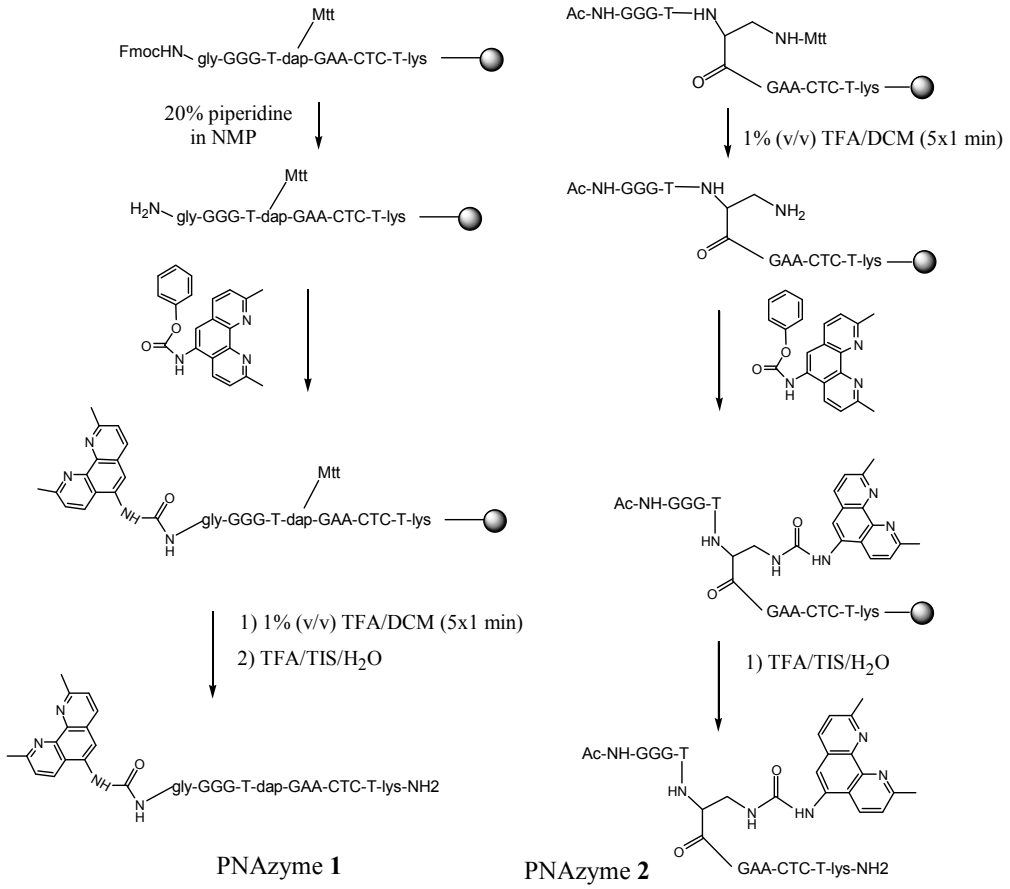


Figure 17. RP HPLC profiles of crude PNA (N-Ac-GGGT-Dap-TCCTTAT-Lys-C) A) Removal of Mtt (1% TFA in DCM) from PNA while the PNA remains solid support bound. B) Subsequent conjugation of solid support bound PNA with activated phenanthroline.

Synthesis of PNAzymes **1** and **2** from the assembled and capped PNA-dap hybrids were carried out manually but on solid support as follows (Scheme 3).

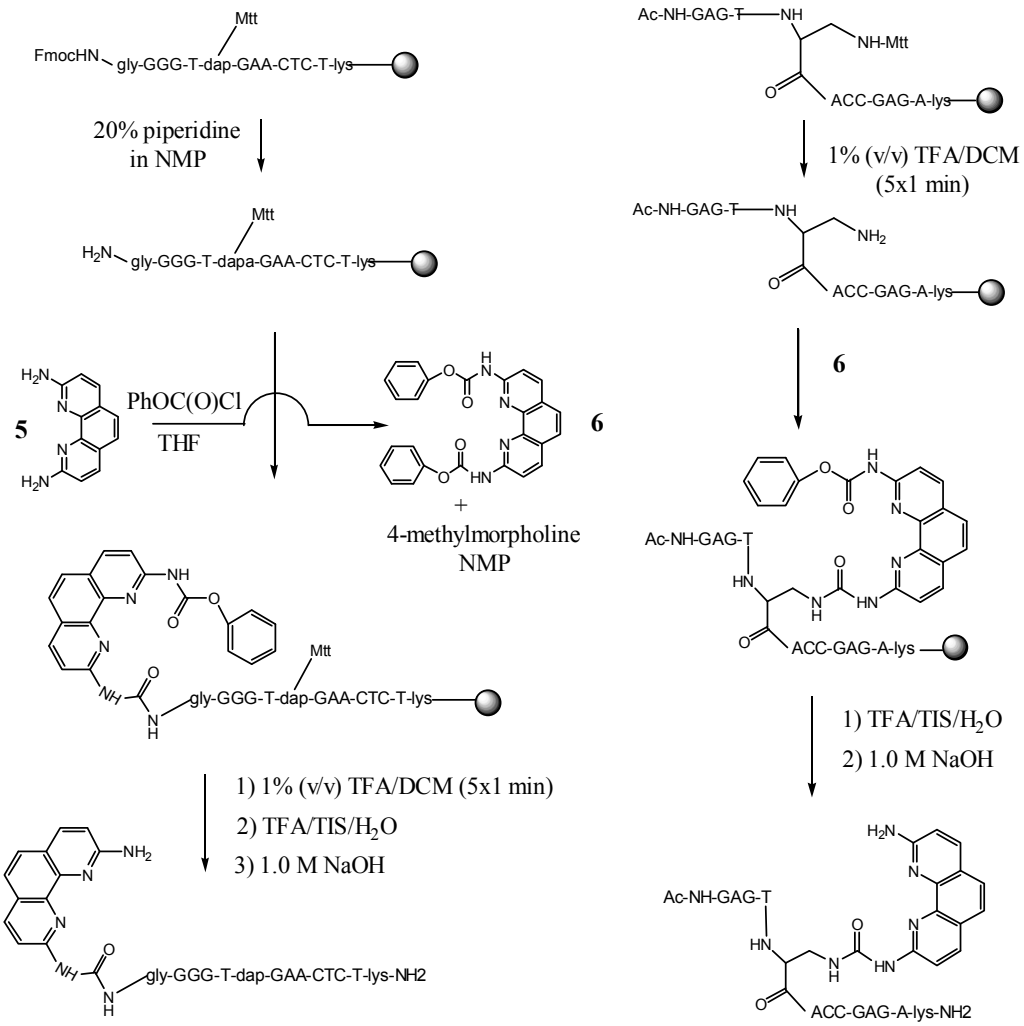


Scheme 3. Synthesis of PNA 2,9-dimethyl-1,10-phenanthroline conjugates, PNAzyme **1** and **2**.

At this state the ability of the PNAzymes to cleave target RNA was investigated. The artificial ribonuclease, PNAzyme **1**, display slow cleavage of target M-BCR/ABL RNA without site-selectivity, suggesting that positioning of the cleaving group to the 5'-end with PNA based compounds is not optimal. On the other hand PNAzyme **2** gave cleavage rates and selectivity comparable to the previously presented 2'-*O*-MeOBANs ($t_{1/2}$ 11 h compared to $t_{1/2}$ 14 h, Table 1 and Table 2).

As a first choice of catalytic group we had chosen to use a Zn^{2+} complex of the conjugated 5-amino-2,9-dimethyl-1,10-phenanthroline. This Zn^{2+} complex has been shown to work as an RNA cleaving group in different studies.(*107, 122, 146, 147*) On the other hand, the Cu^{2+} complex of the 2,9-diamino-1,10-phenanthroline has been reported to hydrolyze 2',3'-cyclic AMP substantially, almost 3 orders of magnitude, faster than the Cu^{2+} complex of the 2,9-dimethyl-1,10-phenanthroline.(*22*) It was speculated that the amino groups in 2,9-diamino-1,10-phenanthroline are acting as hydrogen bond donors to the metal-bound water molecules, thereby lowering their pKa value and thus making it easier for this group to deprotonate and facilitate the expulsion of the leaving group in 2',3'-cyclic AMP.(*22*) Inspired by the rate enhancements obtained in these studies we decided to extend our studies by using 2,9-diamino-1,10 phenanthroline conjugates for PNA-based artificial ribonucleases.

Synthesis of 2,9-diamino-1,10 phenanthroline was performed in our laboratory according to published methods.(*156-158*) 2,9-diamino-1,10-phenanthroline (**5**) was converted into the bisphenylcarbamate (**6**) by reaction with phenyl chloroformate (Scheme 4). Cleavage from the support was performed as previously described and the crude product was treated with NaOH to cleave off remaining phenyl carbamate. The target RNAs were chosen to be the M-BCR/ABL model RNA (for PNazyme **3**) and the initial model RNA (for PNAzyme **4**) forming 4-A bulges opposite to the diaminopropionic acid (dap) unit with a closing G-T wobble base pair on one side.



Scheme 4. Synthesis of PNA 2,9-diamino-1,10-phenanthroline conjugates.

The 5'-linked 2,9-diamino-1,10 phenanthroline conjugate, PNAzyme **3**, gave more than two times faster cleavage than the 5'-linked 2,9-dimethyl-1,10 phenanthroline conjugate (PNAzyme **1**), but with cleavage rate still being only modest ($t_{1/2}$ 60 h). The centrally conjugated 2,9-diamino-1,10 phenanthroline conjugate, PNAzyme **4**, using the initial model RNA as target, gave at the same time considerably lower cleavage rate compared to PNAzyme **2**. It can be concluded that the positioning and alignment of the phenanthroline groups is all-important and is likely to be far from ideal when attached from the 2-amino position. Linking 2,9-diamino-1,10 phenanthroline from the 5 position, as for the 5-amino-2,9-dimethyl-1,10 phenanthroline in PNAzyme **2**, would be highly interesting and would provide a fair comparison of the activities of these catalytic units in the catalytic site of a PNAzyme system.

2.4 Catalytic PNAzymes *(Paper IV)*

2.4.1 Design and synthesis of catalytic PNAzymes

Peptide nucleic acids (PNA) generally gives more stable complexes with RNA than DNA or 2'-*O*-MeRNA does, are stable in biological fluids and are also readily conjugated by use of peptide type chemistry.(*139, 159*) However, PNA has been sparingly used as carrier for RNA cleaving agents and only when using excess of the cleaver or giving stoichiometric cleavage without turnover of the target RNA, i.e., without enzyme like catalysis.(*99, 145, 146, 148, 149*) Our preliminary studies with PNAzymes indicated that these PNA constructs, complexes with centrally placed RNA bulges, were able to cleave target RNA in a similar fashion to the 2'-*O*-MeRNA based OBANs. Therefore we expected that we could create PNA-based artificial nucleases that give catalytic cleavage of RNA with turnover of substrate. Thus, several PNA-based systems directed towards the M-BCR/ABL mRNA model target were synthesized and the cleavage rates were determined (Figure 18, Table 2). 2,9-dimethyl-1,10-phenanthroline conjugation was performed either directly or after extension of the Dap unit with a glycine moiety in order to obtain a longer linker arm.

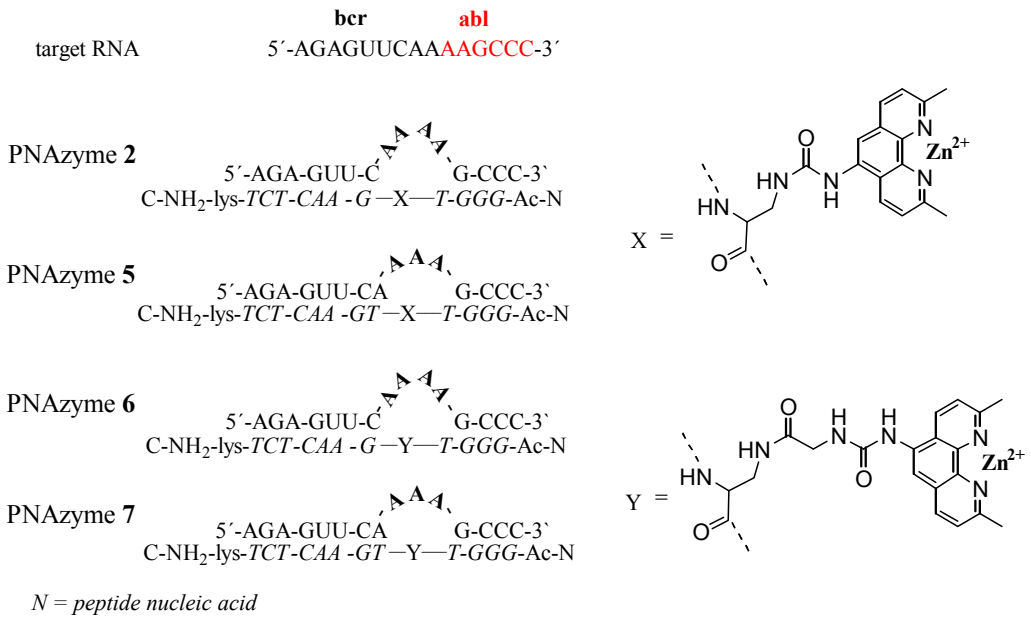


Figure 18. Complexes of PNAzymes (**2**, **5-7**) with the M-BCR/ABL derived RNA model target.

2.4.2 Results and discussion

The efficiency of PNAzymes (**2**, **5-7**) in promoting cleavage of the RNA model was evaluated by analyzing aliquots from the reaction mixture with anion-exchange HPLC (Figure 19). Analysis with IE-HPLC gave higher resolution of the cleavage products than RP-HPLC and, in addition, the PNA conjugate is not disturbing the analysis since it is not retarded on the column (as it is with RP-HPLC).

Table 2. Rate constants and half-lives for cleavage of the M-BCR/ABL RNA model by PNAzymes at a 1:1 ratio (4 μM of each).[a]

	k_{obs} (10^{-6} s^{-1})	$t_{1/2}$ (h)
PNAzyme **2**	17.6 ± 0.6	11
PNAzyme **5**	9.2 ± 0.2	21
PNAzyme **6**	16.0 ± 0.4	12
PNAzyme **7**	13.1 ± 0.7	15

[a] The experiments were performed in 100 μM Zn^{2+}, 10 mM HEPES buffer, 0.1 M NaCl at pH 7.4, t = 37 °C. In the same buffer single stranded M-BCR/ABL RNA was cleaved at a rate of $2x10^{-6}$ s^{-1} and rates of cleavage of bcr/abl RNA in presence of non-conjugated PNAzyme precursors (PP**1** and **2**) (1:1) were less than $1x10^{-6}$ s^{-1}. The stability constant for the complex with Zn (II) is only about 10^4, which means that an excess of zinc ion is necessary to keep the chelate nearly saturated.

The RNA target is cleaved in all investigated systems at a rate comparable to that of 2'-*O*-methyl-RNA based systems carrying the same catalytic group (Table 1). The scission takes place only within the formed bulge and the maximal cleavage rate was observed for a 4-nt bulge forming system (PNAzyme **2**, $t_{1/2}$=11h) with a direct linkage, closely followed by the rate observed for the 3-A and 4-A bulges (PNAzyme **6**, $t_{1/2}$=12h and PNAzyme **7**, $t_{1/2}$=15h) with the longer glycine linker.

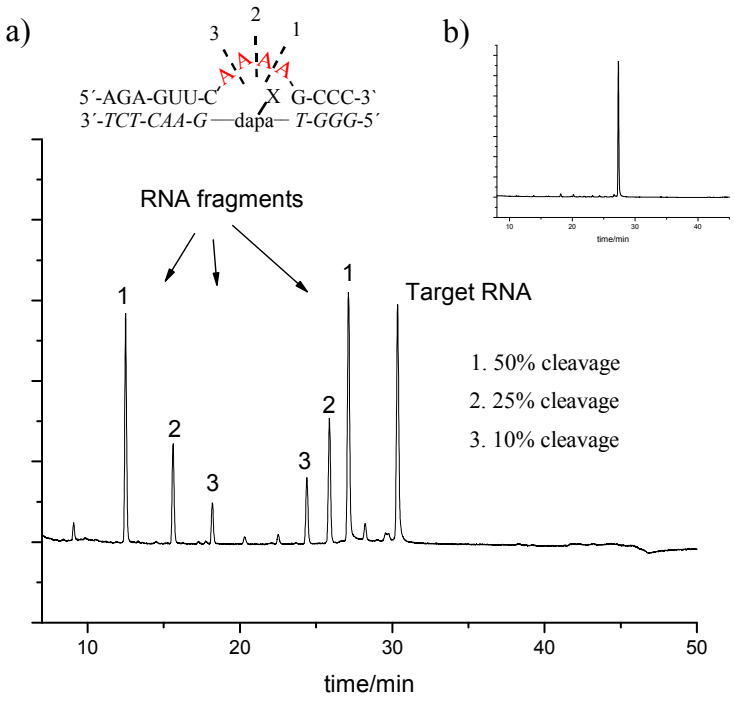


Figure 19. a) IE-HPLC analysis of the PNAzyme **2** promoted cleavage of the M-BCR/ABL RNA model, after 22 h at 37 ºC. Cleavage takes place exclusively in the bulged out region and the cleavage sites are determined by MS of fragments (ESI-TOF MS: Site 1: 1529 + 3251 ; 2: 1857 + 2923 ; 3: 2593 + n.d.), b) HPLC analysis of the background reaction with PNAzyme precursor (unconjugated PNA) and M-BCR/ABL RNA model. Both reactions were carried out using the same conditions (100 μM Zn^{2+}) and were analyzed at the same time points (22 h).

The differences in rates between these systems are not large. There is quite some flexibility in both linkers and bulges, and it is not unlikely that proximity to the cleavage site is mostly governed by the metal phosphate interaction, as suggested for the 2'-*O*-methyloligoribonucleotide based OBANs.(*122*) This means that the intramolecularity, and hence overall rates, will be relatively modest. With a short linker and a 3-A bulge a slightly more restricted system is obtained but this is less active. Thus, the system with PNAzyme **5** gives a significantly lower cleavage rate and it is likely that the geometric restrictions are such that it is energetically more demanding to position the catalytic group in a productive fashion in this system. The phosphate to metal ion interaction would neither, on its own, be strong enough to hold the metal ion in vicinity of only one phosphate nor be able to force the bulge into a single conformation. This is also supported by the observation of more than a single cleavage site even in the more selective systems (Figure 19). It is also clear that the cleavages at all sites are governed by the presence of the Zn(II)-neocuproine moiety since the cleavage in presence of non-conjugated PNA-Dap-PNA is very slow (insert Figure 19).

There is a correlation between site selectivity and overall rate, the higher the cleavage rate is the more site-selective cleavage is obtained. This could be an indication of a somewhat higher proximity at the more prominent cleavage sites.

Studies with ribozymes have revealed that common cationic metal ions, like Mg^{2+}, Ca^{2+} and K^{+}, can play an essential role in RNA folding and catalysis. For example direct contacts between Mg^{2+} ions and heteroatoms in nucleobases, phosphate oxygens or the ribose 2'-function are important, but not only specific metal ion sites in three dimensional RNA structure, but also weakly bound metal ions play an significant role, e.g. in charge screening. Mg^{2+} ions often bind to RNA without forming a direct contact, but instead the water ligands ($Mg^{2+}\times 6H_2O$) interact with RNA base and backbone substituents to stabilize specific motifs, like RNA bulges and loops.(*19, 160*) For these reasons we wanted to examine how Mg^{2+}-ions affects the catalytic activity of PNAzymes. Different PNAzymes and two magnesium ion concentrations were tested, while the experiment conditions were otherwise kept constant (20 or 100 μM Mg^{2+}, 100 μM Zn^{2+}, 10 mM HEPES buffer, 0.1 M NaCl at pH 7.4, t = 37 °C). The results with and without different Mg^{2+} ion concentrations present did not substantially affect the PNAzyme cleaving efficiency.

We performed turnover experiment using PNAzyme **2** with an excess of RNA substrate (ratio 1:2, 1:4, 1:10 PNAzyme:RNA) in order to investigate whether the bulge design concept would enable catalytic behavior in these PNA based systems, although PNA hybridization to RNA fragments is stronger than for the corresponding 2'-*O*-methyl-RNA based OBANs. Reactions were carried out using 50 μM effective concentration of Zn^{2+} ions. Turnover experiments confirmed that this PNA based system is indeed capable of catalytically cleaving the target M-BCR/ABL model RNA with turnover of the substrate, that is present in excess, and is thus truly an enzyme (Figure 20). The PNA based artificial enzyme display a dependence of cleavage rate on the PNAzyme concentration. The background reaction (of zinc aquo ion alone) is also low, even when compared to the reaction with 10 times excess of RNA substrate relative to PNAzyme **2** present. These are the first reported RNA-cleavers based on a PNA backbone that are catalytically active, i.e., that do give turnover of substrate.

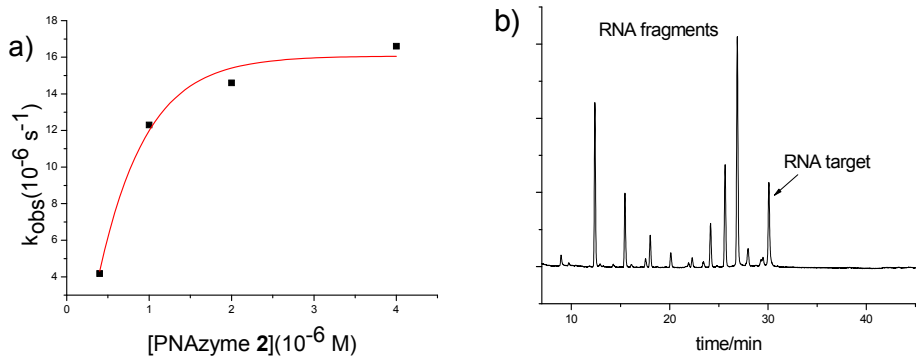


Figure 20. a) PNAzyme concentration dependence of the rate constant for cleavage of the M-BCR/ABL model with PNAzyme **2**. b) HPLC analysis of M-BCR/ABL cleavage by PNAzyme **2** when using a 1:4 ratio of PNAzyme to substrate (44 h reaction time)

The contamination by natural ribonucleases is a real threat to all experiments with artificial ribonucleases, specially when using longer RNA targets. Some earlier publications report difficulties in ruling out false positive effects.(*98, 161*) In our case, the reached sequence-specificity, reproducibility and partial site-selectivity are strong evidences against contaminations (Figure 21). In the case of non-complementary RNA targets and in the absence of PNAzyme, the background reaction also stays low as shown in Figure 21.

We can conclude that PNA-Dap-PNA neocuproine conjugates (PNAzymes) cleave a Leukemia related M-BCR/ABL mRNA model in a catalytic fashion in the presence of zinc ions. For real use in disease therapeutics the overall rate of RNA cleavage is likely to be insufficient for efficient suppression of gene expression. However it is a beginning of a development of PNAzymes that when efficient enough could become potential therapeutic agents. We have thus developed the first peptide nucleic acid based artificial RNA cleavers that really act as enzymes (PNAzymes). Taking both this work and our previous studies on 2'-*O*-methyl-OBANs, it seems that the OBAN sequence can be altered according to the specific target with essentially retained cleavage rates as long as base-pairing in the stems next to the bulge is governing formation of the complex. If base-pairing is not kept, however, the rates fall down to background levels which means that the OBANs/PNAzymes are selective with respect to target sequence.

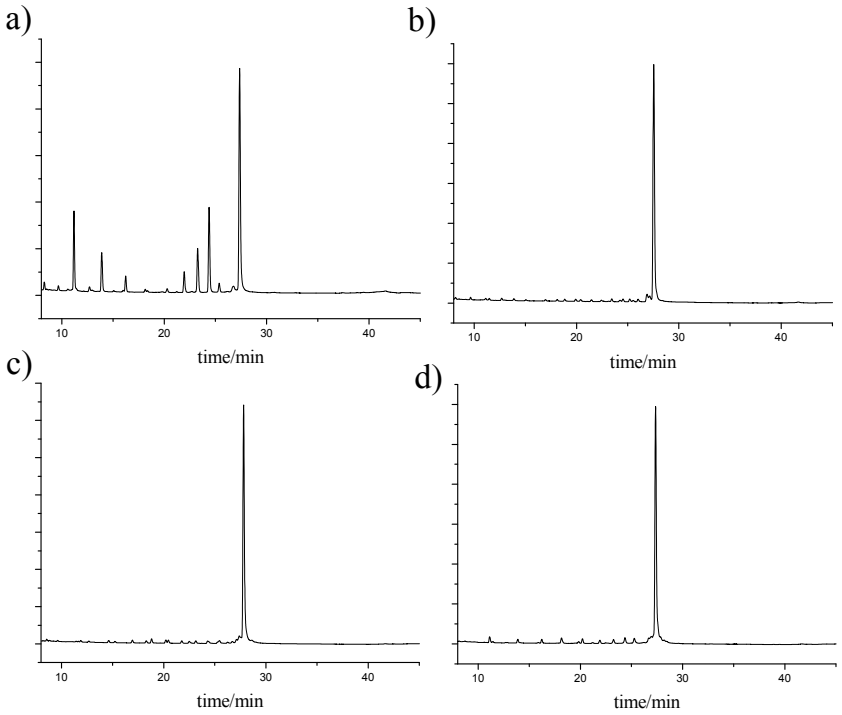


Figure 21. HPLC analysis of the incubation of different RNA sequences with PNAzyme **2** and 100 μM Zn^{2+} for 11 h at 37 ºC. a) PNAzyme **2** and M-BCR/ABL RNA model 5'-AGAGUUCAAAAGCCC-3' b) PNAzyme **2** and RNA 5'-AUAAGGAAGAAGCCC-3' (partly non-complementary) c) PNAzyme **2** and RNA 5'-UCUCGGUAAAAGCGC-3' d) only M-BCR/ABL RNA model 5'-AGAGUUCAAAAGCCC-3'.

It seems that PNAzymes can provide all the basic properties needed to create artificial nucleases for use as tools *in vitro* and with more progress as potential regulators of gene expression. *In vivo* use of metal dependent artificial ribonucleases, either need to utilize metal ions available at sufficient concentrations in biological fluids or could also be possible using metal chelates with sufficiently high binding constants ($>10^8$-10^{12} M^{-1}), although tight binding could hamper the catalytic activity of the metal ion. For example the stability constants of the Cu^{2+} and Zn^{2+} complexes of small azacrowns seems to be high enough to guarantee the binding under intracellular conditions and several groups are working with the development of selective RNA cleavers utilizing these ligands.(*109, 162-164*) In collaboration with Professor Paolo Scrimins research group some preliminary tests were performed with 1,4,7-triazacyclononane-Zn^{2+}/Cu^{2+} complexes as catalytic groups in PNAzymes (Figure 22).

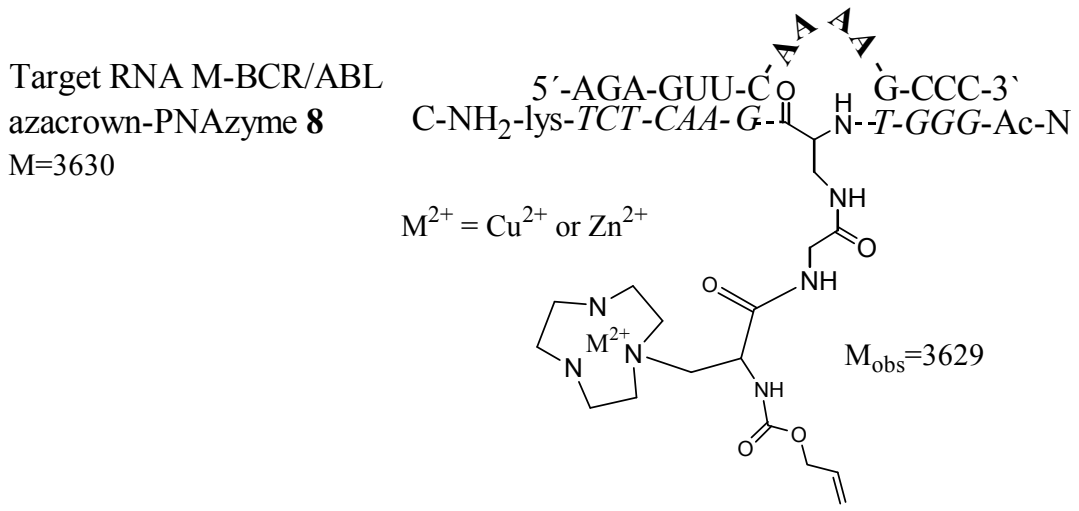


Figure 22. Structure of azacrown-PNA/target M-BCR/ABL RNA complex.

The experiments were done at pH 7.4 in the presence of either Cu^{2+} or Zn^{2+}-ions (50 μM Zn^{2+} or 10 μM Cu^{2+}). Samples were taken after 3 h reaction time and indicated only very modest cleavage rates or no cleavage at all for all tested RNA sequences (RNA **1**, **5-16**, Table 3). This is likely to be an indication that the metal ion can not readily reach the phosphodiester functions in the bulge when using this construct.

2.5 PNAzymes that are artificial RNA restriction enzymes *(Paper V)*

2.5.1 Cu(II)-PNAzymes

Our major goal in a development of artificial ribonucleases is to achieve PNAzymes that are stable compounds, give higher rates of cleavage, multiple turnover and that give virtually specific cleavage at one site, thus creating only two RNA fragments. It was already at an early stage clear that the proximity/linker played a role in determining the rate of cleavage as well as of the site selectivity. It is, however, a quite cumbersome approach to try to optimize this linker and it is also likely that the metal ion plays a major role in anchoring to the cleaved phosphate. To utilize a metal ion with higher acidity and different ligand geometry preferences than zinc ions seemed like a possibility to affect both rate of cleavage and selectivity. Cu(II) ions fulfill this requirement but creates solubility difficulties if the concentration and pH is similar to the concentration of Zn(II) ions used for both 2'-*O*-MeOBAN's and PNAzymes.(*165, 166*) However, the complex constant for binding to 2,9-dimethyl-1,10-phenanthroline is higher(*167*) which enables the use of a lower concentration of copper ions and thus

to explore Cu(II)-based PNAzymes. Cu^{2+} is also at least marginally present in the intracellular environment and very tightly binding ligands could be expected to occur as Cu^{2+} complexes.(*168, 169*) Among 2,9-dimethylphenanthroline derived cleavers, Zn^{2+} chelates has proven to be somewhat less efficient than its Cu^{2+} counterparts, but more efficient than other Cu^{2+} ter and bi pyridyl complexes.(*108, 147, 170*) It was suggested that the reason for the higher reactivity of the copper 2,9-dimethylphenanthroline (neocuproine) relative to the ter- and bipyridylcomplexes is not only due to suppression of bis(μ-hydroxide)dimers by the methyl groups of neocuproine. It was suggested that the complex could coordinate both non-bridging phosphoryl oxygens, thus "doubly activating" the phosphate and that the two methyl groups in the ligand should decrease the O-Cu-O bond angle facilitating the chelation of a phosphate diester.(*170*) Thus, we decided to extend our studies of PNAzymes to employ neocuproine-Cu^{2+}-complexes.

The target RNA was first selected to be the same as for the previous 2'-*O*-MeOBAN and PNAzyme studies retaining the cleavage conditions aside from the metal ion and its concentration (10 mM HEPES, 0.1 M NaCl, pH 7.4, t = 37 °C, Figure 23). To avoid the problems related to Cu precipitation, and made possible by that binding constant of the neocuproine-Cu complex is higher than with Zn^{2+}, the concentration of Cu^{2+}-ions was decreased to 10 μM instead of the previously used 100 μM for Zn^{2+}. This corresponds to 2.5 equivalents excess of metal ions compared to PNAzyme-RNA duplex. Special attention was directed towards preparations of the reaction mixtures in order to avoid PNA aggregations and Cu^{2+} precipitation. Some Cu^{2+}-chelate complexes are known to be in equilibrium to form inactive inter- or intramolecular dimers (*171, 172*) The step wise stability constants of Cu^{2+} and 2,9-dimethyl-1,10-phenanthroline (dmp) complexes are 1.6×10^{6} M^{-1} ($Cu(dmp)^{2+}$ aq) and 6.3×10^{6} M^{-1} ($Cu(dmp)_2{}^{2+}$ aq), indicating that the first ligand facilitates chelation of the second 2,9-dimethyl-1,10-phenanthroline.(*167*) The cooperative binding of the two 2,9-dimethyl-1,10-phenanthroline units could lead to dimerization of PNAzymes and inactivation of the RNA cleaving activity. We did, however, not observe any problems related to the dimerization of the PNAzymes.

The first RNA cleavage experiments with PNAzyme-Cu^{2+} complex showed a remarkable selectivity, with the reaction creating exclusively only two fragments, and which is retained even when target M-BCR/ABL RNA (RNA **1**) is used in multiple-

turnover conditions (Figure 23). Target RNA **1** is cleaved uniquely at one scissile phosphodiester within the RNA-bulge created by PNAzyme **2** hybridization and the cleavage position is the same as the main cleavage site with the analogous Zn^{2+} construct. Improvement in efficiency is nearly 10-fold when compared with analogous PNAzyme-Zn^{2+} complex ($t_{1/2}$ = 70 min for PNAzyme-Cu^{2+} complex, $t_{1/2}$ = 660 min for PNAzyme-Zn^{2+} complex).

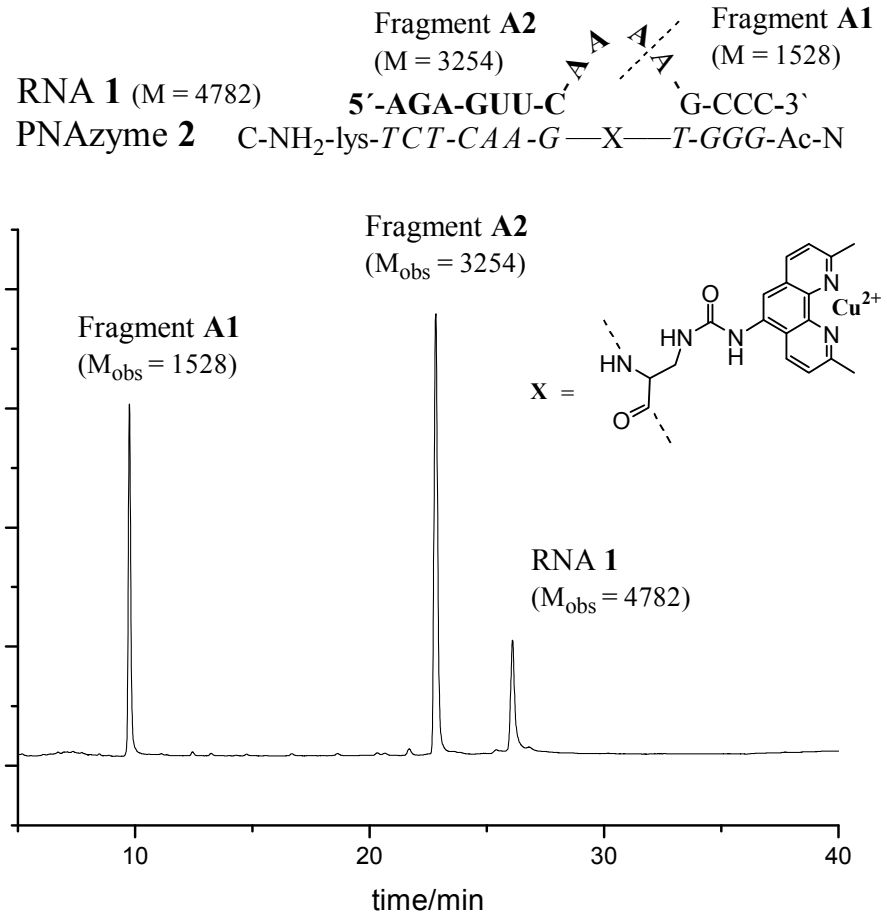


Figure 23. Sequence and secondary structure of the PNAzyme **2** and its substrate RNA **1**. IE-HPLC chromatogram shows the selective cleavage of the target RNA after 3 h incubation time (1:1 PNAzyme **2**:RNA **1**, 10 μM Cu^{2+}, pH 7.4, 37 °C). The cleavage site is indicated by the dashed line. M designates calculated molecular weight and M_{obs} molecular weights of isolated fragments as measured by masspectrometry (ESI-TOF).

Accurate comparisons of metal ion dependent artificial ribonucleases reported by various research groups is often impossible for the reason that results often refer to a specific target, and only a single pH, temperature and ionic strength. Quite often it is assumed that the metal catalyzed cleavage of RNA is retarded when lowering the pH, which could be expected if the catalysis is mediated purely through a metal hydroxide

and/or by an attacking 2'-nucleophile. The activity a couple of different PNAzyme-Cu^{2+}-target RNA complexes is, however, not much affected by lowering the pH (pH=5.0, 20mM MES-buffer), which at least suggest that a metal hydroxide is not a major contributor to catalysis. Although copper-1,10-phenanthroline complex (with hydrogen peroxide) has also been used as oxidative nuclease for scission of RNA,(*173*) showing a preference for single-stranded loops over double-stranded regions, there is no evidence for that the PNAzyme mediated cleavage would go through an oxidative mechanism. This would involve oxidative attack on the ribose, producing distinguishable fragments. These fragments were absent from the reaction mixtures, even after prolonged incubation times, only 2',3'-cyclic-phosphate and 5'-hydroxy containing fragments could be detected thus providing evidence for a transesterification mechanism. Although RNA is hydrolytically much more labile than DNA, it is oxidatively more stable, simply because the additional electron withdrawing hydroxy group destabilizes the cationic transition state necessitated by oxidation.(*174*)

2.5.2 Sequence variation

The base sequence of linear single-stranded RNAs has been reported to make a substantial contribution to the reactivity of the phosphodiesters within these compounds. Transesterification reactions of phosphodiesters within single-stranded RNA molecules are retarded by stacking interactions between the neighboring nucleic acid bases. It is predicted that often the most labile linkages are those between a 3'-*O*-linked pyrimidine and 5'-*O*-linked purine nucleoside (5'-$N^{Py}pN^{Pu}$-3'),(*5, 118*) but this is strongly affected by neighboring nucleic acid bases and even by those further apart in the molecule.(*7*) An interesting question is if the base sequence within the flexible internal RNA-bulge affects the reactivity of the scissile bond. Could we specifically force the phosphodiester backbone of the RNA-strand to adopt a favorable conformation for a catalyzed cleavage reaction by having a specific nucleotide base order in the bulge when combined with the structural effect of the RNA cleaving element? In order to investigate this several RNA sequences (RNA **2**-**22**), where base sequence of the internal 4-nucleotide bulge was varied, were subjected to PNAzyme mediated cleavage (Table 3). By systematically varying the 4-adenosine bulge in the substrate by replacing one adenine by one of the other three natural bases (RNA **5** to **16**) a strong dependence of rate of cleavage on the bulge composition is revealed. All sequences displaying enough cleavage to evaluate site selectivity exhibit almost

exclusive scission between nucleoside 5 and 6 (as in Figure 23) counted from the 3'-end of the RNA.

Table 3. Cleavage extent of RNA sequences **1**-**22** in the presence of PNAzyme **2**. Reaction were carried out with a 1:1 ratio of PNAzyme **1** and RNA target, (4μM each) at pH 7.4, 37 °C and in the presence 10 μM Cu^{2+}. For sequences **2**-**22** only the varied RNA-bulge is indicated, except for sequences **2**-**4** where the base replacing the G neighboring the bulge is also shown.

		% of cleavage		
RNA	RNA sequence	1h	3h	7h
1	5'-*AGAGUUC*- AAAA -*GCCC*-3'	50	80	
2	- AAAA -*U*	-	-	4
3	- AAAA -*C*	-	< 3	< 3
4	- AAAA -*A*	-	16	28
5	- AAA**U** -	-	< 3	< 3
6	- AAA**C** -	-	-	< 3
7	- AAA**G** -	-	25	49
8	- AA**U**A -	-	7	10
9	- AA**C**A -	-	7	11
10	- AA**G**A -	45	80	
11	- A**U**AA -	82		
12	- A**G**AA -	36	72	
13	- A**C**AA -	62		
14	- **U**AAA -	25	64	
15	- **C**AAA -	22	46	
16	- **G**AAA -	23	52	
17	- AA**UU** -	-	-	< 3
18	- AA**GG** -	-	15	41
19	- AA**CC** -	-	-	-
20	- A**UG**A -	56	88	
21	- A**CG**A -	67		
22	- **CUG**A -	46	74	

Replacing the adenine neighboring the GT wobble pair (RNA **5**-**7**) substantially decreases the rate of cleavage and while a guanine gives a lengthening in half life to about 7h, a pyrimidine base virtually kills the activity. Replacing the base of the adenosine in position 6 (RNA **8**-**10**), which is the nucleoside providing the 2'-hydroxyl nucleophile in the reaction, causes less disturbance, but again pyrimidines in this position lead to drastic drops in cleavage rates. If, on the other hand, the A in position

7 (RNA **11-13**) is changed for a pyrimidine the rate of cleavage goes up leading to the bulge sequence –AUAA- that give the highest rate observed (k_{obs} 3.6 $\times 10^{-4}$ s^{-1}, $t_{1/2}$=32 min). RNA scission is virtually site-specific, showing less than 1 % off site cleavage, and the cleavage position is the phosphodiester linkage between nucleotides 5 and 6 from the 3'-end. This is more than twice the rate of the original A4-construct (PNAzyme **2** – RNA **1**; $t_{1/2}$= 70 min). The thermodynamic stabilities of these PNAconjugate/RNA complexes (without Cu^{2+} present) were determined as the UV thermal melting points (T_m). As expected, complexes had very similar melting points, i.e., the sequence variation within the RNA-bulge did not affect the Tm substantially (T_m with RNA **1** = 55 °C, T_m with RNA **11** = 54 °C). The binding equilibrium should then be almost completely shifted towards the complexes at the reaction conditions (at 37 °C, 1:1 ratio PNAzyme:RNA, 4 µM conc. of each). Replacing the A in position 8 (RNA **14-16**) appears acceptable but does lead to lower rates of transesterification. As could be expected from the results with RNA **10**,**11**,**13** and **15**, RNA's **20-22** are cleaved at rates within the same range. The overall most prominent sequence preferences are summarized in a schematic fashion in Figure 24.

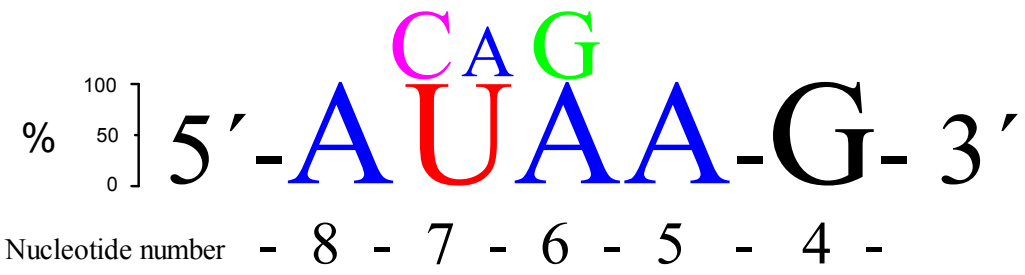


Figure 24. A schematic presentation of how the rate of cleavage in the fastest systems is dependent on the sequence in the central bulge of the RNA target, and the neighboring nucleoside on the 3'-side, upon cleavage with PNAzyme **2**.

All reactions display high site selectivity, but the rate on the other hand is quite sensitive to the sequence which indicates that the structure of the bulge plays a major role for the accessibility and/or proximity to the cleaved phosphate. The G wobble is preferred and the bases neighboring the cleaved phosphate should not be pyrimidines for effective transesterification. This would correlate with that stacking between the bases 4-6 promotes cleavage. Interestingly this stacking is also seen in molecular dynamics studies of a similar bulge (different stem sequences but an A4 bulge neighboring a GU wobble pair).(*132*) In the mentioned model there is also stacking

between A-8 and the opposite strand which reduces the flexibility of the bulge. That an A is the most preferred base at this position, for obtaining efficient cleavage, suggests that this reduced mobility could be contributing towards the scission site being kept closer to the catalyst. That position 7 preferably should be a pyrimidine could perhaps be explained by the need for the nucleoside 6 to make a conformational change upon transesterification and that stacking with a purine at position 7 would raise the barrier to do so. In the modeling study mentioned it was also found that A-7 over the time course studied did stack upon either of A-8 or A-6. Overall this indicates that even what may seem as slight changes in the bulge can alter the dynamics of the bulge in such a way that the rate of the PNAzyme catalyzed cleavage of the RNA target is substantially and sometimes dramatically affected. An important aspect of this with regard to target selectivity is that this means that apart from discriminating targets on the basis of Watson-Crick base pairing of the stems the PNAzymes will also give selectivity for variation in the bulge sequence due to kinetic resolution.

With the new considerably faster and site selective Cu(II)-based system (where also background reaction is barely detectable) the turnover of excess of substrate can be pushed much closer to what natural enzymes can master. Thus PNAzyme **2** (4μM with $[Cu^{2+}]$ = 10 μM) was reacted with a 100-fold excess of RNA substrate **11** (400 μM) and followed over 75 cycles of conversion. PNAzyme **2** is capable of multiple turnover of RNA **11**, the reaction is clean with little background reaction, and the cleavage reaction occur with retained site-selectivity also with excess of substrate. Dependency on the bulge sequence and requirement of the wobble-base pair at the 3'-side of the RNA bulge makes PNAzyme **2** an sequence selective artificial ribozyme with an excellent target selectivity. The PNAzyme **2** in presence of Cu^{2+} is the fastest site-selective artificial ribonuclease reported which is capable of multiple turnover reactions. The next step was to investigate the ability of the PNAzyme to discriminate the target with respect to mismatches, i.e., would PNAzyme cleave a target with a mismatch in the Watson-Crick base-pairing and if so would it be much slower. As can be seen in Table 4, the rate of cleavage with a single mismatch is severely retarded and the cleavage products are below the detection limit after 1h, at which time only 18% of the matched substrate remains and with a mismatch in both stems there is no reaction detectable even after 7 h reaction time. This shows that the PNAzyme has excellent mismatch rejection on the complementary part of the RNA chain and is thus likely to be highly sequence selective even when a similar bulge region can be formed.

Table 4. Mismatch study. The internal RNA-bulge is indicated with italic letters and mismatch nucleotides are marked with bold letters.

		% cleaved		
Entry	Sequence	1h	3h	7h
RNA **11**	5'-AGAGUUC-*AUAA*-GCCC-3'	82		
MIS **1**	5'-AGAGUUC-*AUAA*-GC**G**C-3'	-	4	7
MIS **2**	5'-AGAGU**A**C-*AUAA*-GCCC-3'	-	16	24
MIS **3**	5'-AGAGU**A**C-*AUAA*-GC**G**C-3'	-	-	-

As a further test we constructed two longer RNA target sequences (36mers) both containing two potential cleavage sites with potential to form 4-nucleotide bulge vicinal to a G-T wobble pair, but with one of the sites having a base substitution which would form a mismatch in the base pairing when binding to PNAzyme **2** and one forming a "mismatch-bulge" structure (-AUAU- *vs* –AUAA-). These situations mimic having longer RNA targets that can carry several possible competing target sites. In both cases PNAzyme **2** exhibited excellent site-selectivity, cleaving the targets exclusively at the site with no mismatch (also under turnover conditions) and cleavage at the mismatch site was absent. Figure 25 shows the cleavage of RNA target with "mismatch-bulge" structure.

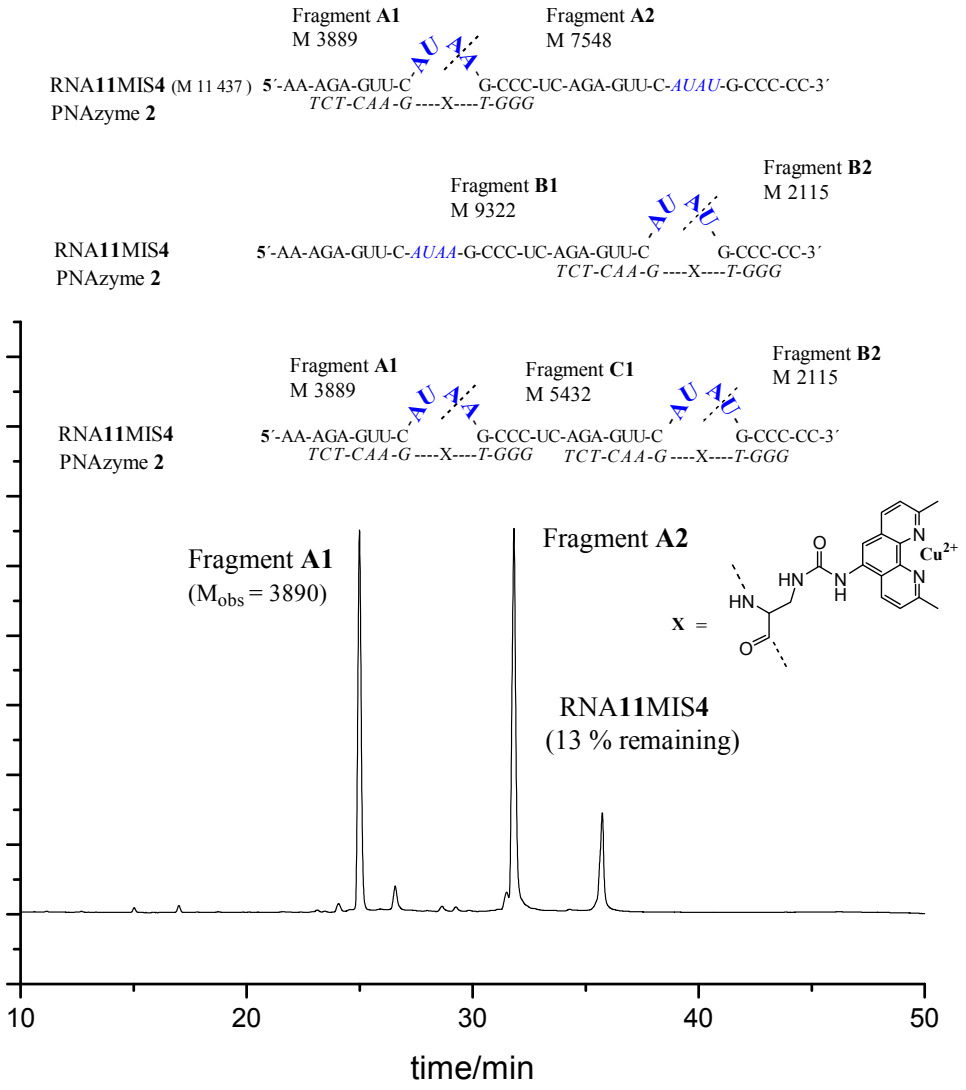


Figure 25. IE-HPLC chromatogram of cleavage of RNA**11**MIS**4** by PNAzyme **2**. The two possible binding sites are shown. Potential cleavage sites are indicated by dashed lines. The chromatogram displays selective cleavage only at one site of the target RNA after 5 h incubation time (4 µM each of PNAzyme **2** and RNA**11**MIS**4**, 10 µM Cu^{2+}, pH 7.4, 37 °C). M designates calculated molecular weight and M_{obs} molecular weight of isolated fragment as measured by masspectrometry (ESI-TOF).

2.5.3 Discussion

This new class of Cu^{2+} dependent PNA-based artificial ribonucleases (PNAzymes) is reasonably fast, display multiple turnover and highly site selective cleavage of the RNA targets. They are considerably faster than their Zn^{2+} dependent counterparts, which at least partly is likely to be due the higher acidity of copper(II) ions. What is more remarkable is the much higher site selectivity. One could speculate on various interactions with the Cu^{2+} and different nucleobases and that this would direct the

catalytic metal ion towards particular phosphate linkage. This is however not supported by the findings that base substitution in the targeted bulge or in the neighbouring GU wobble base pair does not diminish the site selectivity substantially. The rate of reaction is however in many cases of base substitution greatly affected and this is likely to be due to structural changes in the bulge which affect its rigidity and probably also the likelyhood for the Cu^{2+} to reach a productive position. It is well known that Cu^{2+} complexes have different geometry preferences than Zn^{2+} complexes. Therefore it does not seem unlikely that this could be a reason why the copper ions seem to be able to only reach a particular phosphate linkage while the zinc ions are less discriminating. At the moment we do not have the structural resolution to suggest a detailed mechanism for the selectivity nor for the actual cleavage reaction. It seems plausible, however, that one contributing factor could be that the Cu^{2+} complex could anchor to the phosphate and Cu-bound water could protonate the leaving 5'-hydroxyl while the 2'-hydroxyl is attacking (Figure 26), as suggested for metal aquo ion promoted cleavage of RNA.(*15*) According to preliminary studies with PNAzyme **2** the reaction rate is not slowed down when lowering the pH from 7.4 to 5.0, which suggests that Cu^{2+}-ions can facilitate the hydrolysis of 2',3'-cyclic phosphates to a mixture of 2'- and 3'-phosphates. In the case of PNAzyme **2**-Cu^{2+} mediated catalysis further cleavage of the produced 2',3'-cyclic phosphates remains absent. This could simply be due RNA fragment dissociation from the PNAzyme complex, causing a decrease in concentration of active Cu^{2+} ions in the vicinity of the 2',3'-cyclic phosphate. This indicates that the substrate dissociate from the active site of the enzyme and the free concentration of Cu^{2+} ions in the reaction mixture is far too low to induce cleavage of the 2',3'-cyclic phosphate.

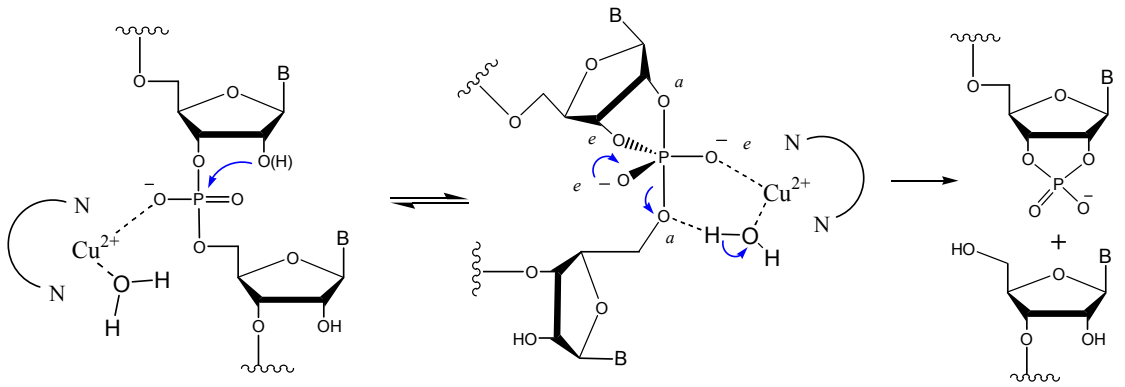


Figure 26. Plausible mechanism for PNAzyme **2**-Cu^{2+} mediated catalysis of RNA phosphodiester bonds (*a* = apical ligand, *e* = equatorial ligand).

The Cu^{2+} dependent PNAzymes display rates corresponding to half-lives in the minute range which should enable this class of artifical enzymes to become useful tools. Moreover, the combination of single site cleavage, excellent mismatch rejection, discrimination of bulge sequence and requirement of the wobble-base pair at the 3'-side of the RNA bulge makes PNAzyme **2** a highly sequence selective artificial ribonuclease that can be considered an RNA restriction enzyme. We have earlier shown that catalysis by oligonucleotide based artificial nucleases can be retained although the Watson-Crick recognition part in the stems are changed which suggests that the RNA restriction enzymes developed here can be tailor-made to target a desired sequence with the only requirement being that for optimum performance a central portion of the target should preferably be APyPuA-G (Py = Pyrimidine, Pu= Purine, see also Figure 24). These RNA restriction enzymes recognize a longer sequence than DNA restriction enzymes normally do, which in principle makes them even more selective in an RNA pool perspective. For some applications it may be desired to obtain a larger number of cleavage sites and for this it may be necessary to either shorten one of the base pairing stems (it should be possible to remove one or two base pairs and still get sufficient binding at 37 °C) or alternatively use a mixture of PNAzymes with different base pairing regions.

2.6 Conclusions and future perspectives

We have developed several efficient sequence-selective RNA cleaving artificial enzymes. The most efficient system is a peptide nucleic acid based artificial ribonuclease, called a PNAzyme, where the catalytic unit 2,9-dimethylphenanthroline-Cu(II) is tethered to the PNA backbone. The PNAzyme gives a half-live for target RNA cleavage of 32 min at pH 7.4, 37 °C, under equimolar conditions. Development of 2'-*O*-MeOBANs and PNAzymes is now at the stage where these artificial enzymes are efficient enough to be potentially useful as tools in biotechnological studies. PNAzymes could be used as conformational probes in the determination of large RNA structures or as highly sequence specific RNA restriction enzymes in molecular biology. It is, however, also clear that for therapeutic use we need to improve on overall rate of RNA cleavage and the nature of the cleaving group has to be changed towards cleavers that bind metal ions more tightly than 2,9-dimethylphenanthroline conjugates

do or utilize other metals ions in order to obtain a high potential for an efficient suppression of gene expression.

Our concept of using RNA bulge creating artificial ribonucleases has several advantages, the cleavage within the duplex will result in sufficient complex destabilization and fragment release, resulting in true artificial ribonucleases capable of catalytic turnover, RNA bulges also offers additional possibilities for interactions compared to fully duplexed RNA. An interesting possibility is to combine the catalytic group with other oligonucleotide modifications that rigidifies the bulged out region of the RNA substrate in a position favorable for cleavage. If non-Watson-Crick interaction occurs, this could not only increase the affinity but could also increase binding specificity to the target RNA. Stabilization of the conformation of the bulged out part of the RNA would be expected to rigidify the structure, which could give rise to a higher intramolecularity and thus faster cleavage. Subsequent work with these constructs could include adding stabilizing elements to rigidify the bulged out part of the RNA into a suitable conformation for intramolecular attack by a 2'-hydroxyl function, testing of different modified phenanthroline chelates (e.g. for higher binding constant or other functions) and possibly to combine different catalytic groups (e.g., non-metal ion with metal ion catalysts) to improve overall cleavage rates. Also conjugation with appropriate cell penetrating peptides to investigate the possible *in vivo* activity of PNAzymes is highly interesting.

3. Cationic peptides *(Paper VI)*

3.1 Introduction to oligonucleotide binding peptides

Cationic peptides are commonly used as DNA compacting agents for gene delivery.(*175*) The compacting ability of these peptides is usually directly related to the size of the cationic peptides, larger oligopeptides being more efficient while smaller peptides end up creating larger and less tightly packed dsDNA particles. The binding and compacting ability is largely due to electrostatic interactions between cationic peptide side-chains and the negatively charged phosphodiesters of oligonucleotides.(*176*) Numerous studies have utilized cationic polymers as components of multi-faceted non-viral gene transfer vehicles. The cationic polymer functions at two different levels within the vector. Firstly, by promoting the condensation of the anionic DNA and thereby reducing the size of the nucleic acid

whilst also protecting it from potential enzymatic and physical degradative processes. Secondly, through providing a platform by which the properties of the vector can be manipulated via the attachment of various functional moieties, e.g. receptor ligands, endosomolytic peptides and nuclear-targeting peptides. Most frequently used carriers for gene delivery are based on poly-L-lysine, polyethyleneimine or cationic lipids. Interestingly the cationic poly-L-ornithine has been shown to be up to 10-fold more efficient than poly-L-lysine in transfection of variety of cell lines, indicating that a relatively slight change in carrier structure can have a significant effect on in-vitro transfection efficiency.(*177*) Different kinds of short cationic peptides are used to enhance the delivery of AS-ONs (as conjugates or complexes).(*87, 178-183*) However, antisense oligonucleotides and siRNA´s are short oligonucleotides and shortening the nucleic acid segment weakens electrostatic cohesion of these complexes, which can then no longer withstand competition with other polyanions present in biological fluids or cell surfaces.(*184*) One solution for exploiting this pathway is designing the cationic peptide structures with high and specific affinity to oligonucleotides. Nevertheless, only a few reports describe the ability of cationic peptides as a means to bind and increase the thermal stability of duplexes of nucleic acids and these are typically cases where the oligonucleotide is conjugated to the peptide and in some cases where the target is longer than the sequence carrying the peptide, hence enabling interaction of the peptide with nucleic acid outside the hybridized region.(*184, 185*) Results for some peptide-oligonucleotide conjugates are also contradictory, some reporting clear duplex stabilization by cationic amino acids and others showing no significant effect.(*186, 187*) Some peptide–oligonucleotide conjugates have been shown not to hybridize to complementary RNA chains with the same affinity as that shown for DNA.(*188, 189*) There seems to be little reported on the thermal melting of oligonucleotide duplexes in the presence of non-conjugated cationic peptides and in particular on any apparent difference between effects on a DNA/DNA duplex (B-type helix) and an RNA/RNA or 2'-O-alkylRNA/RNA (A-type helix). Since the latter helices may represent siRNA and antisense constructs, effects on these could prove valuable in the design of delivery vehicles, for hybridization enhancement and/or nuclease protection.

3.2 Cationic peptides that affect 2'-*O*-MeRNA/RNA hybridization

Interaction between cationic peptides and oligonucleotides is in general often considered to be mainly electrostatic,(*190*) although even with single stranded fragments it appears to be more complex.(*191*) In view of this it appeared interesting to study cationic peptides with different side chain lengths to see how these influence thermal melting of both 2'-*O*-alkyl-RNA/RNA or DNA/DNA duplexes. To investigate peptides ability to increase thermal melting of duplexes several different cationic nonapeptides have been synthesized. We chose to study nonapeptides containing eight consecutive basic D-amino acids with a *C*-terminal L-phenylalanine to provide a UV-label. D-amino acids were used to offer additional protection towards proteases in future applications. These peptides consist of either of the amino acids D-diaminopropionic acid [both α (D-Dap) or β (β-D-Dap) linked], D-diaminobutyric acid (D-Dab), D-lysine or D-histidine (Peptides **1**-**5**, Figure 27). A histidine peptide was included in the studies for the reason that these oligopeptides have been proposed as efficient transfecting agents for gene therapy due to the dual capability of compacting DNA and promoting endosomal membrane fusion and leakage (proton-sponge effect).(*192*)

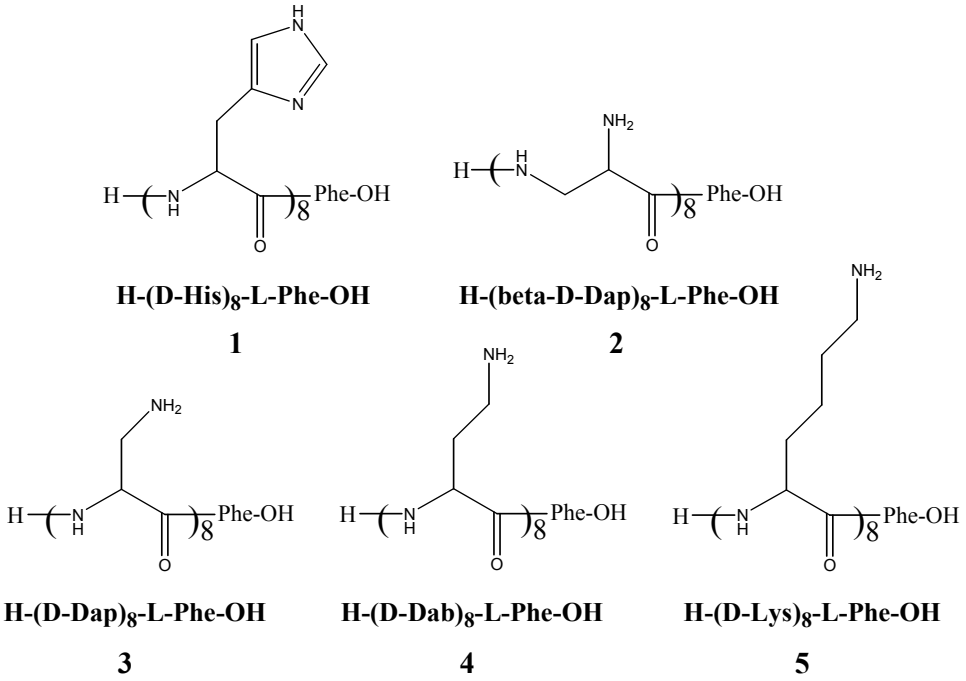


Figure 27. Peptides investigated for their influence on thermal melting of oligonucleotide complexes.

Double-stranded systems composed of fully complementary 2'-*O*-MeRNA/RNA and DNA/DNA oligonucleotides were chosen as targets and thermal meltings of these oligonucleotide duplexes (Table 5) with complementary sequences was measured in both the absence and presence of equimolar amounts of the peptides **1**-**5** at pH 7 and at pH 6.

Table 5. Influence on thermal melting points (Tm) for oligonucleotide duplexes by peptides **1**-**5** (Tm in °C).

Sequence		pH 7.0	D-His **1**	βDDa **2**	D-Dap **3**	D-Dab **4**	D-Lys **5**
DNA **1** DNA **2**	5'-GAGTACCGAGA-3' 3'-CTCATGGCTCT-5'	46.8[a]	47.0	46.4[a]	47.0	47.6[a]	47.6[a]
2'-*O*MeRNA **1** RNA **23**	5'-GAGUACCGAGA-3' 3'-CUCAUGGCUCU-5'	69.0	69.1	70.0	77.0	84.5[d]	75.5[a]
DNA **3** DNA **4**	5'-GAGTACCGA-3' 3'-CTCATGGCT-5'	35.5[a]	36.4[c]	36.3[b]	36.3[b]	38.0	38.0
2'-*O*MeRNA **2** RNA **24**	5'-GAGUACCGA-3' 3'-CUCAUGGCU-5'	57.0	59.2	59.0	64.9	73.5[d]	62.0
		pH 6.0	**1**	**2**	**3**	**4**	**5**
2'-*O*MeRNA **2** RNA **24**		53.2	56.4	67.2[d]	75.7[c,d]	78.0[d]	64.9[d]

Tm values are reported as the mean of duplicate or triplicate measurements. Standard errors of the mean for Tm values are ≤ ± 0.2°C [a] Standard error ≤ ± 0.5°C [b] Standard error ≤ ± 1.0 °C [c] Single measurement [d]A biphasic thermal profile

For the measurements at pH 7 involving the 11-mer DNA/DNA duplex what is immediately striking is that there is virtually no effect on the thermal melting by any of the peptides. This is in direct contrast to the corresponding measurement with the 2'-*O*-methyl-RNA/RNA duplex. The His and β-linked Dap showed very little influence but the lysine peptide did give an increase in melting and both the diaminopropionic acid (D-Dap) peptide and especially the diaminobutyric acid (D-Dab) peptide gave even higher thermal melting (with a ΔTm of nearly + 16 °C, Figure 28).

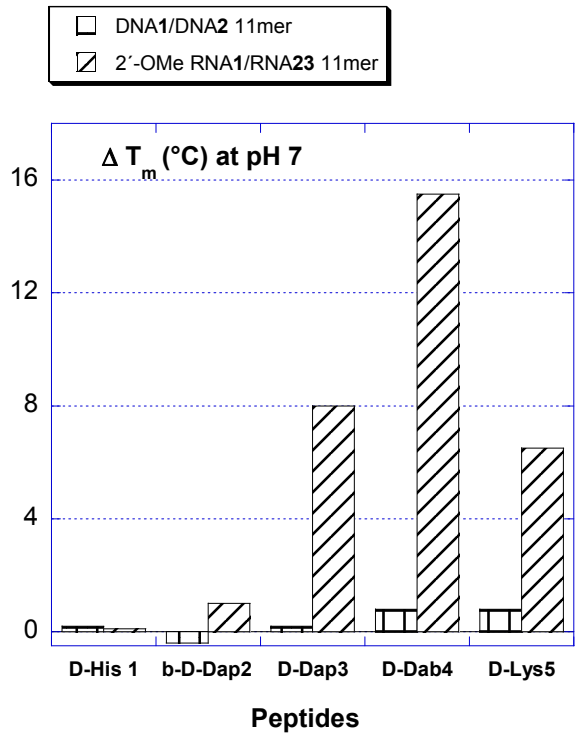


Figure 28. Melting temperature enhancements for 11-mer duplexes at pH 7 in the presence of peptides **1**-**5**.

The basicity of the different side chains varies with lysine being the most basic. The histidine peptide **1** would be only partially protonated as the pKa value of the conjugate acid of the side chain typically varies between 6 and 7.(*193*) The Dab peptide **4** is probably mostly protonated as the pKa of the conjugate acid in polymers containing Dab at every third position has been determined to be 8.85,(*194*) while the corresponding Dap peptide displayed a pKa of 7.49. Although these values could possibly be slightly lower due to vicinity of residues and statistical effects it is likely that both the lysine- (**5**) and Dab-peptides (**4**) are likely to carry largely the same charge, yet the thermal melting is considerably higher for the Dab-peptide. In view of their basicities, the other three peptides (**1**-**3**) are not likely to be more than partially protonated at pH 7, and that the Dap peptide (**3**) still gives a larger effect on thermal melting than the lysine peptide makes it even more evident that shorter side chain gives a larger influence on the melting point. At pH 6 the increased protonation of the Dap (**3**) and β-Dap (**2**) peptides causes a considerable increase in melting point for the 2'-*O*-MeRNA/RNA duplex which now is higher than for the lysine peptide (**5**), and for **3**

almost as high as for the Dab peptide (Figure 29). Truncated 9-mer duplex sequences were used for melting point determinations.

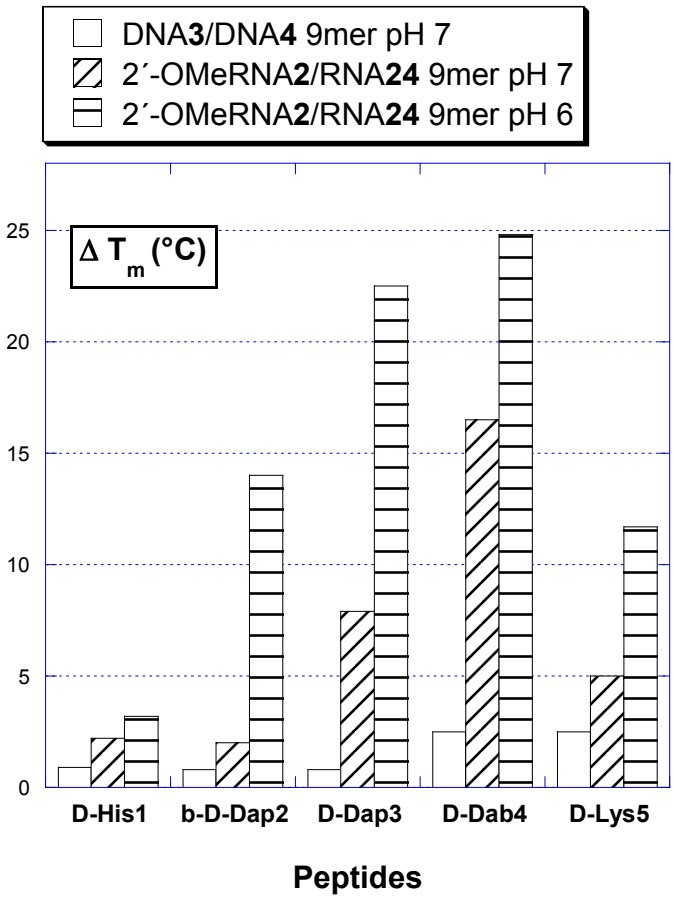


Figure 29. Melting temperature enhancements for 9-mer duplexes at pH 7 and pH 6 in the presence of peptides **1**-**5**.

Since there is higher increase in thermal melting with a shorter side chain than with the Lysine peptide it seems that not only electrostatic stabilization *per se* is at play but also that there is a structural influence.

We envisioned that interaction between peptides and the oligonucleotide duplexes could be observed by perturbation of the dichroic signal upon addition of peptides. In order to see this we analyzed the 11-mer duplexes by circular dichroism (CD) spectroscopy, with and without either of Dab peptide **4** and Dap peptide **3** (Figure 30). At the concentrations used (the same as in the thermal melting studies) the peptides by themselves did not give signals above background. The oligonucleotide duplexes

without peptide gave CD-spectra that for the DNA/DNA pair was typical for a B-type helix and for the 2'-*O*-MeRNA/RNA hybrid characteristic for an A-type structure.(*195, 196*) A 2'-*O*-MeRNA/DNA duplex indicated formation of an A-type structure but slightly less pronounced than for the 2'-*O*-MeRNA/RNA hybrid (The Tm for complexes between 2'-*O*-MeRNA/DNA without and with peptides **3** or **4** was 53, 59.9 and 65.9 °C respectively).

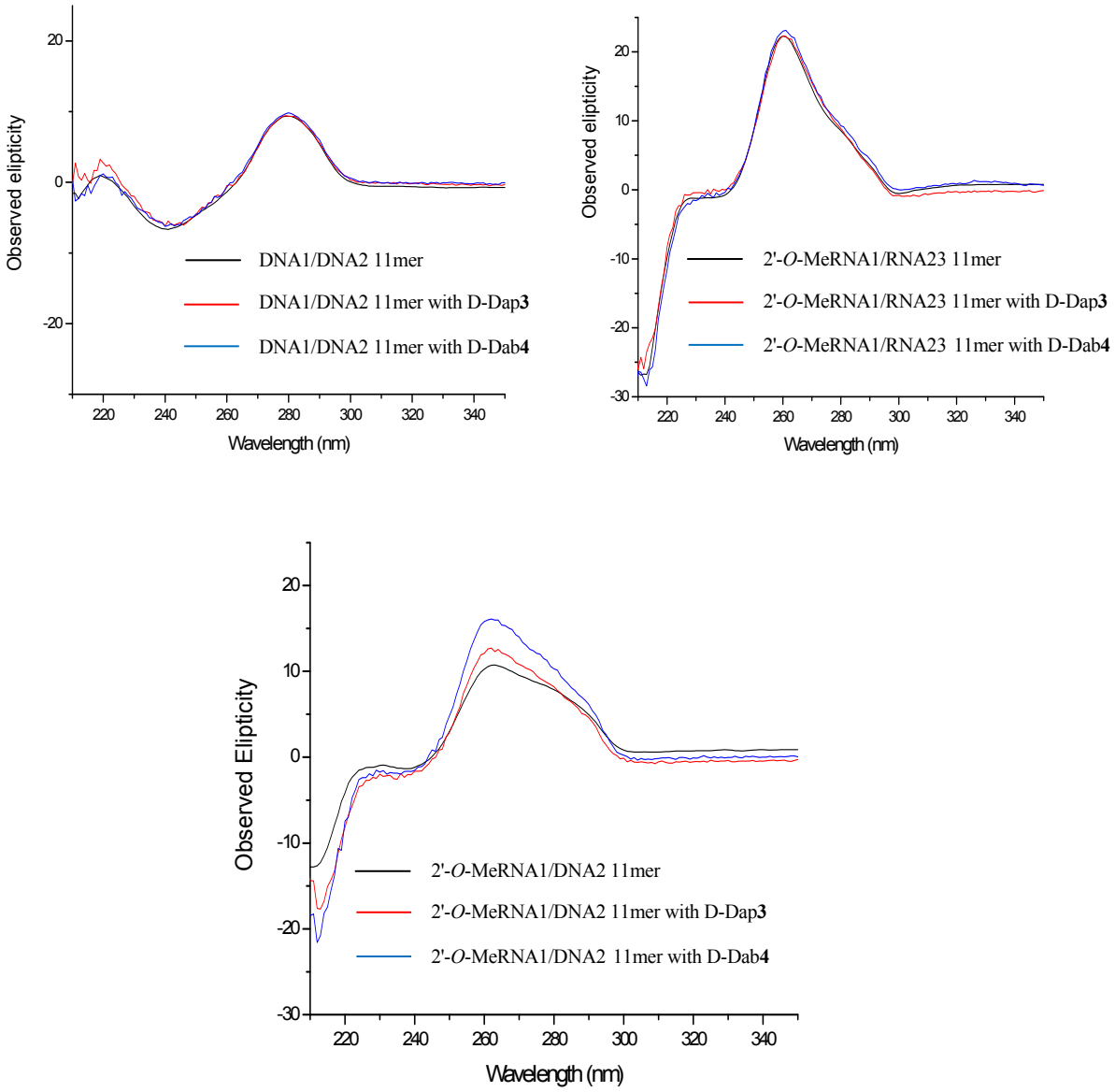


Figure 30. Circular dichroism spectra of DNA/DNA, 2'-*O*-MeRNA/RNA and 2'-*O*-MeRNA/DNA 11mer duplexes with and without the presence of equimolar amounts of peptides **3** or **4** (at 25 °C, pH 7, 4 µM each of peptide and duplex).

In the presence of peptide there was no significant difference in CD signals compared to without, neither for the DNA/DNA nor for the 2'-*O*-MeRNA/RNA duplexes. We can not see any indication of interaction of the peptides with the DNA (B-type) duplex, since the thermal melting is also hardly affected, although we can not exclude an interaction that does affect either CD or Tm. For the 2'-*O*-MeRNA/RNA duplex it is more surprising not see an effect on the CD-spectra.

As it is hard to explain the effects on melting point without an interaction it seems plausible that the interaction is face-on without alteration of the A-type duplex structure, but making it harder to dissociate. An indication that this may be so can be found in the behavior of the 2'-*O*-MeRNA/DNA duplex that in the presence of the peptides **3** and **4** adopts a more pronounced A-type conformation, thus suggesting that the peptides preferably interact with an A-type helix. DNA/DNA binding is then either in another mode and the barrier for changing from B to A is too high or the peptides do not bind at all. Nevertheless, it seems like these peptides selectively increase the melting of A-type duplexes but not of B-helices.

To further explore the influence of the cationic peptides **1**-**5** on oligonucleotide complexes we investigated thermal meltings of complexes with partially unpaired regions, i.e., internal bulges. Such structures have been the focus of attention both for the possibility to selectively stabilize RNA-bulges by additional non-Watson-Crick interaction as well as for being targets for OBAN's and PNAzymes that forces formation of bulges upon binding the target RNA. Bulge systems relevant to the 2'-*O*-MeOBAN and PNAzyme studies were explored by investigating the effect of peptides **2**-**5** on four different RNA-bulge forming modified 2'-*O*-MeRNA/RNA and PNA/RNA complexes (Figure 31). The UV-thermal data of the complexes with and without the presence of peptides is presented in Table **6**.

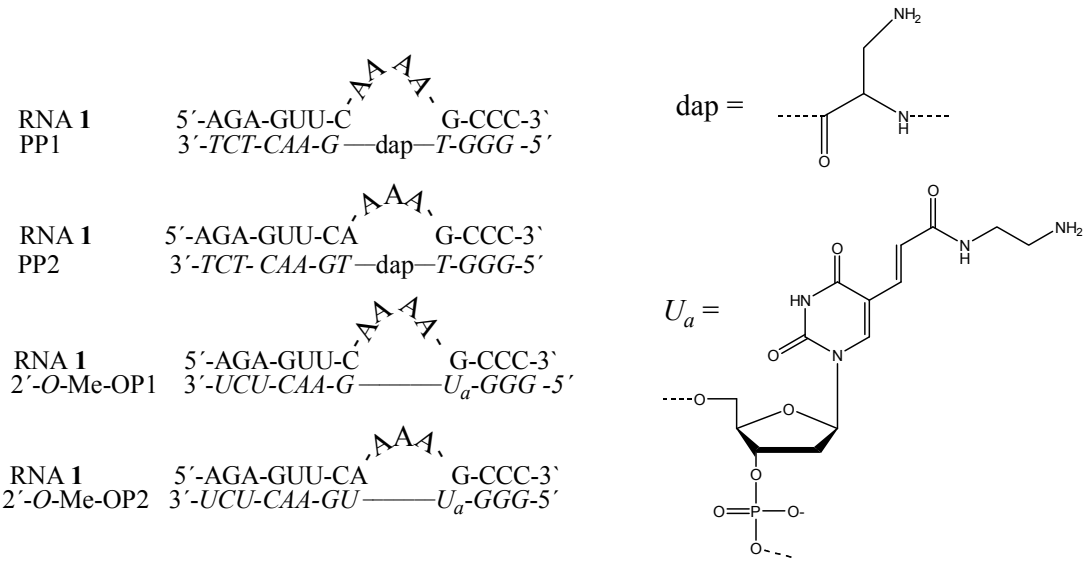


Figure 31. Complexes of PNAzyme and 2'-*O*-MeOBAN precursors with 15mer RNA**1**.

Table 6. Melting points for complexes between PNAzyme and 2'-*O*-MeOBAN precursors with 15mer RNA **1** (Tm in °C).

	pH 7.0	β-D-Dap **2**	D-Dap **3**	D-Dab **4**	D-Lys **5**
RNA**1**/PP1	54.4[a]	53.3[a]	53.8[a]	54.3	52.9
RNA**1**/PP2	55.9[a]	56.0	55.9[a]	56.1	56.4
RNA**1**/ 2'-*O*-Me-OP1	48.5[a]	50.1	55.4[a,b]	59.1[b]	52.7
RNA**1**/ 2'-*O*-Me-OP2	51.0	51.9	59.8	64.7	57.2

Tm values are reported as the mean of duplicate or triplicate measurements. Standard errors of the mean for Tm values are ≤ ± 0.2°C [a] Standard error ≤ ± 0.5°C [b]A biphasic thermal profile

With the bulge forming 2'-*O*-MeOBAN precursors (P1 and P2) the overall picture looks quite similar to that for the 2'-*O*-MeRNA/RNA duplex with little effect on the Tm from the β-linked Dap (**2**) and increase in melting point by the other peptides in

the order **5**<**3**<**4** (Figure 32). On the 5'-side of the RNA, the stem is likely to form an A-type structure that is almost the size of the 9-mer studied above. It is then possible that this would be the attraction for the peptide, (perhaps with some contribution from the bulge region that is single stranded) which could explain why the complex with the smaller bulge had a slightly higher raise in Tm. The PNA-precursors are unlikely to be in a strict A-type structure with the RNA and there will also be a considerably lower total negative charge so it is perhaps not surprising to see that these complexes, like the DNA/DNA duplexes, are little affected by the presence of the peptides.

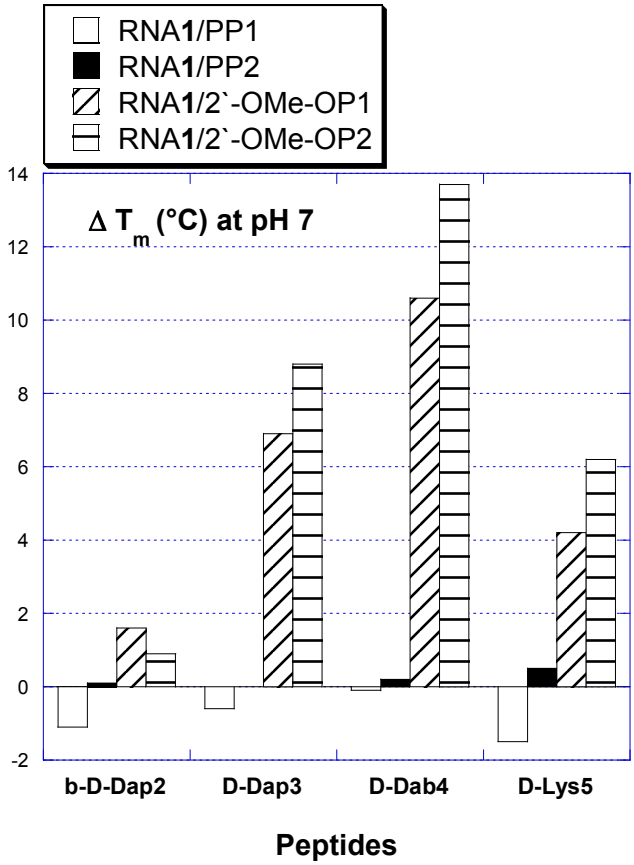


Figure 32. Melting temperature enhancements of bulge containing 2'-*O*-MeOBAN-precursor/RNA and PNAzyme-precursor/RNA complexes at the presence of peptides **2-5** at pH7.

3.3 Conclusions and future perspectives

Although a double stranded oligonucleotide in solution encounters a peptide carrying multiple opposite charges, it appears far from obvious that this will necessarily have a positive effect upon thermal stability of the complex. It is fascinating that these relatively flexible short peptides, with expected charge-charge interaction possibilities, discriminate between affecting DNA/DNA B-type helices and 2'-*O*-MeRNA/RNA A-type duplexes. It is also intriguing that the peptide side-chain flexibility/length appears to have an important role in the increase of thermal stability. Clearly not only the presence of the positive charges in the peptide chain is influencing the thermal stability but also other factors like secondary structure of the duplex and the nature of the amino acids. The cationic peptides studied here seem to selectively affect the properties of A-type duplexes. This unique selectivity and the substantial effect on thermal stability suggest that it is worthwhile to explore these further e.g., in the antisense and siRNA field where typically A-like duplexes are formed. These findings could be useful in future design of serum-resistant peptide oligonucleotide complexes or oligonucleotide conjugates that at the same time affect oligonucleotide duplex hybridization.

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